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1. Cover Letter
2. Title Page
3. Whole Manuscript
4. Submission acknowledgement
5. Editor Decision
6. Author Response (Revisions attachment)
7. Final Decision (Acceptance statement/letter)

Cover Letter

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Semarang, March 13th 2024

The Editor-in-Chief: Biodiversitas

Dear Sir,

Attached, please find our manuscript entitled:

Microscopic and molecular detection of microfilaria L3 and insecticide resistance among wild-caught mosquito vectors in endemic areas of Lymphatic filariasis

which we would like to submit to the scientific journal that you run as an original research paper.

Information regarding microfilaria infections and insecticide resistance among Lymphatic filariasis vectors in endemic areas in Indonesia is hardly accessible to the broad scientific community and the health policy planner. As a part of our attempts to determine and map the magnitude of the microfilaria circulation among mosquito vectors and the susceptibility of mosquito vectors to insecticide in Indonesia, we would like to share our data that might be important for the establishment of the Lymphatic filariasis vector control program in the area and also provide the scientific information for the mosquito-borne disease vulnerability in Indonesia.

We do believe that the manuscript would fill the data unavailability and is also very relevant to your reader.

I am looking forward to hearing your favorable reply

Sincerely yours,
S. Sayono
On behalf of the authors

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Title Page

Microscopic and molecular detection of microfilaria L3 and insecticide resistance among wild-caught mosquito vectors in endemic areas of Lymphatic filariasis

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Microscopic and molecular detection of microfilaria L3 and insecticide resistance among wild-caught mosquito vectors in endemic areas of Lymphatic filariasis

ABSTRACT

The decline in the global prevalence of Lymphatic filariasis (LF) has left areas of endemic focus in Africa and Southeast Asia. The presence of infectious and insecticide-resistant mosquitoes and the annual biting rate in the area are key to microfilaria transmission. This study aims to determine insecticide resistance and microfilariae infection in mosquito vectors in endemic areas. Twice entomological surveys based on six FL cases were carried out in Jenggot and Medono villages where indoor and outdoor mosquito capturing was carried out in ten houses within a 100m-radius of each case as well as household interviews about insecticide use. Laboratory works were done for species identification and detection of ovarian dilatation, microfilariae infection, and knockdown resistance mutations. A total of 1,197 and 581 mosquitoes were distributed to five species, namely *Culex quinquefasciatus* (69.59 and 65.40%), *Cx. tritaeniorhyncus* (5.76 and 0.00%), *Cx. vishnui* (5.85 and 27.54%), *Anopheles vagus* (0.58 and 0.00%), and *Aedes aegypti* (18.21 and 7.06%). The percentage of female mosquito infection, parity, and microfilaria were 66.25 and 86.00%, 63.37 and 70.95%, and 0.00 and 1.34%, respectively. Three base substitutions (TTA-TTT, TTA-CTA, and TTA-TGT) were found in codon 1014 of *Cx. quinquefasciatus* VGSC gene with proportions of 81.66%, 1.67%, and 26.67% showed resistance status to pyrethroid insecticides. These findings indicate the vulnerability of the research location to transmission so early detection, vector control, and further studies regarding the susceptibility of microfilariae to antiparasitic drugs are needed.

Keywords: Microfilaria, microscopic, molecular detection, mosquito vectors, Lymphatic filariasis

INTRODUCTION

Lymphatic Filariasis (LF) has shown a drastic decline in global incidence in the last two decades, although it remains a focus area in Africa and Southeast Asia (NTD Collaborators, 2020). The prevalence of LF in Asia is still stable at 3%, and only four countries are free of this disease, namely China, Japan, Vietnam, and South Korea (Bizhani et al. 2021), even microfilaria infections were still 2.63% after six rounds of mass drug administration (MDA) in Myanmar (Dickson et al. 2018). The reduction in LF prevalence after MDA in endemic areas is very low due to low community understanding and participation in the effort (Aboagye et al. 2022). LF transmission is influenced by the abundance of mosquito vectors where rainfall, temperature, and air humidity play a major role (Sinha et al. 2023). Mosquito vectors that pick up microfilariae when sucking a microfilaremia blood can become infectious and transmit the parasite after passing an extrinsic incubation period of around 13 days (Dharmarajan et al. 2019). The presence, density, and annual biting rate of the infectious mosquito vector are key parameters for parasite transmission to humans (Davis et al. 2019). This data is useful for developing priorities for controlling infectious mosquito vectors to support the success of controlling LF through mass treatment (MDA) in endemic areas. Further research on mosquito-pathogen interactions accompanied by corrective action and strengthening mosquito control methods and strategies needs to be carried out (Famakinde 2018) by developing interaction models of risk factors for LF transmission and designing public health interventions (Zerbo et al. 2021).

The results of entomological surveys in several countries report varying results. Three mosquito species of LF vector were found in two different regions in Tanzania where the results of catching 35,534 mosquitoes in the endemic area of LF Mafia Island found three species with a proportion of 1.3% *Anopheles gambia*e, *An. funestus* 0.5%, and *Cx. quinquefasciatus* 98.2% and 0.25% of the 7,936 mosquitoes examined were infected with *W. bancrofti* (Derua et al. 2017). Similar findings were reported from Masasi District, Tanzania where the study found the same three species with respective proportions of 0.1%, 0.7%, and 99.2%, and a *W. bancrofti* infection rate of 0.5% for *Cx. quinquefasciatus* (Lupenza et al. 2021). Studies in Indonesia also show different findings. A study in Bogor Regency (West Java) captured 8,087 mosquitoes distributed into four genera, namely *Culex*, *Mansonia*, *Aedes*, and *Armigeres*, but there were no microfilariae detected (Nirwan et al. 2022). A study in Pekalongan Regency (Central Java) captured 4,547 mosquitoes from three species: *Cx. quinquefasciatus*, *Cx. vishnui*, and *Ae. aegypti*, and 0.43% of mosquito samples were positive for microfilaria. (Nurjazuli et al. 2022).

The burden of LF vector control efforts is compounded by the resistance of *Cx. quinquefasciatus* mosquitoes to insecticides as reported in Bengal, India (Rai et al. 2019) and Uganda (Silva-Martins et al. 2019). Resistance of *Cx. quinquefasciatus* mosquitoes to insecticide classes, deltamethrine 0.05% and temephos 0.75% occur widely in Sri Lanka accompanied by the L1014F knockdown (kdr) mutation (Chandrasiri et al. 2020). The L1014S and L1014F mutations were also found in the *Cx. pipiens pallens* mosquito in China (Liu et al. 2019). Research in Indonesia also reported resistance to *Cx. quinquefasciatus* mosquitoes from Grobogan and Pekalongan in Central Java to 0.75% permethrin (Chakim et al. 2017) based on bioassay tests, however detection of VGSC gene mutations related to kdr resistance has not been found. This study aims to determine the biological characteristics, insecticide resistance, and the presence of microfilariae in mosquito vectors in LF endemic areas.

MATERIAL AND METHODS

Study sites

Jenggot and Medono subdistricts were chosen as research locations based on filariasis case surveillance data from the Pekalongan City Health Service in 2020. Jenggot subdistrict has the highest microfilaria rate (5.4%) among the eleven LF endemic subdistricts in Pekalongan City and every time a finger blood survey is carried out, sufferers are always found. new. There were five new cases detected in 2020. Previously, Medono Village was not an endemic area for LF, but two new filariasis sufferers were found in a finger blood survey in 2020. This cross-sectional study involved six cases (four in Jenggot and two in Medono) and 10 houses around the case within a radius of 100m.

Ethical Clearance, Data Collection and Analysis

Data collection was conducted after the ethical review was done. This research has received a letter of recommendation with the issuance of Ethical Clearance number: 419/EA/KEPK-FKM/2021 from the health research ethics committee KEPK FKM Diponegoro University Semarang. Twice entomological and household survey was conducted in Jenggot and Medono villages. The data collected in this study was analyzed descriptively to describe each research variable in the form of tables, pictures, and maps.

Mosquito catching procedures

Mosquito catching is carried out at night from 18.00-06.00 the following day according to standard procedures (WHO 2013) in two stages, each stage carried out by 6 people who have been trained using the Human Landing Catch (HLC) and Resting Collection methods (RC). The team was divided into two to catch mosquitoes inside and outside the house for 40 minutes, and 10 minutes to change the paper cup for the mosquitoes, as well as time to rest. Every 1 hour, caught mosquitoes are put in a paper cup with a code and time of capture. RC is carried out from 06.00 – 07.30, to catch mosquitoes that are resting on walls, window curtains, mosquito nets, and hanging clothes, using an aspirator and net. All mosquitoes that are caught are put into a paper cup that is filled with cotton filled with sugar solution. The paper cup is placed in a container covered with banana stems and covered with a wet towel to maintain optimum temperature and humidity of $27\pm2\ ^0$C and 80 ± 10 % respectively. The captured mosquito samples were held for twelve days and fed a 10% sugar solution before examination.

Identification of mosquito species and ovary dilatation

Identify mosquito species using previously published identification keys (Rattanarithikul et al. 2005, Nugroho et al. 2021). Mosquito parosity was determined using a surgical method to determine the amount of ovarian dilatation. The mosquito that has been identified is placed in a petri dish, the wings and legs are separated from the body, then the specimen is placed on a glass object and physiological NaCl is dropped. After that, surgery is performed using a surgical needle. Surgery is carried out microscopically using a stereo microscope. The surgical needle in the left hand presses the chest and the surgical needle in the right hand presses the 7^{th} segment and is then moved slowly to the right until the abdominal contents and ovaries are pulled out. The ovaries were placed on a glass object that had just been given distilled water to view the tracheoles using a stereo microscope with a magnification of 40x10.

Microfilaria detection

The wings of the mosquito to be dissected are cut so that the scales do not contaminate the microscope's field of view. Mosquitoes were placed on glass slides dripped with physiological saline. The mosquito's thorax and abdomen were cut into pieces with a dissecting needle and observed under a microscope at 40X magnification. If there are filarial worms, they will appear to move depending on their stage. Stages 1-2 are short, fat, and slow-moving, while stage 3 (infective) appears long and fast-moving (Laney et al. 2010). Apart from surgery, microfilaria detection also uses molecular methods with the stages of DNA extraction, DNA amplification, electrophoresis, and imaging.

DNA Extraction.

Female mosquitoes were pooled based on species as many as 10-20 mosquitoes. Each pool was homogenized with a pestle in a microcentrifuge tube containing 180 µl of ATL buffer (pH 7.2; PBS) and ground, 20 µl of proteinase K, vortexed, and incubated for 24 hours. In the next step, the specimen was vortexed for 15 seconds, and then 200 µl AL buffer was added, and vortexed again. A total of 600 µl of extract samples were taken put into a mini-column and centrifuged for 1 minute at a speed of 8000 rpm. Add 500 µl of Aw1 to the specimen, centrifuge again for 1 minute, add 500 µl of AW2 buffer, and centrifuge at 14,000 rpm for 3 minutes. The sample was transferred to a 1.5 ml tube and 60 µl AE buffer was added, incubated for 1 minute, and centrifuged at 8000 rpm for 1 minute.

DNA Amplification with Polymerase-Chained Reaction

The extracted mosquito DNA was amplified using a thermal cycler (Perkin-Elmer Cetus, Norwalk, Connecticut, USA) with 2 oligonucleotide primers, NV-l: 5' CGTGATGGCATCAAAGTAGCG 3' (21-mer) and NV-2: 5'CCCTCACTTACCATAAGACAAC 3' (22- mer). Each amplification reaction was carried out in a final volume of 50 and contained 10 ~ I-IMT ri-HCl pH 9.2, 1.5 mM MgCl, 75 mM KCl, 1.25 mM each deoxy-nucleotide triphosphate, 10 pmol each primer NV-l and NV -2, and 2 units of Taq polymerase. The temperature program for PCR was 5 minutes at 95 ^{0}C, followed by 35 cycles of 1 minute each at 94 ^{0}C, 55 ^{0}C, and 72 0C, and elongation of 10 minutes at 72 ^{0}C. A total of 20% of the PCR product from each sample was electrophoresed on a 2% agarose gel and stained with ethidium bromide to confirm amplification (Ramzy et al. 1997).

RESULTS AND DISCUSSION

In total, a total of 1,678 mosquitoes were obtained in two capture periods, 1,197 and 581 respectively, with a proportion of female sex and parity of 66.25 and 86.00% and 63.37 and 70.95%, respectively. The majority (61.49 and 65.72%) of mosquitoes showed ≥ 4 ovarian dilatations or a lifespan of 12-16 days, which is an estimate based on the understanding that one gonotropic cycle is approximately 3-4 days (Fereda 2022). This study applied the mosquito-catching method with HLC and Resting Catch and obtained a high composition of female mosquitoes and parosity. Gravid female mosquitoes suck blood to meet the protein needs for the development of embryos in eggs. Blood-feeding behavior among mosquitoes in tropical regions occurs throughout the year, whereas in temperate regions gravid mosquitoes only appear in spring (Siperstein et al. 2023). This fact shows that air temperature influences mosquitoes in their mating and pregnancy behavior. A total of five mosquito species were found in the first fishing period, and only three mosquito species were found in the second period with the proportion for each species being Cx. quinquefasciatus (69.59 and 65.40%), *Cx. tritaeniorhyncus* (5.76 and 0.00%), Cx. vishnui (5.85 and 27.54%), An. Vagus (0.58 and 0.00%), and Ae. aegypti (18.21 and 7.06%). The proportion of female mosquitoes in the first and second captures based on species is *Cx. quinquefasciatus* (60.78 and 59.78%), *Cx. tritaeniorhyncus* (8.70 and 0.00%), *Cx. vishnui* (8.83 and 32.06%), An. Vagus (0.88 and 0.00%), and *Ae. aegypti* (20.81 and 8.22%) (**Tables 1 and 3**). These data indicate that *Cx quinquefasciatus* is the dominant mosquito species in both study locations, while *Cx. tritaeniorhyncus* and An. vagus was not found on the second arrest. The dominance of *Cx. quinquefasciatus* in LF endemic areas was also reported in several studies such as in Tanzania (Derua et al. 2017, Lupenza et al. 2021), and Gamapaha, Sri Lanka (Pilagolla and Amarasinghe 2021). Similar findings were also reported from several studies in Indonesia, such as in Bogor Regency, West Java (Nirwan et al. 2022), Pekalongan Regency, Central Java (Nurjazuli et al. 2022), and Tangerang (Prasetyowati et al. 2019). However, several studies report different dominant vector species of LF in endemic areas such as *Armigeres subalbatus* in Musi Rawas, South Sumatra (Mulyaningsih et al. 2019), *Ae. scutellaris* and *Ae. kochi* in the South Pacific while *An. gambiae*, *A. funestus*, and *An. punctulatus* in rural Asia and Africa (Bhuvaneswari et al. 2023). Differences in species dominance are influenced by habitat conditions, especially the presence of aquatic plants (Pratiwi et al. 2019), chloride content, and water temperature (Amini et al. 2020).

The results of catching mosquitoes showed different results, wherein the first period they produced greater numbers. This indicates that mosquito abundance varies over time. However, mosquito abundance did not correlate with microfilaria findings. The surgery and PCR examination results of mosquito samples from the first capture period did not reveal microfilariae (**Table 2**). In contrast, in the second period of capture, fewer numbers and species of mosquitoes were obtained, namely 581 mosquitoes from three species (**Table 3**), but the results of dissection of female mosquitoes after holding for 12 days found that 4 out of 298 (1.34%) mosquitoes contained L3 microfilariae, with species *W. bancrofti* (**Fig.** 1). All microfilariae were detected from mosquito samples aged 12-20 days (ovarian dilatation 4 and 5) with proportions of 25% and 75%. This fact shows that microfilariae are found in mosquitoes that have gone through 4-5 periods of sucking blood. Detection of microfilariae in mosquito vectors in Indonesia is still limited. This study adds to the fact that there is still an infectious vector of LF in Pekalongan City, Central Java, Indonesia, even with a higher percentage compared to findings in other areas (Derua et al. 2017, Lupenza et al. 2021), including in nearby areas (Nujazuli et al. 2022). This finding confirms that this area still has a high potential for LF transmission with the main vector being the *Cx. quinquefasciatus*. Apart from adding data on the number of microfilaria infections in mosquito vectors, these findings also confirm the role of *Cx. quinquefascitus* as the main vector of LF in Pekalongan. *W. bancrofti* is the microfilariae most frequently found in mosquito vectors (Kinyatta et al 2023), especially *Cx. quinquefasciatus*. This finding is also following similar reports in Tanzania where this species is also the main vector of LF although with a much smaller infection rate (Lupenza et al. 2021). The variation in species and proportion of mosquitoes found in this study is also similar to other reported studies where Cx. quinquefasciatus is the dominant species in LF endemic areas (Derua et al. 2017, Lupenza et al. 2021). In the local context, this study found more mosquito species than previous findings at other nearby locations where only three mosquito species were found, namely *Cx. quinquefasciatus*, *Cx. vishnui*, and *Ae. aegypti*, although the proportion is not stated (Nurjazuli et al. 2022). The findings of this study illustrate the high potential for LF transmission in the region. This is supported by the fact that the majority of *Cx. quinquefasciatus* is a female with a high proportion of parity and an estimated lifespan of more than 12 days. These findings indicate a higher proportion of mosquitoes with ovarian dilatations ≥ 4 than the number of dilatations below. This data illustrates that the majority of female Culex mosquitoes in this population have a high potential to become competent vectors if in the first or second gonotrophic cycle they succeed in obtaining microfilariae from the blood of parasitemia sufferers. This possibility is strengthened by the fact that all L3 microfilariae identified in this study came from mosquito samples with an ovarian dilation range of 4-5 times or 12-20 days old. This finding follows other reports which state that the incubation period for extrinsic microfilariae in the mosquito's body to reach the L3 stage and enter the salivary glands takes 13 days (Xu et al. 2018, Dharmarajan et al. 2019). The potential for LF transmission is increasing along with climate change, where increasing the average daily air temperature can shorten the extrinsic incubation period for microfilariae (Simon et al. 2017).

Results of nucleotide substitution analysis of the ace1 and *Cx. quinquefasciatus* VGSC genes as an indication of the insecticide resistance status of the mosquito population. Part (A) in Figure 2 shows the results of nucleotide alignment at codon 119 of the ace1 gene where all DNA samples show the same wildtype base arrangement, namely GGC (Valine amino acid). Part (B) is a chromatogram display of the base arrangement. Part (C) shows the results of aligning the nucleotide sequence in codon 1014 of the VGSC gene where there are three forms of nucleotide substitution, namely from T to C in the first base, T to G in the second base, and A to T in the third base. There are three nucleotide substitution variations in codon 1014 of the VGSC gene of *Cx. quinquefasciatus* from the formation of wild-type leucine (TTA) to phenylalanine (TTT) and Cysteine (TGT), and one silent mutation from TTA to CTA (Table 4). The first change is a form of silent mutation where the base substitution (TTA to CTA) does not change the amino acid, namely Leucine. The second change results in an amino acid change from Leucine (TTA) to Cysteine (TGT) and the third change results in a change in the amino acid Phenylalanine (TTT) (Fig. 2). The L1014F and L1014C mutations (Fig. 3) show homozygous and heterozygous forms. Parts A and B are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Phenylalanine (TTT) and Cysteine (TGT), while C and D are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Cysteine (TGT). Image B is a heterozygous form where the T and G bases are at the same locus, although T is more dominant. The opposite condition occurs in image D. The overall results of mutation analysis of the VGSC codon 1014 gene show that the proportion of mosquitoes resistant to insecticides in this population is 98- 100%. This finding is supported by the fact that the activity of using insecticides by the community is also very high with a range of 85-92.5%, even 63.3-65% for more than two years, with a daily intensity of 40-53.3%, especially the pyrethroid group (80-81.7%) and 58-60% in burnt coil formulations (**Table 5**). Reports of resistance to Culex mosquito species, especially *Cx. quinquefasciatus* in Indonesia is still limited. This finding complements previous data where *Cx. quinquefasciatus* in Central Java, Indonesia has been resistant to pyrethroid insecticides (Chakim et al. 2017), but a study in Jakarta reported that this species is still susceptible to pyrethroids and organophosphates (Subahar et al. 2022). Recent data show that *Cx. quinquefasciatus* in Surabaya has been resistant to both groups of insecticides, and it has even been proven that there are mutations in the VGSC and ace-1 genes (Panjinegara et al. 2022). This condition proves that Culex mosquito resistance to insecticides has become increasingly widespread. The phenomenon of expanding the resistance area of Cx mosquitoes. quinquefasciatus was also reported in Bengal, India (Anju-Viswan et al. 2020, Rai et al. 2019). Cameroon (Talipouo et al. 2021), Brazil (Lopes et al. 2019), Nigeria (Omotayo et al. 2023), and Korea (Jeon et al. 2024). Molecular analysis of mosquito samples only showed four genotypes of the VGSC gene, namely wild type, silent mutation, and mutant (Leu-Phe and Leu-Cis). The Leu-Phe mutation is the most frequently reported mutant form in Indonesia (Panjinegara et al. 2024) and other countries (Talipouo et al. 2021). This study did not find any mutations in the ace-1 gene. This is different from the findings in Surabaya which found wildtype, mutant-heterozygous, and mutant-homozygous genetic variations (Panjinegara et al. 2024). A more serious genetic mutation in this species was reported from Brazil involving esterase, ace-1, and VGSC (Lopes et al. 2019). Studies in Korea also did not find the G119S mutation but did find heterozygous forms of AGC/GGC, L1014F, and L1014S (Jeon et al. 2024). This genetic mutation phenomenon indicates a serious problem that can hamper vector control efforts in LF-endemic areas, so the search for other effective methods is necessary.

In conclusion, *Cx. quinquefasciatus* is the dominant species among the five vector mosquito species in the LF endemic area of Pekalongan Regency, and 1.34% of them were proven to carry *W. bancrofti* microfilariae. This fact shows that the area is still vulnerable to LF transmission. This condition is exacerbated by *Cx. quinquefasciatus* resistance, both against pyrethroid and organophosphate class insecticides. Molecular analysis of the VGSC gene found the wild-type allele and three mutant alleles, namely silent mutation (TTA to CTA), TTA to TTT mutation, and TTA to TGT. Further investigation is needed to detect the susceptibility of microfilariae to various antiparasitic drugs and educate the public to implement methods of self-protection from exposure to mosquitoes accompanied by environmental cleaning movements to eradicate the habitat of LF vector mosquitoes.

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CONFLICT OF INTEREST

All of the authors state that there was no conflict of interest.

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Table 1. Distribution of Mosquitos Based on Characteristics and Study Sites (1st Period of Mosquito Capture)

No.	Variables	Study Sites			
		Medono		Jenggot	
		n	%	n	%
1	Mosquito species and sex				
	Cx. quinquefasciatus	**280**	**33.61**	**553**	**67.39**
	Male	124	44.39	227	41.09
	Female	156	55.61	326	58.91
	Cx. tritaeniorhyncus	**31**	**44.93**	**38**	**55.07**
	Male	0	0.00	0	0.00
	Female	31	100.00	38	100.00
	Cx. vishnui	**29**	**41.43**	**41**	**58.57**
	Male	0	0.00	0	0.00
	Female	29	100.00	41	100.00
	An. vagus	**0**	**0.00**	**7**	**100.00**
	Male	0	0.00	0	0.00
	Female	0	0.00	7	100.00
	Ae. aegypti	**83**	**38.07**	**135**	**61.93**
	Male	17	20.62	36	26.67
	Female	66	79.38	99	73.33
2	**Parity**	**103**	**33.99**	**200**	**66.01**
	Nulliparous	23	22.33	88	44.00
	Parous	80	77.67	112	56.00
3	**Number of Ovary Dilatation (age in days)**	**80**	**41.67**	**112**	**58.33**
	1 (4)	0	0.00	8	7.14
	2 (8)	5	6.25	14	12.50
	3 (12)	18	22.50	29	25.89
	4 (16)	14	20.48	7	6.25
	5 (20)	18	22.50	10	8.93
	6 (24)	6	7.50	26	23.21
	7 (28)	19	23.75	18	16.07

Table 2. Results of Microscopic Microfilariae Detection of Female Mosquitoes (1st Period of Mosquito Capture)

No.	Species	Study Sites			
		Medono		Jenggot	
		n	%	n	%
1	***Cx. quinquefasciatus***	**50**	**50.00**	**50**	**50.00**
	Positive	0	0.00	0	0.00
	Negative	50	100.00	50	100.00
2	***Cx. tritaeniorhyncus***	**10**	**50.00**	**10**	**50.00**
	Positive	0	0.00	0	0.00
	Negative	10	100.00	10	100.00
3	***Cx. vishnui***	**10**	**50.00**	**10**	**50.00**
	Positive	0	0.00	0	0.00
	Negative	10	100.00	10	100.00
4	***An. vagus***	**-**	**-**	**7**	**100.00**
	Positive	-	-	0	00.00
	Negative	-	-	7	100.00
5	***Ae. aegypti***	**10**	**50.00**	**10**	**50.00**
	Positive	0	0.00	0	00.00
	Negative	10	100.00	10	100.00

Figure 1. *Wuchereria bancrofti* larvae were detected from four mosquito samples of *Cx quinquefasciatus* collected from Jenggot village, Pekalongan City.

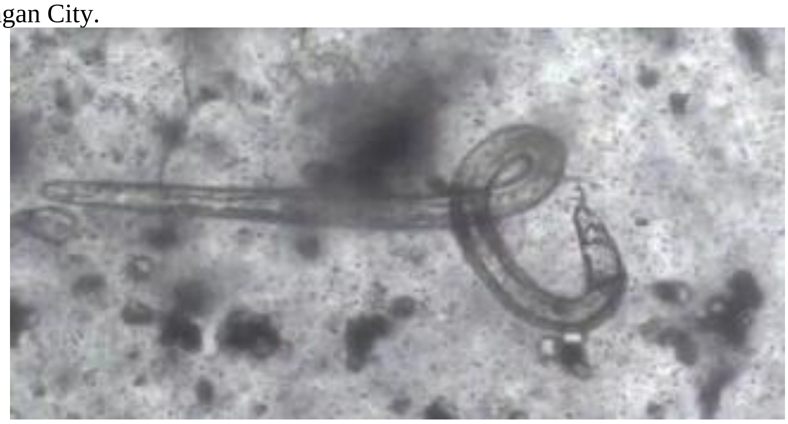

Table 3. Distribution of Mosquito Characteristics from Jenggot Village (2nd Period of Mosquito Capture)

No.	Characteristic of Mosquitoes	n	%
1	Mosquito species and sex		
	Cx. quinquefasciatus	**380**	**65.40**
	Male	82	21.58
	Female	298	78.42
	Cx. vishnui	**160**	**27.54**
	Male	0	0.00
	Female	160	100.00
	Ae. aegypti	**41**	**7.06**
	Male	0	0.00
	Female	41	100.00
	Total	**581**	**100.00**
2	**Parity**		
	Nulliparous	86	29.05
	Parous	210	70.95
	Total	**296**	**33.99**
3	**Number of Ovary Dilatation (Age of Mosquito; days)**		
	1 (4)	7	3.33
	2 (8)	18	8.57
	3 (12)	47	22.38
	4 (16)	43	20.48
	5 (20)	47	22.38
	6 (24)	23	10/95
	7 (28)	25	11.90
	Total	**210**	**100.00**
4	**Microscopic detection of microfilariae in female mosquitoes**		
	Cx. quinquefasciatus	**298**	**59.72**
	Positive	4	1.34
	Negative	294	98.66
	Cx. vishnui	**160**	**32.06**
	Positive	0	0.00
	Negative	160	100.00
	Ae. aegypti	**41**	**8.22**
	Positive	0	0.00
	Negative	41	100.00
	Total	**499**	**100.00**

Table 4. Frequency of VGSC gene mutations among *Cx. quinquefasciatus* mosquito

No.	Nucleotide Substitution	Type of Mutation			
		Homozygous	%	Heterozygous	%
1	TTA to TTT	33	55.00	16	26.67
2	TTA to CTA	1	1.67	0	0.00
3	TTA to TGT	4	6.67	6	10.00

Table 5. The Insecticide Resistance Status and History of Insecticide Use in Study Sites

No	Variabel	Study Sites			
		Medono		Jenggot	
		n	%	n	%
1	**Resistance Status**				
	Sensitive	0	0	1	2
	Resistance	20	100	59	98
2	**The Use of Household Insecticide**				
	Yes	37	92.50	51	85
	No	3	7.50	9	15
3.	**Insecticide Group**				
	Organophosphate	8	20	11	18.33
	Pyrethroid	32	80	49	81.67
4.	**History of Insecticide Use (years)**				
	< 2	14	35	22	36.67
	≥ 2	26	65	38	63.33
5.	**Insecticide Use Intensity**				
	Not everyday	24	60	28	46.67
	Everyday	16	40	32	53.33
6.	**Insecticide Formulation**				
	Coil	24	60	35	58.33
	Spray	6	15	10	16.67
	Electric	2	5	4	6.67
	Repellent	8	20	11	18.33

Figure 2. Molecular analysis of codon 119 of ace-1 gene of *Cx. quinquefasciatus*

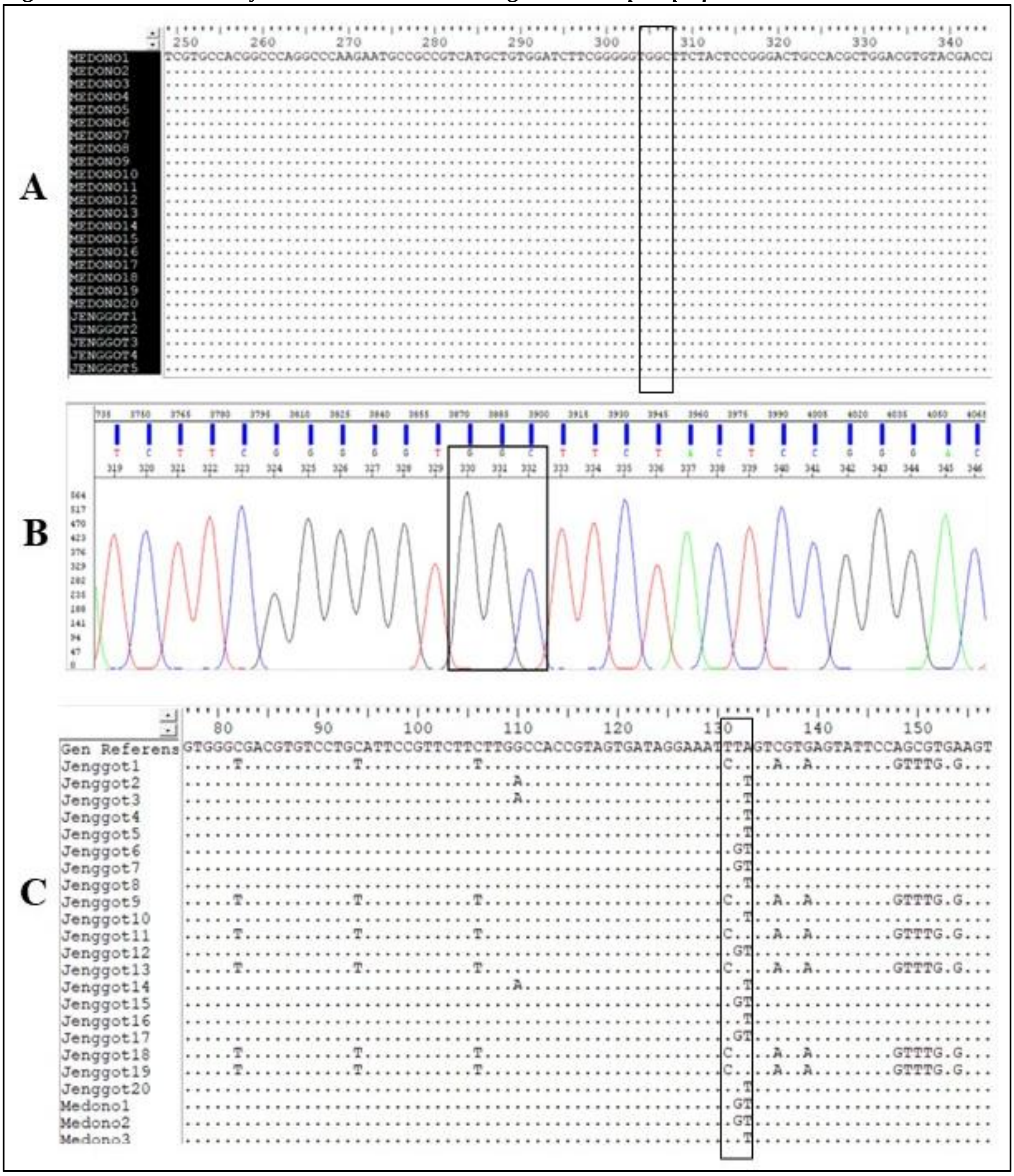

Fig. 2. Results of nucleotide substitution analysis of the ace1 and VGSC gene of *Cx. quinquefasciatus* as an indication of the insecticide resistance status of the mosquito population. No nucleotide substitutions were found in codon 119 of the ace1 gene (A) and the chromatogram shape of codon 119 (B). Part C is a variation of nucleotide substitution in codon 1014 of the VGSC gene which shows three variant changes, namely TTA-CTA (silent mutation), TTA-TTT, and TTA-TGT.

Figure 3. Homozygous and heterozygous nucleotide substitution in codon 1014 VGSC gene of *Cx. quinquefasciatus*

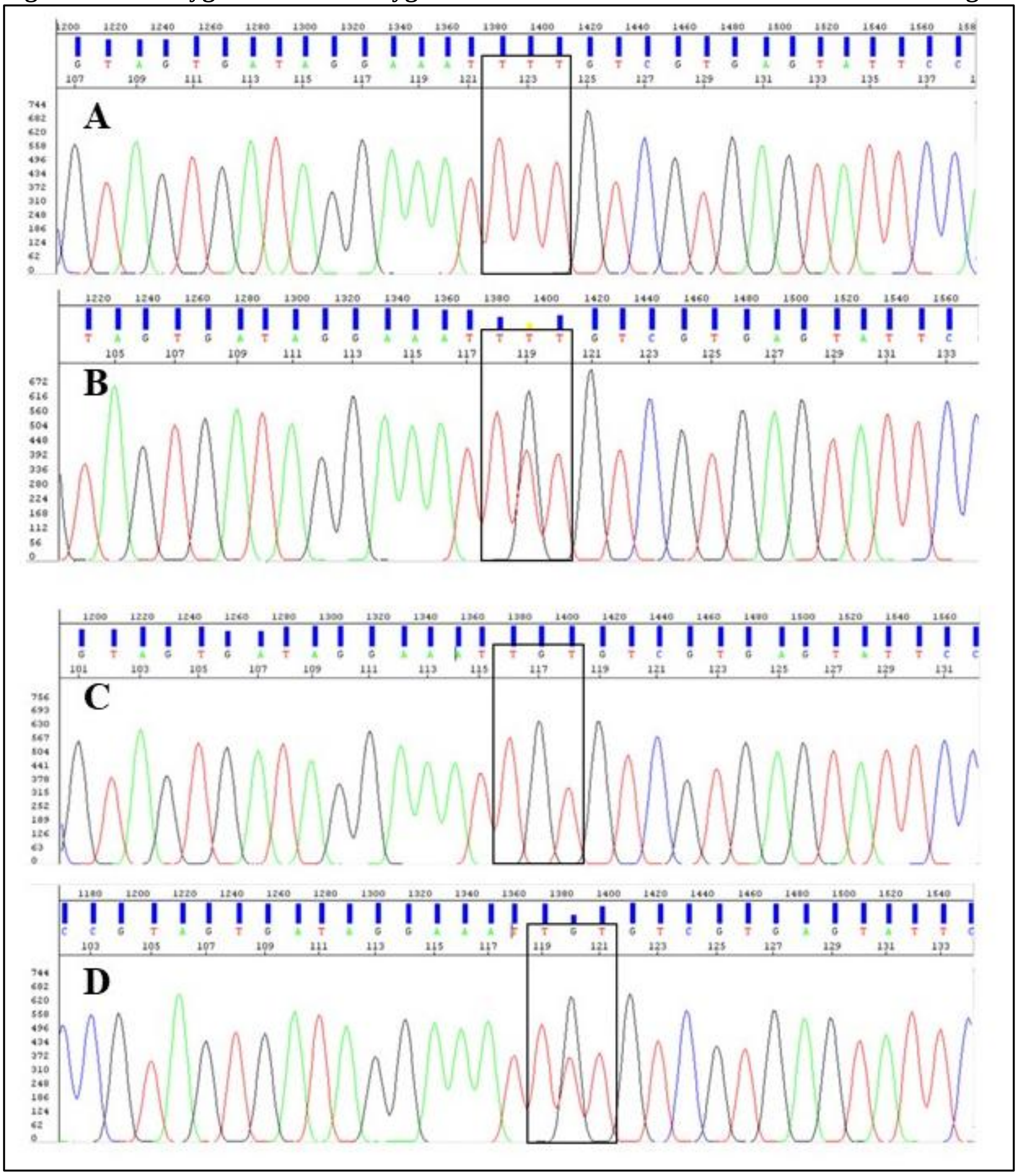

Fig.3. Homozygous and heterozygous nucleotide substitution in codon 1014 of VGSC gene of *Cx. quinquefasciatus*. A and B are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Phenylalanine (TTT) and Cysteine (TGT), while C and D are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Cysteine (TGT). Image B is a heterozygous form where the bases T and G are at the same locus, although T is more dominant. The opposite condition occurs in image D.

Editor Acknowledgement

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~~Microscopic and molecular detection~~Detection of ~~microfilaria~~Microfilaria L3 and ~~insecticide resistance~~Insecticide Resistance among ~~wild~~Wild-caught ~~mosquito vectors~~Mosquito Vectors in ~~endemic areas~~Endemic Areas of Lymphatic ~~filariasis~~Filariasis

ABSTRACT

The decline in the global prevalence of Lymphatic filariasis (LF) ~~has left areas of~~is evident in the persistent endemic ~~focus~~foci in Africa and Southeast Asia. ~~The~~In this context, the presence of infectious and insecticide-resistant mosquitoes and the annual biting rate in the area are key to microfilaria transmission. ~~This study aims~~Therefore, this research aimed to determine insecticide resistance and microfilariae infection in mosquito vectors in endemic areas. ~~Twice~~In Jenggot and Medono villages, twice entomological surveys were conducted based on six ~~FL~~LF cases~~ were carried out in Jenggot and Medono villages~~, where indoor and outdoor mosquito capturing was carried out in ten houses within a 100m~~-~~ radius of each case as well as household interviews about insecticide use. ~~Laboratory~~In addition, laboratory works were ~~done~~performed for species identification and detection of ovarian dilatation, microfilariae infection, and knockdown resistance mutations. A total of 1,197 and 581 mosquitoes were distributed to five species, namely *Culex quinquefasciatus* (69.59 and 65.40%), *Cx. tritaeniorhyncus* (5.76 and 0.00%), *Cx. vishnui* (5.85 and 27.54%), *Anopheles vagus* (0.58 and 0.00%), and *Aedes aegypti* (18.21 and 7.06%). The percentage of female mosquito infection, parity, and microfilaria were 66.25 and 86.00%, 63.37 and 70.95%, ~~and~~as well as 0.00 and 1.34%, respectively. ~~Three base substitutions (~~Furthermore, TTA-TTT, TTA-CTA, and TTA-TGT~~)~~ base substitutions were ~~found~~reported in codon 1014 of *Cx. ~~quinquefasciatus~~Quinquefasciatus* VGSC gene with proportions of 81.66%, 1.67%, and 26.67~~%~~%, respectively. The result showed ~~resistance status to pyrethroid insecticides. These findings indicate~~that the vulnerability of the research location to transmission ~~so~~emphasized the necessity for early detection, vector control, and further ~~studies regarding~~analyses of the susceptibility of microfilariae to antiparasitic drugs ~~are needed~~.

Keywords: Microfilaria, ~~microscopic, molecular~~Microscopic, Molecular detection, ~~mosquito~~Mosquito vectors, Lymphatic filariasis

INTRODUCTION

~~Foci of endemic areas of~~ The decline in the global prevalence of Lymphatic ~~Filariasis~~filariasis (LF) ~~remain~~is evident in ~~African~~the persistent endemic foci in Africa and Southeast ~~Asian countries even though global incidence has shown a drastic decline in the last two decades~~ Asia (NTD Collaborators, 2020). The prevalence of LF in Asia is ~~still~~ stable at 3%, and only four countries are free of ~~this~~the disease, namely China, Japan, Vietnam, and South Korea (Bizhani et al. 2021~~), even after~~). Despite six ~~periods~~rounds of mass ~~treatment~~drug administration (MDA~~)~~), the ~~stability~~persistence of microfilaria infections ~~this is still the case in~~continues, a situation exemplified by Myanmar (Dickson et al. 2018). This low decrease in LF prevalence after Preventive chemotherapy, Operations, Monitoring, and Participation (POMP) in endemic areas is reported to be due to a lack of community understanding and participation in ~~these~~the efforts (Aboagye et al. 2022). Other ~~studies~~research report that ~~LF~~the transmission is influenced by high mosquito vector density due to favorable rainfall, temperature, and air humidity (Sinha et al. 2023), resulting in the proportion of infectious ~~vector mosquitoes~~ vectors (Dharmarajan et al. 2019~~), and the bite rate. annual transmission of the parasite to humans~~) (Davis et al. 2019).

Data and information on competent vectors and the circulation of microfilariae in mosquitoes are ~~urgently~~ needed to complete ~~mass treatment (~~MDA~~)~~ in endemic areas by prioritizing vector mosquito control efforts. ~~Unfortunately~~However, this data and information is very limited in Indonesia, ~~especially~~specifically in Central Java. ~~This~~The condition requires further research to understand the interaction of pathogens with mosquitoes as input~~ for improving and strengthening~~. This strengthens methods and strategies for controlling LF vector mosquitoes (Famakinde 2018), including ~~for~~ developing predictive models of ~~the interaction of~~ risk factors for LF transmission and public health interventions (Zerbo et al. 2021). ~~The~~Furthermore, the results of entomological surveys in several countries report varying results. ~~A study~~Research in Mafia Island, Tanzania resulted in three mosquito species of LF vector~~ where the mosquito species order based on the proportion were~~, namely *Cx. quinquefasciatus*, *Anopheles gambia*e, and *An.* ~~*funestus*, and~~*Funestus*. In this context, 0.25% of the examined mosquitoes were infected with *W. bancrofti* (Derua et al. 2017). ~~Similar findings were reported from~~In Masasi District, Tanzania~~ where~~, the ~~study~~research found the same three species with a higher ~~of~~ *W. bancrofti* infection rate for *Cx. quinquefasciatus* (Lupenza et al. 2021). ~~Studies in Indonesia also show different findings. A study~~Research in Bogor Regency (West Java) reported five mosquito genera, namely *Culex*, *Mansonia*, *Aedes*, and *Armigeres*, ~~but there were no~~without detecting microfilariae ~~detected~~ (Nirwan et al. 2022). ~~A study~~Another research in Pekalongan Regency (Central Java) captured three mosquito species, namely *Cx. quinquefasciatus*, *Cx. vishnui*, and *Ae. aegypti*, and 0.43% of ~~mosquito~~ samples were positive for microfilaria~~.~~ (Nurjazuli et al. 2022). The results showed a variation in the ~~species of mosquito~~ vectors and the infection rates of microfilaria in different regions~~ so the local specific condition is important to be understood~~.

The burden of LF vector control efforts is compounded by the resistance of *Cx. quinquefasciatus*~~ mosquitoes~~ to insecticides as reported in Bengal, India (Rai et al. 2019) and Uganda (Silva-Martins et al. 2019). Resistance of *Cx. quinquefasciatus* mosquitoes to insecticide classes, deltamethrine 0.05% and temephos 0.75% occur widely in Sri Lanka accompanied by the L1014F knockdown (kdr) mutation (Chandrasiri et al. 2020). The L1014S and L1014F mutations were also ~~found~~reported in ~~the~~ *Cx. pipiens pallens* mosquito in China (Liu et al. 2019). ~~Research in Indonesia also reported resistance~~Resistance to *Cx. quinquefasciatus* ~~mosquitoes~~was also reported from Grobogan and Pekalongan in Central Java to 0.75% permethrin (Chakim et al. 2017) based on bioassay tests~~, however detection of VGSC gene mutations related to kdr resistance has not been found. This study aims~~. Therefore, this research aimed to determine the biological characteristics, insecticide resistance, and the presence of microfilariae in mosquito vectors in LF endemic areas of Pekalongan City, Central Java Province, Indonesia.

MATERIAL AND METHODS

~~Study~~Research sites

Jenggot and Medono subdistricts were ~~chosen~~selected as research locations based on filariasis case surveillance data from the Pekalongan City Health Service in 2020 (**Fig.1**). Furthermore, Jenggot subdistrict ~~has~~possessed the highest microfilaria rate (5.4%) ~~among the eleven LF endemic subdistricts~~ in Pekalongan City and ~~every time a finger blood survey is carried out, sufferers are always found. new. There were~~ five ~~new~~ cases were detected in 2020. ~~Previously,~~ Medono Village was not an endemic area for LF, but two new filariasis sufferers were found in a finger blood survey in 2020. This cross-sectional ~~study involved~~research included six cases ~~(four in Jenggot and two in Medono)~~ and 10 houses around the case within a radius of 100m.

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Ethical Clearance, Data Collection and Analysis

Data collection was conducted after the ethical review was ~~done~~achieved. This research has received a letter of recommendation with the issuance of Ethical Clearance number: 419/EA/KEPK-FKM/2021 from the health ~~research~~ ethics committee KEPK FKM Diponegoro University Semarang. ~~Twice~~Furthermore, twice entomological and household survey was conducted in Jenggot and Medono villages. The data collected ~~in this study~~ was analyzed ~~descriptively~~ to describe each research variable in the form of tables, pictures, and maps.

Mosquito catching procedures

Mosquito catching is carried out at night from 18.00-06.00 the following day according to standard procedures (WHO 2013~~) in two stages, each~~). The stage was carried out by 6 people ~~who have been~~ trained using the Human Landing Catch (HLC) and Resting Collection (RC) methods ~~(RC).~~. The team was divided into two to catch mosquitoes inside and outside the house for 40 ~~minutes,~~ and 10 minutes to change the paper cup ~~for the mosquitoes, as well as time to rest.~~. Every 1 hour, ~~caught~~the mosquitoes are put in a paper cup with a code and time of capture. RC is carried out from 06.00 – 07.30~~,~~ to catch ~~mosquitoes that are~~samples resting on walls, window curtains, mosquito nets, and hanging clothes, using an aspirator and net. ~~All~~The mosquitoes ~~that are~~ caught are put into a paper cup ~~that is~~ filled with cotton filled with sugar solution. The paper cup is placed in a container covered with banana stems and ~~covered with~~ a wet towel to maintain optimum temperature and humidity of 27±2 ^{0}C and 80±10 ~~%~~%, respectively. The captured mosquito samples were held for twelve days and fed a 10% sugar solution before examination.

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Identification of mosquito species and ovary dilatation

~~Identify mosquito~~ Mosquito species were identified using previously published identification keys (Rattanarithikul et al. 2005, Nugroho et al. 2021). ~~Mosquito~~The parosity was determined using a surgical method to determine the amount of ovarian dilatation. The ~~mosquito that has been~~ identified mosquito is ~~placed in~~carefully positioned within a petri dish, where the wings and legs are separated from the body~~, then the~~. The specimen is ~~placed on~~delicately positioned onto a glass ~~object and~~ apparatus, where physiological NaCl solution is ~~dropped. After that~~precisely administered. Additionally, surgery is performed using a surgical needle~~. Surgery is~~ and carried out ~~microscopically~~ using a stereo microscope. The surgical needle in the left ~~hand~~and right hands presses the chest and the ~~surgical needle in the right hand presses the~~ 7th segment ~~and is then moved slowly to the right until the abdominal contents and ovaries are pulled out.~~. The ovaries were placed on a glass object ~~that had just been~~ given distilled water to view the tracheoles using a stereo microscope with a magnification of 40x10.

Microfilaria detection

The wings of the mosquito ~~to be dissected are cut so that the scales do not contaminate~~slated for dissection were incised with precision to prevent the dispersion of scales, ensuring the integrity of the microscope's field of view. Mosquitoes were placed on glass slides dripped with physiological saline. The ~~mosquito's~~ thorax and abdomen were cut into pieces with a dissecting needle and observed under a microscope at 40X magnification. ~~If there are~~ Meanwhile, the presence of filarial worms~~, they will appear~~ appears to move depending on ~~their~~the stage. ~~Stages~~ In this context, stages 1-2 are short, fat, and slow-moving, while stage 3 ~~(infective)~~ appears long and fast-moving (Laney et al. 2010). ~~Apart from surgery,~~

~~microfilaria~~Microfilaria detection also uses molecular methods with the stages of DNA extraction,~~ DNA~~ amplification, electrophoresis, and imaging.

DNA Extraction.

~~Female~~ A total of 10-20 female mosquitoes were pooled based on species~~ as many as 10-20 mosquitoes~~. Each pool was homogenized with a pestle in a microcentrifuge tube containing 180 µl of ATL buffer (pH 7.2; PBS) and ground, 20 µl of proteinase K, vortexed, and incubated for 24 hours. In the ~~next~~subsequent step, the specimen was vortexed for 15 seconds, and ~~then~~ 200 µl AL buffer was added~~, and vortexed again.~~. A total of 600 µl of extract samples were ~~taken~~ put into a mini-column and centrifuged for 1 minute at a speed of 8000 rpm. ~~Add~~Approximately 500 µl of Aw1 was added to the specimen, ~~centrifuge again~~and centrifuged for 1 minute, ~~add~~before adding 500 µl of AW2 buffer~~,~~ and ~~centrifuge~~centrifuged at 14,000 rpm for 3 minutes. The sample was transferred to a 1.5 ml tube and 60 µl AE buffer was added, incubated for 1 minute, and centrifuged at 8000 rpm for 1 minute.

DNA Amplification with Polymerase-Chained Reaction

The extracted mosquito DNA was amplified using a thermal cycler (Perkin-Elmer Cetus, Norwalk, Connecticut, USA) with 2 oligonucleotide primers, NV-l: 5' CGTGATGGCATCAAAGTAGCG 3' (21-mer) and NV-2: 5'CCCTCACTTACCATAAGACAAC 3' (22- mer). Each amplification reaction was carried out in a final volume of 50 and contained 10 ~ I-IMT ri-HCl pH 9.2, 1.5 mM MgCl, 75 mM KCl, 1.25 mM each deoxy-nucleotide triphosphate, 10 pmol each primer NV-l and NV -2, and 2 units of Taq polymerase. ~~The~~Furthermore, the temperature program for PCR was 5 minutes at 95 ^{0}C, followed by 35 cycles of 1 minute each at 94 ^{0}C, 55 ^{0}C, and 72 0C, and an elongation of 10 minutes at 72 ^{0}C. A total of 20% of the PCR product from each sample was electrophoresed on a 2% agarose gel and stained with ethidium bromide to confirm amplification (Ramzy et al. 1997).

RESULTS AND DISCUSSION

~~In total, a~~A total of 1,678 mosquitoes were obtained in two capture periods, namely 1,197 and 581~~ respectively~~, with a proportion of female sex and parity of 66.25 and 86.00% ~~and~~as well as 63.37 and 70.95~~%, respectively.~~%. The majority (61.49 and 65.72%) of mosquitoes showed ≥ 4 ovarian dilatations or a lifespan of 12-16 days, which is an estimate based on the understanding that ~~one~~a gonotropic cycle is approximately 3-4 days (Fereda 2022). This ~~study~~research applied the mosquito-catching method with HLC and Resting Catch ~~and obtained~~to obtain a high composition of female mosquitoes and parosity. Gravid female mosquitoes suck blood to meet the protein needs for the development of embryos in eggs. ~~Blood~~In tropical regions, blood-feeding behavior ~~among mosquitoes in tropical regions occurs throughout the~~ persists year~~,~~ ~~whereas~~ round, but in temperate regions, gravid mosquitoes are breeded only ~~appear in~~during the spring season (Siperstein et al. 2023). ~~This fact shows that~~Therefore, air temperature influences mosquitoes in ~~their~~the mating and pregnancy behavior. A total of five ~~mosquito~~ species were found in the first fishing period, and only three ~~mosquito species~~ were ~~found~~reported in the second period with the proportion ~~for each species~~ being *Cx. quinquefasciatus* (69.59 and 65.40%), *Cx. tritaeniorhyncus* (5.76 and 0.00%), Cx. vishnui (5.85 and 27.54%), *An. Vagus* (0.58 and 0.00%), and *Ae. aegypti* (18.21 and 7.06%). The proportion of female mosquitoes in the first and second captures based on species is *Cx. quinquefasciatus* (60.78 and 59.78%), *Cx. tritaeniorhyncus* (8.70 and 0.00%), *Cx. vishnui* (8.83 and 32.06%), *An. Vagus* (0.88 and 0.00%), and *Ae. aegypti* (20.81 and 8.22%) (**Tables 1 and 3**). These data ~~indicate~~show that *Cx quinquefasciatus* is the dominant ~~mosquito~~ species in ~~both study~~the locations, while *Cx. tritaeniorhyncus* and *An.* ~~vagus was not~~*Vagus* are found on the second arrest. The dominance of *Cx. quinquefasciatus* in LF endemic areas was also reported in several ~~studies~~research such as in Tanzania (Derua et al. 2017, Lupenza et al. 2021), and Gamapaha, Sri Lanka (Pilagolla and Amarasinghe 2021). Similar findings were ~~also~~ reported~~ from several studies in Indonesia, such as~~ in Bogor Regency, West Java (Nirwan et al. 2022), Pekalongan Regency, Central Java (Nurjazuli et al. 2022), and Tangerang (Prasetyowati et al. 2019). However, several ~~studies~~research report different dominant vector species of LF in endemic areas such as *Armigeres subalbatus* in Musi Rawas, South Sumatra (Mulyaningsih et al. 2019), *Ae. scutellaris* and *Ae. kochi* in the South Pacific~~ while~~, as well as *An. gambiae*, *A. funestus*, and *An. punctulatus* in rural Asia and Africa (Bhuvaneswari et al. 2023). Differences in species dominance are influenced by habitat conditions, ~~especially~~specifically the presence of aquatic plants (Pratiwi et al. 2019), chloride content, and water temperature (Amini et al. 2020).

The results of catching mosquitoes showed different results, ~~wherein~~where the first period ~~they~~ produced greater numbers. This ~~indicates~~shows that mosquito abundance varies over time~~. However, mosquito abundance did~~ but does not correlate with microfilaria findings. The surgery and PCR examination results of mosquito samples from the first capture period did not ~~reveal~~report microfilariae (**Table 2**). In~~ contrast, in~~ the second period of capture, fewer numbers and species ~~of mosquitoes~~ were obtained, namely 581 mosquitoes ~~from three species~~ (**Table 3**~~), but the~~). The results of dissection~~ of female mosquitoes~~ after holding for 12 days found that 4 out of 298 (1.34%) mosquitoes contained L3 microfilariae, with species *W. bancrofti* (**Fig.** 2). ~~All~~The microfilariae were detected from mosquito samples aged 12-20 days (ovarian dilatation 4 and 5) with proportions of 25% and 75%. ~~This fact shows that~~Therefore, microfilariae are found in mosquitoes ~~that have gone through~~subjected to 4-5 periods of sucking blood. Detection of microfilariae in mosquito vectors in Indonesia is still

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limited. ~~This study adds to the fact that there~~There is still an infectious vector of LF in Pekalongan City, Central Java~~, Indonesia~~, even with a higher percentage compared to findings in other areas (Derua et al. 2017, Lupenza et al. 2021~~), including in nearby areas~~) (Nujazuli et al. 2022). ~~This finding confirms that this~~In addition, the area still has a high potential for LF transmission with the main vector being the *Cx. quinquefasciatus*. Apart from adding data on the number of microfilaria infections in mosquito vectors, ~~these~~the findings also confirm the role of *Cx. quinquefascitus* as the main vector of LF in Pekalongan. *W. bancrofti* is the microfilariae most frequently found in mosquito vectors (Kinyatta et al 2023), ~~especially~~specifically *Cx. quinquefasciatus*. ~~This~~The finding ~~is~~ also ~~following~~follows similar reports in Tanzania where ~~this~~the species is ~~also~~ the main vector of LF ~~although with a much smaller infection rate~~ (Lupenza et al. 2021). The variation in species and proportion of mosquitoes ~~found in this study~~ is also similar to other ~~reported studies~~reports where *Cx. quinquefasciatus* is the dominant species in LF endemic areas (Derua et al. 2017, Lupenza et al. 2021). In the local context, this ~~study~~research found more mosquito species than previous findings at other nearby locations ~~where only three mosquito species were found, namely *Cx. quinquefasciatus*, *Cx. vishnui*, and *Ae. aegypti*, although the proportion is not stated~~ (Nurjazuli et al. 2022). ~~-~~The findings ~~of this study illustrate~~show the high potential for LF transmission in the region. ~~This is supported by the fact that the~~The majority of *Cx. quinquefasciatus* is a female with a high proportion of parity and an estimated lifespan of more than 12 days. These findings ~~indicate~~show a higher proportion of mosquitoes with ovarian dilatations ≥ 4 than the number ~~of dilatations below. This~~ . The data ~~illustrates~~reports that the majority of female Culex mosquitoes ~~in this population~~ have a high potential to become competent vectors ~~if~~when in the first or second gonotrophic cycle ~~they succeed in obtaining~~to obtain microfilariae from the blood of parasitemia sufferers. ~~This possibility~~The probability is ~~strengthened~~supported by the ~~fact~~observation that all L3 microfilariae ~~identified in this study came~~originated from mosquito samples ~~with~~showing an ovarian dilation range of 4-5 times or 12-20 days old. ~~This finding follows other reports which state that the~~ This discovery is consistent with previous findings showing an incubation period of 13 days for extrinsic microfilariae ~~in~~within the ~~mosquito's~~ body to ~~reach~~transition to the L3 stage and ~~enter~~migrate to the salivary glands ~~takes 13 days~~ (Xu et al. 2018, Dharmarajan et al. 2019). The potential for LF transmission is increasing ~~along~~ with climate change, where ~~increasing~~ the average daily air temperature ~~can shorten~~shortens the extrinsic incubation period for microfilariae (Simon et al. 2017).

~~Results~~The results of nucleotide substitution analysis ~~of~~conducted on the ace1 and *Cx. quinquefasciatus* VGSC genes serve as ~~an indication of~~indicative markers for assessing the insecticide resistance status ~~of~~within the mosquito population. Part (A) in **Figure 3** shows the results of nucleotide ~~alignment~~consistency at codon 119 of the ace1 gene where ~~all~~ DNA samples show the same wildtype base arrangement, namely GGC (Valine amino acid). ~~Part~~Meanwhile, part (B) is a chromatogram ~~display~~analysis of the base arrangement. Part (C) ~~shows~~is the results of ~~aligning~~consistency in the nucleotide sequence in codon 1014 of the VGSC gene where there are three forms of nucleotide substitution~~, namely~~ from T to C ~~in the first base~~, T to G, and A to T in the first, second base, and ~~A to T in the~~ third ~~base~~bases, respectively. There are three nucleotide substitution variations in codon 1014 of the VGSC gene of *Cx. quinquefasciatus* from the formation of wild-type leucine (TTA) to phenylalanine (TTT) and Cysteine (TGT), ~~and one~~as well as a silent mutation from TTA to CTA (**Table 4**). The first change is a form of silent mutation where ~~the base substitution (~~TTA to CTA~~)~~ does not change the amino acid, namely Leucine. The second change results in an amino acid change from Leucine (TTA) to Cysteine (TGT) ~~and~~while the third ~~change results in~~leads to a change in ~~the amino acid~~ Phenylalanine (TTT) (**Fig. 2**). The L1014F and L1014C mutations (**Fig. 4**) show homozygous and heterozygous forms. Parts A and B are homozygous and heterozygous forms of changing ~~the amino acid~~ Leucine (TTA) to Phenylalanine (TTT) and Cysteine (TGT), while C and D are homozygous and heterozygous forms of changing ~~the amino acid~~ Leucine (TTA) to Cysteine (TGT). Image B is a heterozygous form where ~~the~~ T and G bases are at the same locus~~, although~~ but T is more dominant. The ~~opposite condition occurs in image D. The overall results of~~ mutation analysis of ~~the~~ VGSC codon 1014 gene ~~show~~shows that the proportion of mosquitoes resistant to insecticides in ~~this~~the population is 98-~~-~~100%. ~~This finding is supported by the fact that the activity of using~~ The utilization of insecticides ~~by the community is also very high~~remains prevalent, with ~~a range of~~rates ranging from 85-% to 92.5%, ~~even~~persisting at 63.3-% to 65% for ~~more than~~durations exceeding two years~~, with a~~. Moreover, daily usage intensity ~~of~~ranges from 40-% to 53.3%, ~~especially~~with particular emphasis on the pyrethroid group ~~(~~at 80-% to 81.7~~%) and 58-60% in~~% as well as burnt coil formulations, which account for 58% to 60% of usage (**Table 5**). Reports of resistance to Culex mosquito species, ~~especially~~specifically *Cx. quinquefasciatus* ~~in Indonesia~~ is still limited. This finding complements previous data where *Cx. quinquefasciatus* in Central Java~~, Indonesia~~ has been resistant to pyrethroid insecticides (Chakim et al. 2017~~), but a study~~). However, research in Jakarta reported that ~~this~~the species ~~is~~was still susceptible to pyrethroids and organophosphates (Subahar et al. 2022). ~~Recent~~According to recent data ~~show that~~, *Cx. quinquefasciatus* in Surabaya has been resistant to both groups of insecticides~~, and it has even been proven that there are~~ with mutations in the VGSC and ace-1 genes (Panjinegara et al. 2022). ~~This condition proves that~~Therefore, Culex mosquito resistance to insecticides has become increasingly widespread. The phenomenon of expanding the resistance area of *Cx* ~~mosquitoes.~~ *quinquefasciatus* was also reported in Bengal, India (Anju-Viswan et al. 2020, Rai et al. 2019~~).~~), Cameroon (Talipouo et al. 2021), Brazil (Lopes et al. 2019), Nigeria (Omotayo et al. 2023), and Korea (Jeon et al. 2024). Molecular analysis of mosquito samples ~~only~~ showed four genotypes of the VGSC gene, namely wild type, silent mutation, and mutant (Leu-Phe and Leu-Cis). The Leu-Phe mutation is the most frequently reported mutant form in Indonesia (Panjinegara et al. 2024) and other countries (Talipouo et al. 2021). ~~This study did not find any mutations in the ace-1 gene.~~ This is different from the findings in Surabaya which ~~found~~reports wildtype, mutant-heterozygous, and mutant-homozygous genetic variations (Panjinegara et al. 2024). A ~~more~~

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serious genetic mutation ~~in this species~~ was reported ~~from~~in Brazil ~~involving~~including esterase, ace-1, and VGSC (Lopes et al. 2019). ~~Studies~~Research in Korea ~~also~~ did not ~~find~~obtain the G119S mutation but ~~did find~~reported heterozygous forms of AGC/GGC, L1014F, and L1014S (Jeon et al. 2024). This genetic mutation phenomenon ~~indicates~~shows a serious problem ~~that can hamper~~affecting vector control efforts in LF-endemic areas~~, so the search for other effective methods is necessary.~~
.

In conclusion, *Cx. quinquefasciatus* ~~is~~was the dominant species among the five vector ~~mosquito species~~mosquitos in the LF endemic area of Pekalongan Regency, and 1.34% ~~of them~~ were proven to carry *W. bancrofti* microfilariae. ~~This fact shows that~~Therefore, the area ~~is still~~was vulnerable to LF transmission~~. This~~ and the condition ~~is exacerbated~~was increased by *Cx. quinquefasciatus* resistance~~, both~~ against pyrethroid and organophosphate class insecticides. Molecular analysis of the VGSC gene found the wild-type allele and three mutant alleles, namely ~~silent mutation (~~TTA to CTA~~)~~,, TTA to TTT ~~mutation~~, and TTA to TGT mutations. Further investigation ~~is needed~~should be conducted to detect the susceptibility of microfilariae to various antiparasitic drugs and educate the public ~~to implement~~in implementing methods of self-protection from exposure to mosquitoes accompanied by environmental cleaning movements~~ to eradicate the habitat of LF vector mosquitoes~~.

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CONFLICT OF INTEREST

~~All of the~~The authors state that there was no conflict of interest.

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Figure 1. The Map of ~~Study~~Research Sites

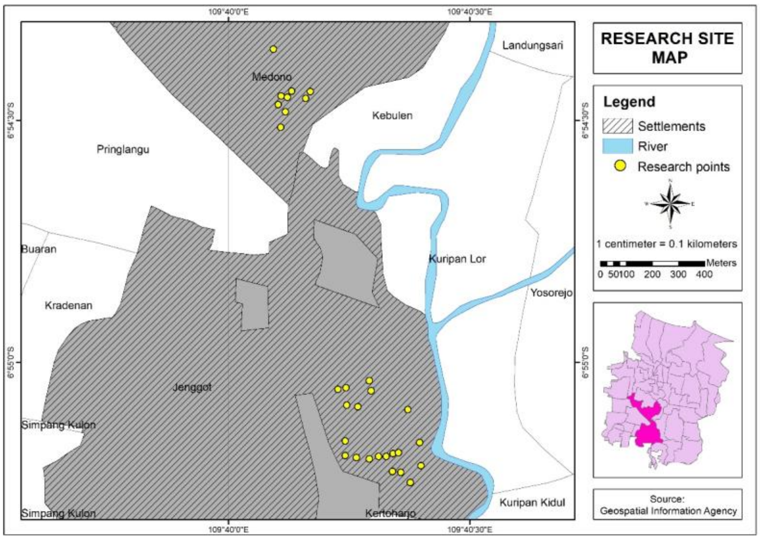

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Table 1. Distribution of Mosquitos Based on Characteristics and ~~Study~~Research Sites (1st Period of Mosquito Capture)

No.	Variables	~~Study~~Research Sites			
		Medono		Jenggot	
		n	%	n	%
1	Mosquito species and sex				
	Cx. quinquefasciatus	**280**	**33.61**	**553**	**67.39**
	Male	124	44.39	227	41.09
	Female	156	55.61	326	58.91
	Cx. tritaeniorhyncus	**31**	**44.93**	**38**	**55.07**
	Male	0	0.00	0	0.00
	Female	31	100.00	38	100.00
	Cx. vishnui	**29**	**41.43**	**41**	**58.57**
	Male	0	0.00	0	0.00
	Female	29	100.00	41	100.00
	An. vagus	**0**	**0.00**	**7**	**100.00**
	Male	0	0.00	0	0.00
	Female	0	0.00	7	100.00
	Ae. aegypti	**83**	**38.07**	**135**	**61.93**
	Male	17	20.62	36	26.67
	Female	66	79.38	99	73.33
2	**Parity**	**103**	**33.99**	**200**	**66.01**
	Nulliparous	23	22.33	88	44.00
	Parous	80	77.67	112	56.00
3	**Number of Ovary Dilatation (age in days)**	**80**	**41.67**	**112**	**58.33**
	1 (4)	0	0.00	8	7.14
	2 (8)	5	6.25	14	12.50
	3 (12)	18	22.50	29	25.89
	4 (16)	14	20.48	7	6.25
	5 (20)	18	22.50	10	8.93
	6 (24)	6	7.50	26	23.21
	7 (28)	19	23.75	18	16.07

Table 2. Results of Microscopic Microfilariae Detection of Female Mosquitoes (1st Period of Mosquito Capture)

No.	Species	~~Study~~Research Sites			
		Medono		Jenggot	
		n	%	n	%
1	***Cx. quinquefasciatus***	**50**	**50.00**	**50**	**50.00**
	Positive	0	0.00	0	0.00
	Negative	50	100.00	50	100.00
2	***Cx. tritaeniorhyncus***	**10**	**50.00**	**10**	**50.00**
	Positive	0	0.00	0	0.00
	Negative	10	100.00	10	100.00
3	***Cx. vishnui***	**10**	**50.00**	**10**	**50.00**
	Positive	0	0.00	0	0.00
	Negative	10	100.00	10	100.00
4	***An. vagus***	**-**	**-**	**7**	**100.00**
	Positive	-	-	0	00.00
	Negative	-	-	7	100.00
5	***Ae. aegypti***	**10**	**50.00**	**10**	**50.00**
	Positive	0	0.00	0	00.00
	Negative	10	100.00	10	100.00

Figure 2. *Wuchereria bancrofti* larvae were detected from four mosquito samples of *Cx quinquefasciatus* collected from Jenggot village, Pekalongan City.

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Table 3. Distribution of Mosquito Characteristics from Jenggot Village (2nd Period of Mosquito Capture)

No.	Characteristics of Mosquitoes	n	%
1	Mosquito species and sex		
	Cx. quinquefasciatus	**380**	**65.40**
	Male	82	21.58
	Female	298	78.42
	Cx. vishnui	**160**	**27.54**
	Male	0	0.00
	Female	160	100.00
	Ae. aegypti	**41**	**7.06**
	Male	0	0.00
	Female	41	100.00
	Total	**581**	**100.00**
2	**Parity**		
	Nulliparous	86	29.05
	Parous	210	70.95
	Total	**296**	**33.99**
3	**Number of Ovary Dilatation (Age of Mosquito; days)**		
	1 (4)	7	3.33
	2 (8)	18	8.57
	3 (12)	47	22.38
	4 (16)	43	20.48
	5 (20)	47	22.38
	6 (24)	23	10/95
	7 (28)	25	11.90
	Total	**210**	**100.00**
4	**Microscopic detection of microfilariae in female mosquitoes**		
	Cx. quinquefasciatus	**298**	**59.72**
	Positive	4	1.34
	Negative	294	98.66
	Cx. vishnui	**160**	**32.06**
	Positive	0	0.00
	Negative	160	100.00
	Ae. aegypti	**41**	**8.22**
	Positive	0	0.00
	Negative	41	100.00
	Total	**499**	**100.00**

Table 4. Frequency of VGSC gene mutations among *Cx. quinquefasciatus* mosquito

No.	Nucleotide Substitution	Type of Mutation			
		Homozygous	%	Heterozygous	%
1	TTA to TTT	33	55.00	16	26.67
2	TTA to CTA	1	1.67	0	0.00
3	TTA to TGT	4	6.67	6	10.00

Table 5. The Insecticide Resistance Status and History of Insecticide Use in ~~Study~~Research Sites

No	Variabel	~~Study~~Research Sites			
		Medono		Jenggot	
		n	%	n	%
1	**Resistance Status**				
	Sensitive	0	0	1	2
	Resistance	20	100	59	98
2	**The Use of Household Insecticide**				
	Yes	37	92.50	51	85
	No	3	7.50	9	15
3.	**Insecticide Group**				
	Organophosphate	8	20	11	18.33
	Pyrethroid	32	80	49	81.67
4.	**History of Insecticide Use (years)**				
	< 2	14	35	22	36.67
	≥ 2	26	65	38	63.33
5.	**Insecticide Use Intensity**				
	Not everyday	24	60	28	46.67
	Everyday	16	40	32	53.33
6.	**Insecticide Formulation**				
	Coil	24	60	35	58.33
	Spray	6	15	10	16.67
	Electric	2	5	4	6.67
	Repellent	8	20	11	18.33

Figure 3. Molecular analysis of codon 119 of ace-1 gene of *Cx. quinquefasciatus*

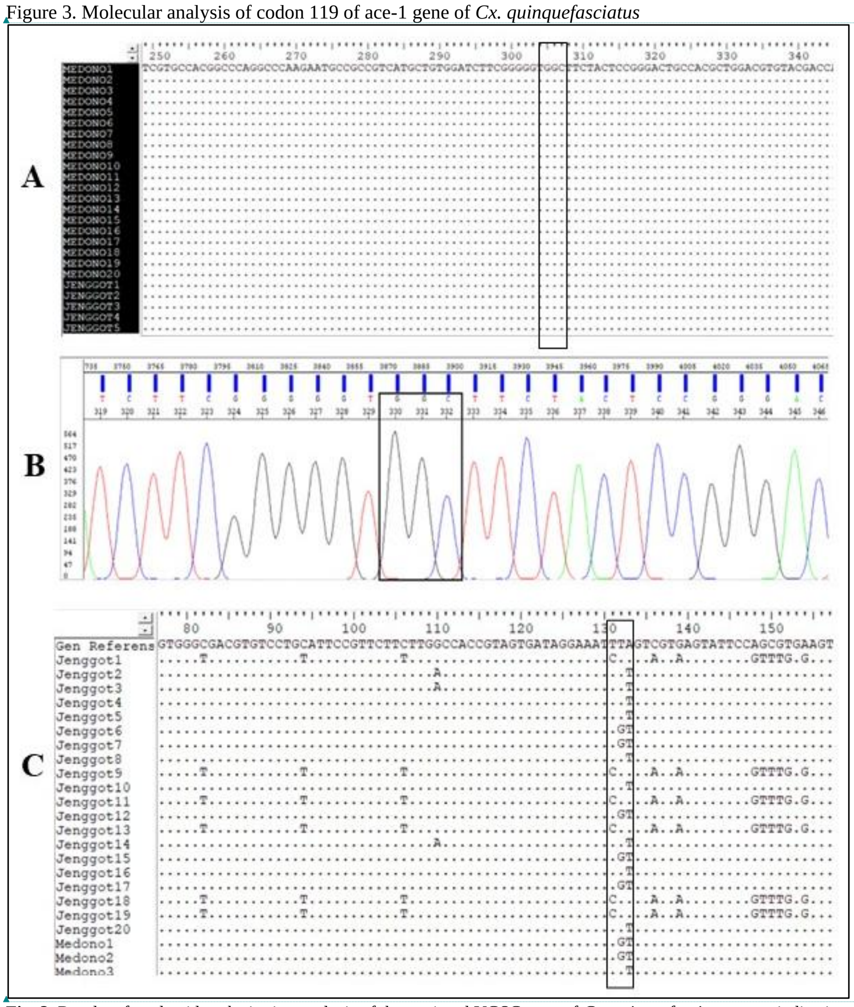

Fig. 2. Results of nucleotide substitution analysis of the ace1 and VGSC gene of *Cx. quinquefasciatus* as an indication of the insecticide resistance status of the mosquito population. No nucleotide substitutions were found in codon 119 of the ace1 gene (A) and the chromatogram shape of codon 119 (B). Part C is a variation of nucleotide substitution in codon 1014 of the VGSC gene which shows three variant changes, namely TTA-CTA (silent mutation), TTA-TTT, and TTA-TGT.

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Figure 4. Homozygous and heterozygous nucleotide substitution in codon 1014 VGSC gene of *Cx. quinquefasciatus*

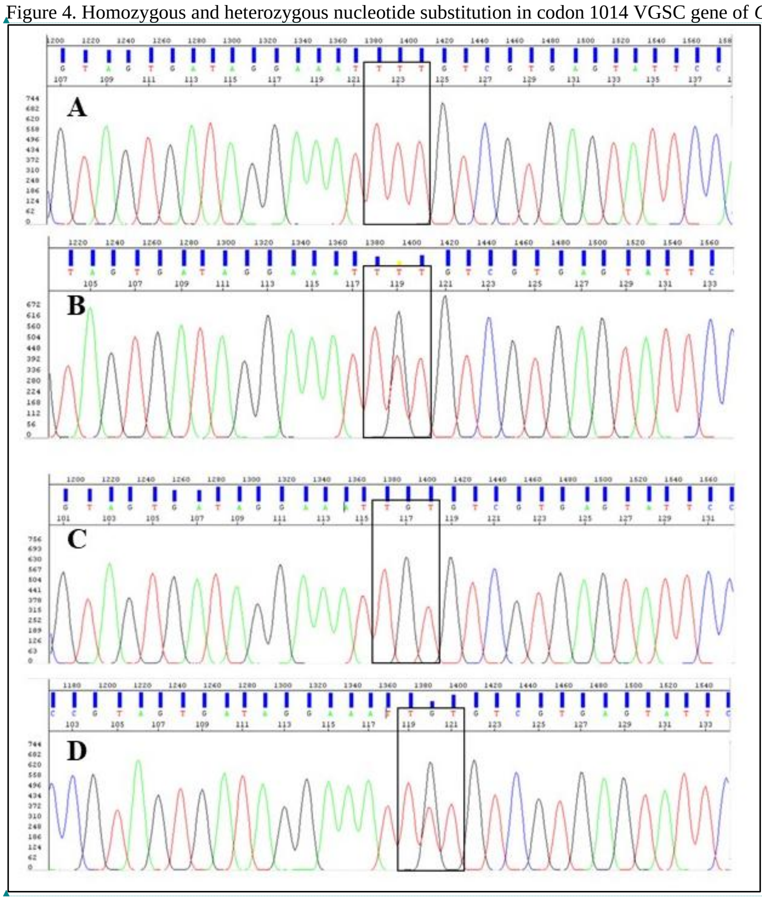

Fig.4. Homozygous and heterozygous nucleotide substitution in codon 1014 of VGSC gene of *Cx. quinquefasciatus*. A and B are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Phenylalanine (TTT) and Cysteine (TGT), while C and D are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Cysteine (TGT). Image B is a heterozygous form where the bases T and G are at the same locus, although T is more dominant. The opposite condition occurs in image D.

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[biodiv] Editor Decision
1 pesan

Smujo Editors via SMUJO <support@smujo.com> 18 Maret 2024 pukul 10.25
Balas Ke: Smujo Editors <editors@smujo.id>
Kepada: Sayono Sayono <say.epid@gmail.com>, Abdul Ghofur <omopung@gmail.com>, Suharyo Hadisaputro <prof_haryo@yahoo.co.id>

Sayono Sayono, Abdul Ghofur, Suharyo Hadisaputro:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Microscopic and molecular detection of microfilaria L3 and insecticide resistance among wild-caught mosquito vectors in endemic areas of Lymphatic filariasis". Complete your revision with a Table of Responses containing your answers to reviewer comments (for multiple comments) or enable Track Changes.

Our decision is: Revisions Required

Reviewer A:

Dear Author(s),

Introduction is about 600-700 words, covering the aims of the research and provide an adequate background, avoiding a detailed literature survey or a summary of the results. Please add some references that support your research background.

Thank you

Recommendation: Revisions Required

Biodiversitas Journal of Biological Diversity

Author's response

Corrected aspects of manuscript number 17500

No.	Aspects	Revised-versions
1	Margins of paper	Top 3cm, bottom 2cm, left & right 1.8cm
2	Title page	The first page contains the title, authors, affiliation, and indication of the corresponding author.
3	Font size	Times New Roman 10 pt, and smaller in tables
4	Conclusion	Embedded at the end of the Result and Discussion Section
5	Table and figure	Resized and placed in 4 pages at the end of the manuscript; font 9 pt size in tables
6	DOI in each reference	DOI is added to each reference; if available. If there is none, replace the article URL

Semarang, March 16th 2024
Corresponding Author

S. Sayono

Revised-manuscript

Detection of microfilaria L3 and insecticide resistance among wild-caught mosquito vectors in endemic areas of lymphatic filariasis

ABSTRACT

The decline in the global prevalence of Lymphatic filariasis (LF) is evident in the persistent endemic foci in Africa and Southeast Asia. In this context, the presence of infectious and insecticide-resistant mosquitoes and the annual biting rate in the area are key to microfilaria transmission. Therefore, this research aimed to determine insecticide resistance and microfilariae infection in mosquito vectors in endemic areas. In Jenggot and Medono villages, twice entomological surveys were conducted based on six LF cases, where indoor and outdoor mosquito capturing was carried out in ten houses within a 100m radius of each case as well as household interviews about insecticide use. In addition, laboratory works were performed for species identification and detection of ovarian dilatation, microfilariae infection, and knockdown resistance mutations. A total of 1,197 and 581 mosquitoes were distributed to five species and their proportions, namely *Culex quinquefasciatus* (69.59 and 65.40%), *Cx. tritaeniorhyncus* (5.76 and 0.00%), *Cx. vishnui* (5.85 and 27.54%), *Anopheles vagus* (0.58 and 0.00%), and *Aedes aegypti* (18.21 and 7.06%). The percentage of female mosquito infection, parity, and microfilaria were 66.25 and 86.00%, 63.37 and 70.95%, as well as 0.00 and 1.34%, [JB1][A2]respectively. Furthermore, TTA-TTT, TTA-CTA, and TTA-TGT base substitutions were reported in codon 1014 of *Cx. q*~~Q~~*uinquefasciatus* VGSC gene with proportions of 81.66%, 1.67%, and 26.67%, respectively. The result showed that the vulnerability of the research location to transmission emphasized the necessity for early detection, vector control, and further analyses of the susceptibility of microfilariae to antiparasitic drugs.

Keywords: ~~Microfilaria~~Culex quinquefasciatus, Microscopic, Molecular detection, Mosquito vectors, ~~Lymphatic filariasis~~Wuchereria bancrofti
[JB3][A4]

INTRODUCTION

The decline in the global prevalence of Lymphatic filariasis (LF) is evident in the persistent endemic foci in Africa and Southeast Asia (NTD Collaborators~~,~~ 2020[JB5]). The prevalence of LF in Asia is stable at 3%, and only four countries are free of the disease, namely China, Japan, Vietnam, and South Korea (Bizhani et al. 2021). Despite six rounds of mass drug administration (MDA), the persistence of microfilaria infections continues, a situation exemplified by Myanmar (Dickson et al. 2018). This low decrease in LF prevalence after Preventive chemotherapy, Operations, Monitoring, and Participation (POMP) in endemic areas is reported to be due to a lack of community understanding and participation in the efforts (Aboagye et al. 2022). ~~Other research report~~Other research reports that the transmission is influenced by high mosquito vector density due to favorable rainfall, temperature, and air humidity (Sinha et al. 2023), resulting in the proportion of infectious vectors (Dharmarajan et al. 2019; ~~) (~~[JB6]Davis et al. 2019).

Data and information on competent vectors and the circulation of microfilariae in mosquitoes are needed to complete MDA in endemic areas by prioritizing vector mosquito control efforts. However, this data and information is very limited in Indonesia, specifically in Central Java. The condition requires further research to understand the interaction of pathogens with mosquitoes as input. This strengthens methods and strategies for controlling LF vector mosquitoes (Famakinde 2018), including developing predictive models of risk factors for LF transmission and public health interventions (Zerbo et al. 2021). Furthermore, the results of entomological surveys in several countries report varying results. Research in Mafia[JB7] Island, Tanzania resulted in three mosquito species of LF vector, namely *Cx. quinquefasciatus*, *Anopheles gambia*e, and *An.* ~~*F*~~*funestus*[JB8]~~. In this context,~~, and 0.25% of the examined mosquitoes were infected with *W. bancrofti* (Derua et al. 2017). In Masasi District, Tanzania, the research found the same three species with a higher *W. bancrofti* infection rate for *Cx. quinquefasciatus* (Lupenza et al. 2021). Research in Bogor Regency (West Java) reported five mosquito genera, namely *Culex*, *Mansonia*, *Aedes*, and *Armigeres*, without detecting microfilariae (Nirwan et al. 2022). Another research in Pekalongan Regency (Central Java) captured three mosquito species, namely *Cx. quinquefasciatus*, *Cx. vishnui*, and *Ae. aegypti*, and 0.43% of samples were positive for microfilaria (Nurjazuli et al. 2022). The results showed a variation in the vectors and the infection rates of microfilaria in different regions.

The burden of LF vector control efforts is compounded by the resistance of *Cx. quinquefasciatus* to insecticides as reported in Bengal, India (Rai et al. 2019) and Uganda (Silva-Martins et al. 2019). Resistance of *Cx. quinquefasciatus* mosquitoes to insecticide classes, deltamethrine 0.05% and temephos 0.75% occur widely in Sri Lanka accompanied by the L1014F knockdown (kdr) mutation (Chandrasiri et al. 2020). The L1014S and L1014F mutations were also reported in *Cx. pipiens pallens* mosquito in China (Liu et al. 2019). Resistance to *Cx. quinquefasciatus* was also reported from Grobogan and Pekalongan in Central Java to 0.75% permethrin (Chakim et al. 2017) based on bioassay tests. Therefore, this research aimed to determine the biological characteristics, insecticide resistance, and the presence of microfilariae in mosquito vectors in LF endemic areas of Pekalongan City, Central Java Province, Indonesia.

MATERIALs AND METHODS

~~Research~~ Study sites

As many as 20 sites in Jenggot and 9 sites in Medono ~~subdistricts~~ villages were selected as research locations[JB9] based on filariasis case surveillance data from the Pekalongan City Health Service in 2020 (**Fig.1**). Furthermore, Jenggot subdistrict possessed the highest microfilaria rate (5.4%) in Pekalongan City and five cases were detected in 2020. Medono Village was not an endemic area for LF, but two new filariasis sufferers were found in a finger blood survey in 2020[JB10] (Anonymus 2021). This cross-sectional research included six cases and 10 houses around the case within a radius of 100m.

Ethical Clearance, Data Collection and Analysis

Data collection was conducted after the ethical review was achieved. This research has received a letter of recommendation with the issuance of Ethical Clearance number: 419/EA/KEPK-FKM/2021 from the health ethics committee KEPK FKM Diponegoro University Semarang. Furthermore, twice entomological and household survey was conducted in Jenggot and Medono villages. The data collected were~~as~~[JB11] analyzed to describe each research variable in the form of tables, pictures, and maps.

Mosquito catching procedures

Mosquito catching wa~~i~~s[JB12] carried out at night from 18.00-06.00 at the house of the new LF case and 9-10 neighboring houses within a 100m radius ~~the following day~~ according to standard procedures (WHO 2013). ~~The stage was carried out by~~ As many as 6 people were trained to ~~us~~apply ~~ing~~ the Human Landing Catch (HLC) and Resting Collection (RC) [JB13]methods. In general, the HLC method involved the mosquito catcher carrying an aspirator and sitting at the capture location with one leg up to the calf open. Mosquitoes that land on their feet are sucked in using an aspirator. In contrast to HLC, the target of the RC method is mosquitoes that land on the walls of the house. The team was divided into two to catch mosquitoes inside and outside the house for 40 and 10 minutes to change the paper cup. Every 1 hour, the mosquitoes are put in a paper cup with a code and time of capture. RC wa~~i~~s[JB14] carried out from 06.00 – 07.30 to catch samples resting on walls, window curtains, mosquito nets, and hanging clothes, using an aspirator and net. The mosquitoes caught are put into a paper cup filled with cotton filled with sugar solution. The paper cup is placed in a container covered with banana stems and a wet towel to maintain optimum temperature and humidity of 27±2 ^{0}C and 80±10 %, respectively. The captured mosquito samples were held for twelve days and fed a 10% sugar solution before examination.

Identification of mosquito species and ovary dilatation

Mosquito species were identified using previously published identification keys (Rattanarithikul et al. 2005~~, –~~; Nug[JB15]roho et al. 2021). The parosity was determined using a surgical method to determine the amount of ovarian dilatation. The identified mosquito is carefully positioned within a petri dish, where the wings and legs are separated from the body. The specimen is delicately positioned onto a glass apparatus, where physiological NaCl solution is precisely administered. Additionally, surgery is performed using a surgical needle and carried out using a stereo microscope. The surgical needle in the left and right hands presses the chest and the 7th segment. The ovaries were placed on a glass object given distilled water to view the tracheoles using a stereo microscope with a magnification of 40x10.

Microfilaria detection

The wings of the mosquito slated for dissection were incised with precision to prevent the dispersion of scales, ensuring the integrity of the microscope's field of view. Mosquitoes were placed on glass slides dripped with physiological saline. The thorax and abdomen were cut into pieces with a dissecting needle and observed under a microscope at 40X magnification. Meanwhile, the presence of filarial worms appears to move depending on the stage. In this context, stages 1-2 are short, fat, and slow-moving, while stage 3 appears long and fast-moving (Laney et al. 2010). Microfilaria detection also uses molecular methods with the stages of DNA extraction, amplification, electrophoresis, and imaging.

DNA Extraction.

A total of 10-20 female mosquitoes from each location were pooled based on species[JB16]. Each pool was homogenized with a pestle in a microcentrifuge tube containing 180 µl of ATL buffer (pH 7.2; PBS) and ground, 20 µl of proteinase K, vortexed, and incubated for 24 hours. In the subsequent step, the specimen was vortexed for 15 seconds, and 200 µl AL buffer was added. A total of 600 µl of extract samples were put into a mini-column and centrifuged for 1 minute at a speed of 8000 rpm. Approximately 500 µl of Aw1 was added to the specimen, and centrifuged for 1 minute, before adding 500 µl of AW2 buffer and centrifuged at 14,000 rpm for 3 minutes. The sample was transferred to a 1.5 ml tube and 60 µl AE buffer was added, incubated for 1 minute, and centrifuged at 8000 rpm for 1 minute.

DNA Amplification with Polymerase-Chained Reaction

The extracted mosquito DNA was amplified using a thermal cycler (Perkin-Elmer Cetus, Norwalk, Connecticut, USA) with 2 oligonucleotide primers, NV-l: 5' CGTGATGGCATCAAAGTAGCG 3' (21-mer) and NV-2:

5'CCCTCACTTACCATAAGACAAC 3' (22- mer). Each amplification reaction was carried out in a final volume of 50 and contained 10 ~ I-IMT ri-HCl pH 9.2, 1.5 mM MgCl, 75 mM KCl, 1.25 mM each deoxy-nucleotide triphosphate, 10 pmol each primer NV-l and NV -2, and 2 units of Taq polymerase. Furthermore, the temperature program for PCR was 5 minutes at 95 ^{0}C, followed by 35 cycles of 1 minute each at 94 ^{0}C, 55 ^{0}C, and 72 0C, and an elongation of 10 minutes at 72 ^{0}C. A total of 20% of the PCR product from each sample was electrophoresed on a 2% agarose gel and stained with ethidium bromide to confirm amplification (Ramzy et al. 1997).

RESULTS AND DISCUSSION

A total of 1,678 mosquitoes were obtained in two cap[JB17]ture ~~periods~~shift, namely 1,197 and 581, with a proportion of female sex and parity of 66.25 and 86.00% as well as 63.37 and 70.95%. The majority (61.49 and 65.72%) of mosquitoes showed ≥ 4 ovarian dilatations or a lifespan of 12-16 days, which is an estimate based on the understanding that a gonotropic cycle is approximately 3-4 days (Fereda 2022). This research applied the mosquito-catching method with HLC and Resting Catch to obtain a high composition of female mosquitoes and parosity. Gravid female mosquitoes suck blood to meet the protein needs for the development of embryos in eggs. In tropical regions, blood-feeding behavior persists year-round, but in temperate regions, gravid mosquitoes are breeded only during the spring season (Siperstein et al. 2023). Therefore, air temperature influences mosquitoes in ~~the~~ their mating and pregnancy behavior. A total of five species were found in the first fishing period, and only three were reported in the second period with the proportion being *Cx. quinquefasciatus* (69.59 and 65.40%)[JB18], *Cx. tritaeniorhyncus* (5.76 and 0.00%), *Cx. vishnui* (5.85 and 27.54%), *An. v~~V~~agus* [JB19](0.58 and 0.00%), and *Ae. aegypti* (18.21 and 7.06%). The proportion of female mosquitoes in the first and second captures based on species is *Cx. quinquefasciatus* (60.78 and 59.78%), *Cx. tritaeniorhyncus* (8.70 and 0.00%), *Cx. vishnui* (8.83 and 32.06%), *An. Vagus* (0.88 and 0.00%), and *Ae. aegypti* (20.81 and 8.22%) (**Tables 1 and 3**). These data show that *Cx quinquefasciatus* is the dominant species in the locations, while *Cx. tritaeniorhyncus* and *An. ~~V~~vagus*[JB20] ~~are~~ was found on the second shift of mosquito catching~~arr~~[JB21]~~est~~. The dominance of *Cx. quinquefasciatus* in LF endemic areas was also reported in several research such as in Tanzania (Derua et al. 2017, Lupenza et al. 2021), and Gamapaha, Sri Lanka (Pilagolla and Amarasinghe 2021). Similar findings were reported in Bogor Regency, West Java (Nirwan et al. 2022), Pekalongan Regency, Central Java (Nurjazuli et al. 2022), and Tangerang (Prasetyowati et al. 2019). However, several research report different dominant vector species of LF in endemic areas such as *Armigeres subalbatus* in Musi Rawas, South Sumatra (Mulyaningsih et al. 2019), *Ae. scutellaris* and *Ae. kochi* in the South Pacific, as well as *An. gambiae*, *A. funestus*, and *An. punctulatus* in rural Asia and Africa (Bhuvaneswari et al. 2023). Differences in species dominance are influenced by habitat conditions, specifically the presence of aquatic plants (Pratiwi et al. 2019), chloride content, and water temperature (Amini et al. 2020).

The results of catching mosquitoes showed different results, where the first period produced greater numbers. This shows that mosquito abundance varies over time but does not correlate with microfilaria findings. The surgery and PCR examination results of mosquito samples from the first capture period did not report microfilariae (**Table 2**). In the second period of capture, fewer numbers and species were obtained, namely 581 mosquitoes (**Table 3**). The results of dissection after holding for 12 days found that 4 out of 298 (1.34%) mosquitoes contained L3 microfilariae, with species *W. bancrofti* (**Fig.** 2). The microfilariae were detected from mosquito samples aged 12-20 days (ovarian dilatation 4 and 5) with proportions of 25% and 75%. Therefore, microfilariae are found in mosquitoes subjected to 4-5 periods of sucking blood. Detection of microfilariae in mosquito vectors in Indonesia is still limited. There is still an infectious vector of LF in Pekalongan City, Central Java, even with a higher percentage compared to findings in other areas (Derua et al. 2017, Lupenza et al. 2021) (Nujazuli et al. 2022). In addition, the area still has a ~~high~~ pot[JB22]ential for LF transmission with the main vector being the *Cx. quinquefasciatus*. Apart from additional~~ng~~ data on the number of microfilaria infections in mosquito vectors, the findings also confirm the role of *Cx. quinquefascitus* as the ~~main~~dominance[JB23] vector of LF in Pekalongan. *W. bancrofti* is the microfilariae most frequently found in mosquito vectors (Kinyatta et al[JB24]. 2023), specifically *Cx. quinquefasciatus*. The finding also follows similar reports in Tanzania where the species is the main vector of LF (Lupenza et al. 2021). The variation in species and proportion of mosquitoes is also similar to other reports where *Cx. quinquefasciatus* is the dominant species in LF endemic areas (Derua et al. 2017, Lupenza et al. 2021). In the local context, this research found more mosquito species than previous findings at other nearby locations (Nurjazuli et al. 2022). The findings show the high potential for LF transmission in the region. The majority of *Cx. quinquefasciatus* is a female with a high proportion of parity and an estimated lifespan of more than 12 days. These findings show a higher proportion of mosquitoes with ovarian dilatations ≥ 4 than the number. The data reports that the majority of female Culex mosquitoes have a high potential to become competent vectors when in the first or second gonotrophic cycle to obtain microfilariae from the blood of parasitemia sufferers. The probability is supported by the observation that all L3 microfilariae originated from mosquito samples showing an ovarian dilation range of 4-5 times or 12-20 days old. This discovery is consistent with previous findings showing an incubation period of 13 days for extrinsic microfilariae within the body to transition to the L3 stage and migrate to the salivary glands (Xu et al. 2018, Dharmarajan et al. 2019). The potential for LF transmission is increasing with climate change, where the average daily air temperature shortens the extrinsic incubation period for microfilariae (Simon et al. 2017).

The results of nucleotide substitution analysis conducted on the ace1 and *Cx. quinquefasciatus* VGSC genes serve as indicative markers for assessing the insecticide resistance status within the mosquito population. Part (A) in Figure[JB25] 3 shows the results of nucleotide consistency at codon 119 of the ace1 gene where DNA samples show the same wildtype base arrangement, namely GGC (Valine amino acid). Meanwhile, part (B) is a chromatogram analysis of the base arrangement. Part (C) is the results of consistency in the nucleotide sequence in codon 1014 of the VGSC gene where there are three forms of nucleotide substitution from T to C, T to G, and A to T in the first, second base, and third bases, respectively. There are three nucleotide substitution variations in codon 1014 of the VGSC gene of *Cx. quinquefasciatus* from the formation of wild-type leucine (TTA) to phenylalanine (TTT) and Cysteine (TGT), as well as a silent mutation from TTA to CTA (**Table 4**). The first change is a form of silent mutation where TTA to CTA does not change the amino acid, namely Leucine. The second change results in an amino acid change from Leucine (TTA) to Cysteine (TGT) while the third leads to a change in Phenylalanine (TTT) (**Fig. 2**). The L1014F and L1014C mutations (**Fig. 4**) show homozygous and heterozygous forms. Parts A and B are homozygous and heterozygous forms of changing Leucine (TTA) to Phenylalanine (TTT) and Cysteine (TGT), while C and D are homozygous and heterozygous forms of changing Leucine (TTA) to Cysteine (TGT). Image B is a heterozygous form where T and G bases are at the same locus but T is more dominant. The mutation analysis of the VGSC codon 1014 gene shows that the proportion of mosquitoes resistant to insecticides in the population is 98-100%. The utilization of insecticides remains prevalent, with rates ranging from 85% to 92.5%, and some families ~~persisting a~~[JB26][A27]~~t~~ (63.3-65.0%) ~~to 65%~~ still use it for ~~durations exceeding~~more than two years. Moreover, daily usage intensity ranges from 40% to 53.3%, with particular emphasis on the pyrethroid group at 80% to 81.7% as well as burnt coil formulations, which account for 58% to 60% of usage (**Table 5**). Reports of resistance to Culex mosquito species, specifically *Cx. quinquefasciatus* is still limited. This finding complements previous data where *Cx. quinquefasciatus* in Central Java has been resistant to pyrethroid insecticides (Chakim et al. 2017). However, research in Jakarta reported that the species was still susceptible to pyrethroids and organophosphates (Subahar et al. 2022). According to recent data, *Cx. quinquefasciatus* in Surabaya has been resistant to both groups of insecticides with mutations in the VGSC and ace-1 genes (Panjinegara et al. 2022). Therefore, Culex mosquito resistance to insecticides has become increasingly widespread. The phenomenon of expanding the resistance area of *Cx quinquefasciatus* was also reported in Bengal, India (Anju-Viswan et al. 2020, Rai et al. 2019), Cameroon (Talipouo et al. 2021), Brazil (Lopes et al. 2019), Nigeria (Omotayo et al. 2023), and Korea (Jeon et al. 2024). Molecular analysis of mosquito samples showed four genotypes of the VGSC gene, namely wild type, silent mutation, and mutant (Leu-Phe and Leu-Cis). The Leu-Phe mutation is the most frequently reported mutant form in Indonesia (Panjinegara et al. 2024) and other countries (Talipouo et al. 2021). This is different from the findings in Surabaya which reports wildtype, mutant-heterozygous, and mutant-homozygous genetic variations (Panjinegara et al. 2024). A serious genetic mutation was reported in Brazil including esterase, ace-1, and VGSC (Lopes et al. 2019). Research in Korea did not obtain the G119S mutation but reported heterozygous forms of AGC/GGC, L1014F, and L1014S (Jeon et al. 2024). This genetic mutation phenomenon shows a serious problem affecting vector control efforts in LF-endemic areas.

In conclusion, *Cx. quinquefasciatus* was the dominant species among the five vector mosquitos in the LF endemic area of Pekalongan Regency, and 1.34% were proven to carry *W. bancrofti* microfilariae. Therefore, the area was vulnerable to LF transmission and the condition was increased by *Cx. quinquefasciatus* resistance against pyrethroid and organophosphate class insecticides. Molecular analysis of the VGSC gene found the wild-type allele and three mutant alleles, namely TTA to CTA, TTA to TTT, and TTA to TGT mutations. Further investigation should be conducted to detect the susceptibility of microfilariae to various antiparasitic drugs and educate the public in implementing methods of self-protection from exposure to mosquitoes accompanied by environmental cleaning movements.

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CONFLICT OF INTEREST

The authors state that there was no conflict of interest.

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Figure[JB41][A42] 1. The Map of Research Sites

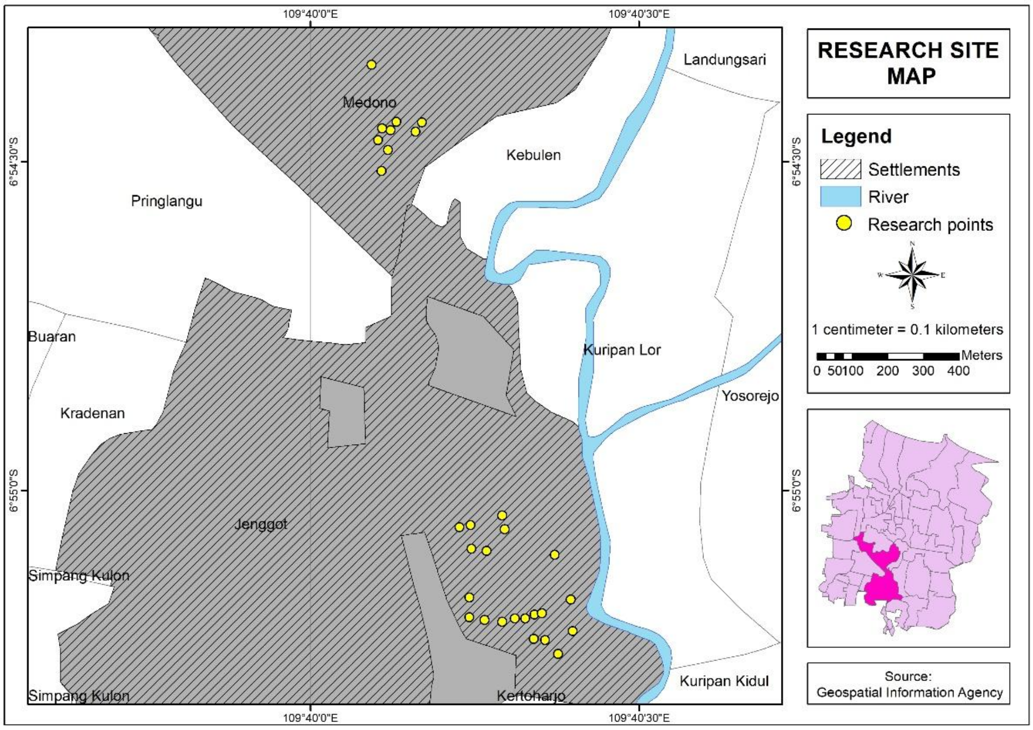


Table 1. Distribution of Mosquitos Based on Characteristics and Research Sites (1st Period of Mosquito Capture)

No.	Variables	Research Sites			
		Medono		Jenggot	
		n	%	n	%
1	Mosquito species and sex				
	Cx. quinquefasciatus	**280**	**33.61**	**553**	**67.39**
	Male	124	44.39	227	41.09
	Female	156	55.61	326	58.91
	Cx. tritaeniorhyncus	**31**	**44.93**	**38**	**55.07**
	Male	0	0.00	0	0.00
	Female	31	100.00	38	100.00
	Cx. vishnui	**29**	**41.43**	**41**	**58.57**
	Male	0	0.00	0	0.00
	Female	29	100.00	41	100.00
	An. vagus	**0**	**0.00**	**7**	**100.00**
	Male	0	0.00	0	0.00
	Female	0	0.00	7	100.00
	Ae. aegypti	**83**	**38.07**	**135**	**61.93**
	Male	17	20.62	36	26.67
	Female	66	79.38	99	73.33
2	**Parity**	**103**	**33.99**	**200**	**66.01**
	Nulliparous	23	22.33	88	44.00
	Parous	80	77.67	112	56.00
3	**Number of Ovary Dilatation (age in days)**	**80**	**41.67**	**112**	**58.33**
	1 (4)	0	0.00	8	7.14
	2 (8)	5	6.25	14	12.50
	3 (12)	18	22.50	29	25.89
	4 (16)	14	20.48	7	6.25
	5 (20)	18	22.50	10	8.93
	6 (24)	6	7.50	26	23.21
	7 (28)	19	23.75	18	16.07

Table 2. Results of Microscopic Microfilariae Detection of Female Mosquitoes (1st Period of Mosquito Capture)

No.	Species	Research Sites			
		Medono		Jenggot	
		n	%	n	%
1	***Cx. quinquefasciatus***	**50**	**50.00**	**50**	**50.00**
	Positive	0	0.00	0	0.00
	Negative	50	100.00	50	100.00
2	***Cx. tritaeniorhyncus***	**10**	**50.00**	**10**	**50.00**
	Positive	0	0.00	0	0.00
	Negative	10	100.00	10	100.00
3	***Cx. vishnui***	**10**	**50.00**	**10**	**50.00**
	Positive	0	0.00	0	0.00
	Negative	10	100.00	10	100.00
4	***An. vagus***	**-**	**-**	**7**	**100.00**
	Positive	-	-	0	00.00
	Negative	-	-	7	100.00
5	***Ae. aegypti***	**10**	**50.00**	**10**	**50.00**
	Positive	0	0.00	0	00.00
	Negative	10	100.00	10	100.00

Figure 2. *Wuchereria bancrofti* larvae were detected from four mosquito samples of *Cx.*[JB43] *quinquefasciatus* collected from Jenggot village, Pekalongan City.

Table 3. Distribution of Mosquito Characteristics from Jenggot Village (2nd Period of Mosquito Capture)

No.	Characteristics of Mosquitoes	n	%
1	Mosquito species and sex		
	Cx. quinquefasciatus	**380**	**65.40**
	Male	82	21.58
	Female	298	78.42
	Cx. vishnui	**160**	**27.54**
	Male	0	0.00
	Female	160	100.00
	Ae. aegypti	**41**	**7.06**
	Male	0	0.00
	Female	41	100.00
	Total	**581**	**100.00**
2	**Parity**		
	Nulliparous	86	29.05
	Parous	210	70.95
	Total	**296**	**33.99**
3	**Number of Ovary Dilatation (Age of Mosquito; days)**		
	1 (4)	7	3.33
	2 (8)	18	8.57
	3 (12)	47	22.38
	4 (16)	43	20.48
	5 (20)	47	22.38
	6 (24)	23	10/95
	7 (28)	25	11.90
	Total	**210**	**100.00**
4	**Microscopic detection of microfilariae in female mosquitoes**		
	Cx. quinquefasciatus	**298**	**59.72**
	Positive	4	1.34
	Negative	294	98.66
	Cx. vishnui	**160**	**32.06**
	Positive	0	0.00
	Negative	160	100.00
	Ae. aegypti	**41**	**8.22**
	Positive	0	0.00
	Negative	41	100.00
	Total	**499**	**100.00**

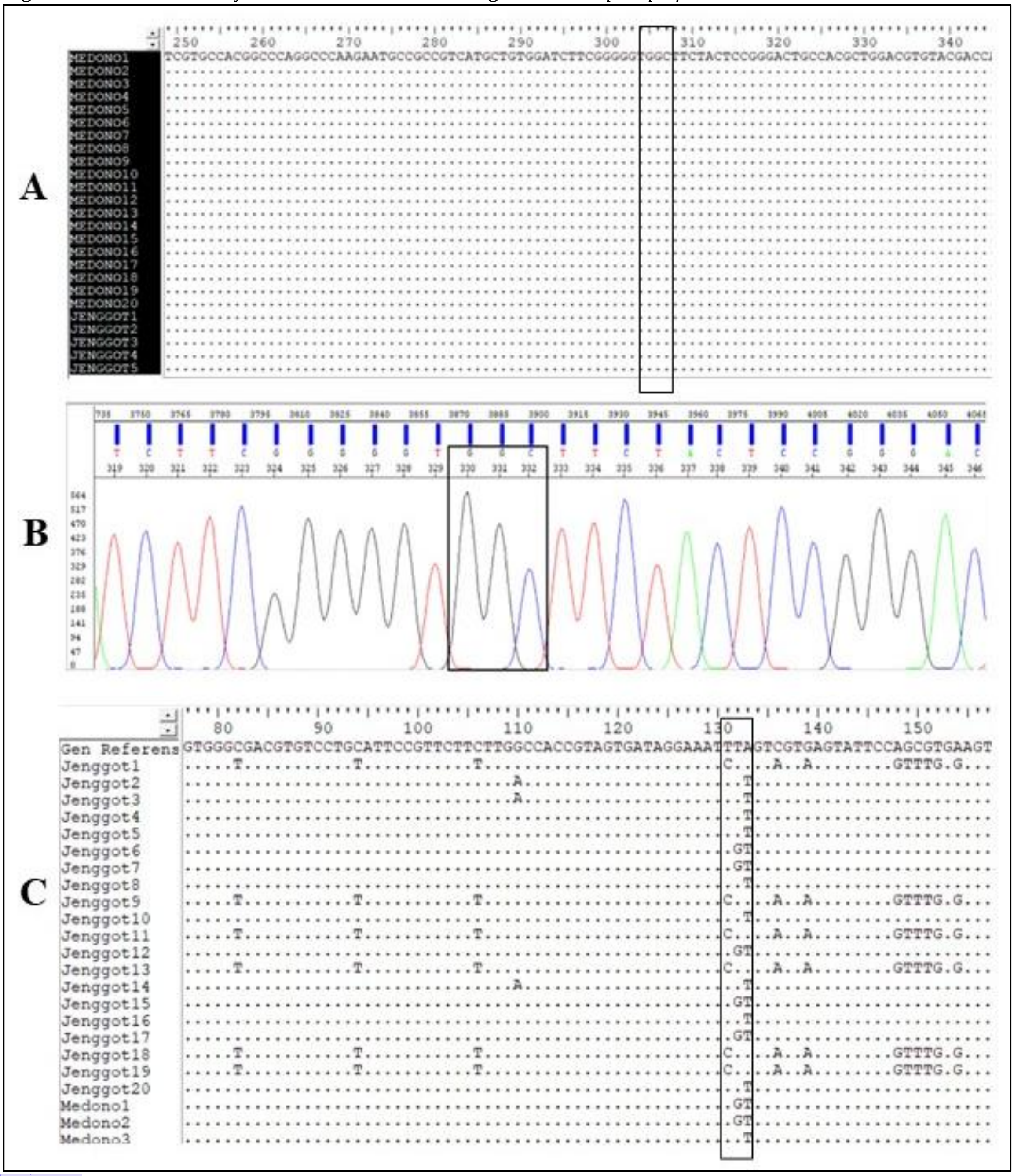
Table 4. Frequency of VGSC gene mutations among *Cx. quinquefasciatus* mosquito

No.	Nucleotide Substitution	Type of Mutation			
		Homozygous	%	Heterozygous	%
1	TTA to TTT	33	55.00	16	26.67
2	TTA to CTA	1	1.67	0	0.00
3	TTA to TGT	4	6.67	6	10.00

Table 5. The Insecticide Resistance Status and History of Insecticide Use in Research Sites

No	Variabel	Research Sites			
		Medono		Jenggot	
		n	%	n	%
1	**Resistance Status**				
	Sensitive	0	0	1	2
	Resistance	20	100	59	98
2	**The Use of Household Insecticide**				
	Yes	37	92.50	51	85
	No	3	7.50	9	15
3.	**Insecticide Group**				
	Organophosphate	8	20	11	18.33
	Pyrethroid	32	80	49	81.67
4.	**History of Insecticide Use (years)**				
	< 2	14	35	22	36.67
	≥ 2	26	65	38	63.33
5.	**Insecticide Use Intensity**				
	Not everyday	24	60	28	46.67
	Everyday	16	40	32	53.33
6.	**Insecticide Formulation**				
	Coil	24	60	35	58.33
	Spray	6	15	10	16.67
	Electric	2	5	4	6.67
	Repellent	8	20	11	18.33

Figure 3. Molecular analysis of codon 119 of ace-1 gene of *Cx. quinquefasciatus*



Fig[JB44]**. 3~~2~~.** Results of nucleotide substitution analysis of the ace1 and VGSC gene of *Cx. quinquefasciatus* as an indication of the insecticide resistance status of the mosquito population. No nucleotide substitutions were found in codon 119 of the ace1 gene (A) and the chromatogram shape of codon 119 (B). Part C is a variation of nucleotide substitution in codon 1014 of the VGSC gene which shows three variant changes, namely TTA-CTA (silent mutation), TTA-TTT, and TTA-TGT.

Figure 4. Homozygous and heterozygous nucleotide substitution in codon 1014 VGSC gene of *Cx. quinquefasciatus*

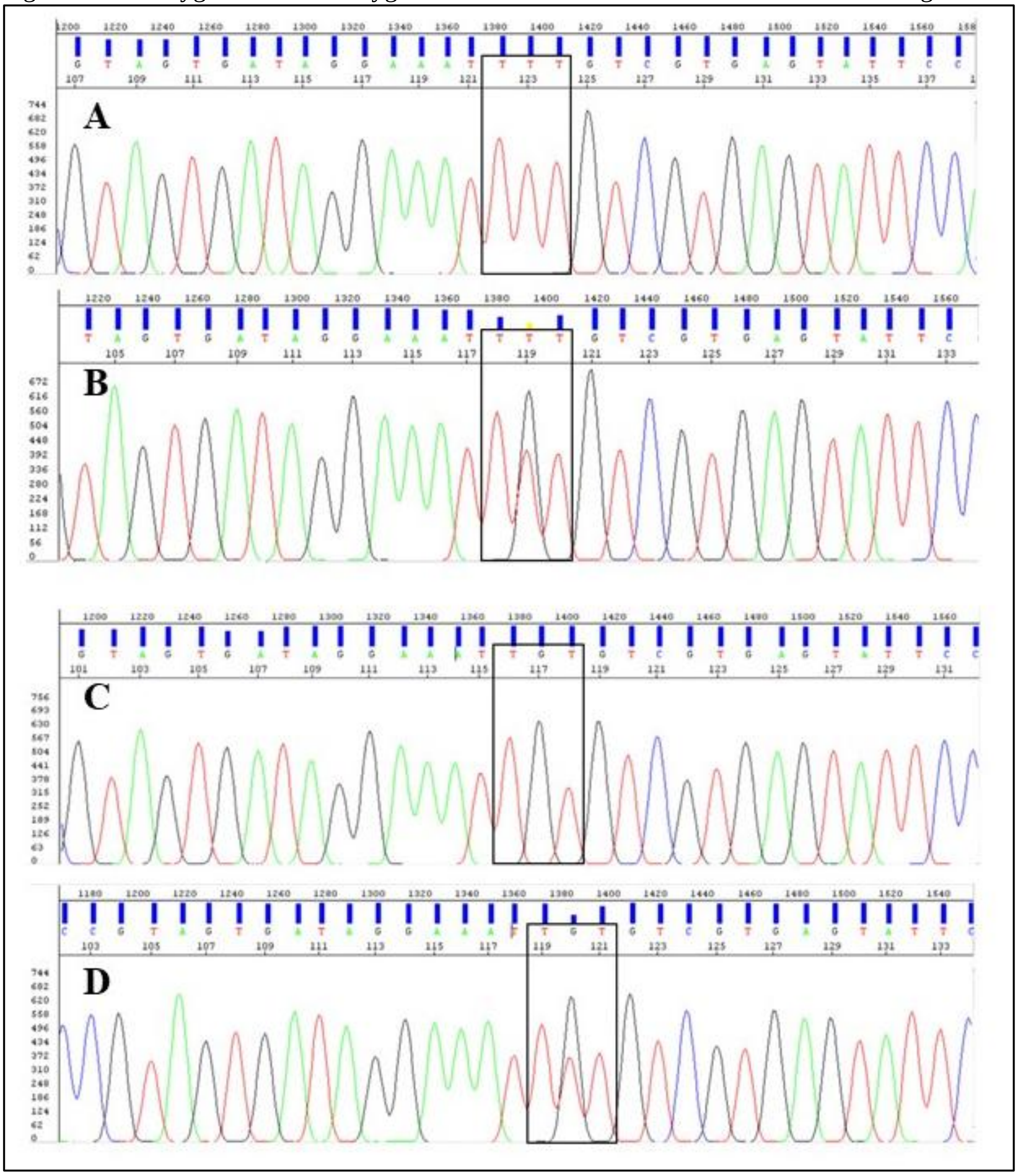


Fig.4. Homozygous and heterozygous nucleotide substitution in codon 1014 of VGSC gene of *Cx. quinquefasciatus*. A and B are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Phenylalanine (TTT) and Cysteine (TGT), while C and D are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Cysteine (TGT). Image B is a heterozygous form where the bases T and G are at the same locus, although T is more dominant. The opposite condition occurs in image D.

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[biodiv] Editor Decision

1 pesan

Smujo Editors via SMUJO <support@smujo.com> 21 April 2024 pukul 04.29
Balas Ke: Smujo Editors <editors@smujo.id>
Kepada: Sayono Sayono <say.epid@gmail.com>, Abdul Ghofur <omopung@gmail.com>, Suharyo Hadisaputro <prof_haryo@yahoo.co.id>

Sayono Sayono, Abdul Ghofur, Suharyo Hadisaputro:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Microscopic and molecular detection of microfilaria L3 and insecticide resistance among wild-caught mosquito vectors in endemic areas of Lymphatic filariasis". Complete your revision with a Table of Responses containing your answers to reviewer comments (for multiple comments) or enable Track Changes.

Our decision is: Revisions Required

Reviewer B:

With respect
The study was checked in terms of language and grammar
- Please follow the stricter essay writing frameworks
- Draw a GIS map for the vectors and study areas.
- The study needs a general review in terms of language and grammar.
Good luck.

Recommendation: Revisions Required

Biodiversitas Journal of Biological Diversity

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Author's response

Corrected aspects of manuscript number 17500

No.	Aspects	Revised-versions
1	Background	Paraphrases and avoiding the detailed results of literature
2	Number of words in Background	604 words
3	Track change use	Yes

Semarang, March 26th 2024
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S. Sayono

2nd Revised-Manuscript

Microscopic and molecular detection of microfilaria L3 and insecticide resistance among wild-caught mosquito vectors in endemic areas of Lymphatic filariasis

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Microscopic and molecular detection of microfilaria L3 and insecticide resistance among wild-caught mosquito vectors in endemic areas of Lymphatic filariasis

ABSTRACT

The decline in the global prevalence of Lymphatic filariasis (LF) has left areas of endemic focus in Africa and Southeast Asia. The presence of infectious and insecticide-resistant mosquitoes and the annual biting rate in the area are key to microfilaria transmission. This study aims to determine insecticide resistance and microfilariae infection in mosquito vectors in endemic areas. Twice entomological surveys based on six FL cases were carried out in Jenggot and Medono villages where indoor and outdoor mosquito capturing was carried out in ten houses within a 100m-radius of each case as well as household interviews about insecticide use. Laboratory works were done for species identification and detection of ovarian dilatation, microfilariae infection, and knockdown resistance mutations. A total of 1,197 and 581 mosquitoes were distributed to five species, namely *Culex quinquefasciatus* (69.59 and 65.40%), *Cx. tritaeniorhyncus* (5.76 and 0.00%), *Cx. vishnui* (5.85 and 27.54%), *Anopheles vagus* (0.58 and 0.00%), and *Aedes aegypti* (18.21 and 7.06%). The percentage of female mosquito infection, parity, and microfilaria were 66.25 and 86.00%, 63.37 and 70.95%, and 0.00 and 1.34%, respectively. Three base substitutions (TTA-TTT, TTA-CTA, and TTA-TGT) were found in codon 1014 of *Cx. quinquefasciatus* VGSC gene with proportions of 81.66%, 1.67%, and 26.67% showed resistance status to pyrethroid insecticides. These findings indicate the vulnerability of the research location to transmission so early detection, vector control, and further studies regarding the susceptibility of microfilariae to antiparasitic drugs are needed.

Keywords: Microfilaria, microscopic, molecular detection, mosquito vectors, Lymphatic filariasis

INTRODUCTION

Foci of endemic areas of Lymphatic Filariasis (LF) are remaining in African and Southeast Asian countries even though global incidence has shown a drastic decline in the last two decades (NTD Collaborators, 2020). The prevalence of LF in Asia is still stable at 3%, and only four countries are free of this disease, namely China, Japan, Vietnam, and South Korea (Bizhani et al. 2021), even after six periods of mass treatment (MDA) the stability of microfilaria infections this is still the case in Myanmar (Dickson et al. 2018). This low decrease in LF prevalence after POMP in endemic areas is reported to be due to a lack of community understanding and participation in these efforts (Aboagye et al. 2022). Other studies report that LF transmission is influenced by high mosquito vector density due to favorable rainfall, temperature, and air humidity (Sinha et al. 2023), resulting in the proportion of infectious vector mosquitoes (Dharmarajan et al. 2019), and the bite rate. annual transmission of the parasite to humans (Davis et al. 2019). Data and information on competent vectors and the circulation of microfilariae in mosquitoes is urgently needed to complete mass treatment (MDA) in endemic areas by prioritizing vector mosquito control efforts. Unfortunately, this data and information is very limited in Indonesia, especially in Central Java. This condition requires further research to understand the interaction of pathogens with mosquitoes as input for improving and strengthening methods and strategies for controlling LF vector mosquitoes (Famakinde 2018), including for developing predictive models of the interaction of risk factors for LF transmission and public health interventions (Zerbo et al. 2021). ~~Lymphatic Filariasis (LF) has shown a drastic decline in global incidence in the last two decades, although it remains a focus area in Africa and Southeast Asia (NTD Collaborators, 2020). The prevalence of LF in Asia is still stable at 3%, and only four countries are free of this disease, namely China, Japan, Vietnam, and South Korea (Bizhani et al. 2021), even microfilaria infections were still 2.63% after six rounds of mass drug administration (MDA) in Myanmar (Dickson et al. 2018). The reduction in LF prevalence after MDA in endemic areas is very low due to low community understanding and participation in the effort (Aboagye et al. 2022). LF transmission is influenced by the abundance of mosquito vectors where rainfall, temperature, and air humidity play a major role (Sinha et al. 2023). Mosquito vectors that pick up microfilariae when sucking a microfilaremia blood can become infectious and transmit the parasite after passing an extrinsic incubation period of around 13 days (Dharmarajan et al. 2019). The presence, density, and annual biting rate of the infectious mosquito vector are key parameters for parasite transmission to humans (Davis et al. 2019). This data is useful for developing priorities for controlling infectious mosquito vectors to support the success of controlling LF through mass treatment (MDA) in endemic areas. Further research on mosquito-pathogen interactions accompanied by corrective action and strengthening mosquito control methods and strategies needs to be carried out (Famakinde 2018) by developing interaction models of risk factors for LF transmission and designing public health interventions (Zerbo et al. 2021).~~

The results of entomological surveys in several countries report varying results. A study in Mafia Island, Tanzania resulted in ~~Three~~three mosquito species of LF vector ~~were found in two different regions in Tanzania~~ where the mosquito species order based on the ~~results of catching 35,534 mosquitoes in the endemic area of LF Mafia Island found three species with a~~ proportion ~~of~~ were *Cx. quinquefasciatus*, ~~1.3%~~ *Anopheles gambiae*, and *An. funestus* ~~0.5%, and~~ ~~*Cx. quinquefasciatus* 98.2%~~ , and 0.25% of the ~~7,936~~ examined mosquitoes ~~examined~~ were infected with *W. bancrofti* (Derua et al. 2017). Similar findings were reported from Masasi District, Tanzania where the study found the same three species with ~~respective proportions of 0.1%, 0.7%, and 99.2%, and~~ a higher of *W. bancrofti* infection rate ~~of 0.5%~~ for *Cx. quinquefasciatus* (Lupenza et al. 2021). Studies in Indonesia also show different findings. A study in Bogor Regency (West Java) ~~captured 8,087 mosquitoes distributed into four genera~~reported five mosquito genera, namely *Culex*, *Mansonia*, *Aedes*, and *Armigeres*, but there were no microfilariae detected (Nirwan et al. 2022). A study in Pekalongan Regency (Central Java) captured ~~4,547 mosquitoes from~~ three mosquito species, namely~~:~~ *Cx. quinquefasciatus*, *Cx. vishnui*, and *Ae. aegypti*, and 0.43% of mosquito samples were positive for microfilaria. (Nurjazuli et al. 2022). The results showed a variation in the species of mosquito vectors and the infection rates of microfilaria in different regions so the local specific condition is important to be understood.

The burden of LF vector control efforts is compounded by the resistance of *Cx. quinquefasciatus* mosquitoes to insecticides as reported in Bengal, India (Rai et al. 2019) and Uganda (Silva-Martins et al. 2019). Resistance of *Cx. quinquefasciatus* mosquitoes to insecticide classes, deltamethrine 0.05% and temephos 0.75% occur widely in Sri Lanka accompanied by the L1014F knockdown (kdr) mutation (Chandrasiri et al. 2020). The L1014S and L1014F mutations were also found in the *Cx. pipiens pallens* mosquito in China (Liu et al. 2019). Research in Indonesia also reported resistance to *Cx. quinquefasciatus* mosquitoes from Grobogan and Pekalongan in Central Java to 0.75% permethrin (Chakim et al. 2017) based on bioassay tests, however detection of VGSC gene mutations related to kdr resistance has not been found. This study aims to determine the biological characteristics, insecticide resistance, and the presence of microfilariae in mosquito vectors in LF endemic areas of Pekalongan city, Central Java Province, Indonesia.

MATERIAL AND METHODS

Study sites

Jenggot and Medono subdistricts were chosen as research locations based on filariasis case surveillance data from the Pekalongan City Health Service in 2020. Jenggot subdistrict has the highest microfilaria rate (5.4%) among the eleven LF endemic subdistricts in Pekalongan City and every time a finger blood survey is carried out, sufferers are always found. new. There were five new cases detected in 2020. Previously, Medono Village was not an endemic area for LF, but two new filariasis sufferers were found in a finger blood survey in 2020. This cross-sectional study involved six cases (four in Jenggot and two in Medono) and 10 houses around the case within a radius of 100m.

Ethical Clearance, Data Collection and Analysis

Data collection was conducted after the ethical review was done. This research has received a letter of recommendation with the issuance of Ethical Clearance number: 419/EA/KEPK-FKM/2021 from the health research ethics committee KEPK FKM Diponegoro University Semarang. Twice entomological and household survey was conducted in Jenggot and Medono villages. The data collected in this study was analyzed descriptively to describe each research variable in the form of tables, pictures, and maps.

Mosquito catching procedures

Mosquito catching is carried out at night from 18.00-06.00 the following day according to standard procedures (WHO 2013) in two stages, each stage carried out by 6 people who have been trained using the Human Landing Catch (HLC) and Resting Collection methods (RC). The team was divided into two to catch mosquitoes inside and outside the house for 40 minutes, and 10 minutes to change the paper cup for the mosquitoes, as well as time to rest. Every 1 hour, caught mosquitoes are put in a paper cup with a code and time of capture. RC is carried out from 06.00 – 07.30, to catch mosquitoes that are resting on walls, window curtains, mosquito nets, and hanging clothes, using an aspirator and net. All mosquitoes that are caught are put into a paper cup that is filled with cotton filled with sugar solution. The paper cup is placed in a container covered with banana stems and covered with a wet towel to maintain optimum temperature and humidity of 27±2 ^{0}C and 80±10 % respectively. The captured mosquito samples were held for twelve days and fed a 10% sugar solution before examination.

Identification of mosquito species and ovary dilatation

Identify mosquito species using previously published identification keys (Rattanarithikul et al. 2005, Nugroho et al. 2021). Mosquito parosity was determined using a surgical method to determine the amount of ovarian dilatation. The mosquito that has been identified is placed in a petri dish, the wings and legs are separated from the body, then the specimen is placed on a glass object and physiological NaCl is dropped. After that, surgery is performed using a surgical needle. Surgery is carried out microscopically using a stereo microscope. The surgical needle in the left hand presses the chest and the surgical needle in the right hand presses the 7th segment and is then moved slowly to the right until the abdominal contents and ovaries are pulled out. The ovaries were placed on a glass object that had just been given distilled water to view the tracheoles using a stereo microscope with a magnification of 40x10.

Microfilaria detection

The wings of the mosquito to be dissected are cut so that the scales do not contaminate the microscope's field of view. Mosquitoes were placed on glass slides dripped with physiological saline. The mosquito's thorax and abdomen were cut into pieces with a dissecting needle and observed under a microscope at 40X magnification. If there are filarial worms, they will appear to move depending on their stage. Stages 1-2 are short, fat, and slow-moving, while stage 3 (infective) appears long and fast-moving (Laney et al. 2010). Apart from surgery, microfilaria detection also uses molecular methods with the stages of DNA extraction, DNA amplification, electrophoresis, and imaging.

DNA Extraction.

Female mosquitoes were pooled based on species as many as 10-20 mosquitoes. Each pool was homogenized with a pestle in a microcentrifuge tube containing 180 µl of ATL buffer (pH 7.2; PBS) and ground, 20 µl of proteinase K, vortexed, and incubated for 24 hours. In the next step, the specimen was vortexed for 15 seconds, and then 200 µl AL buffer was added, and vortexed again. A total of 600 µl of extract samples were taken put into a mini-column and centrifuged for 1 minute at a speed of 8000 rpm. Add 500 µl of Aw1 to the specimen, centrifuge again for 1 minute, add 500 µl of AW2 buffer, and centrifuge at 14,000 rpm for 3 minutes. The sample was transferred to a 1.5 ml tube and 60 µl AE buffer was added, incubated for 1 minute, and centrifuged at 8000 rpm for 1 minute.

DNA Amplification with Polymerase-Chained Reaction

The extracted mosquito DNA was amplified using a thermal cycler (Perkin-Elmer Cetus, Norwalk, Connecticut, USA) with 2 oligonucleotide primers, NV-l: 5' CGTGATGGCATCAAAGTAGCG 3' (21-mer) and NV-2: 5'CCCTCACTTACCATAAGACAAC 3' (22- mer). Each amplification reaction was carried out in a final volume of 50 and contained 10 ~ I-IMT ri-HCl pH 9.2, 1.5 mM MgCl, 75 mM KCl, 1.25 mM each deoxy-nucleotide triphosphate, 10 pmol each primer NV-l and NV -2, and 2 units of Taq polymerase. The temperature program for PCR was 5 minutes at 95 ^{0}C, followed by 35 cycles of 1 minute each at 94 ^{0}C, 55 ^{0}C, and 72 0C, and elongation of 10 minutes at 72 ^{0}C. A total of 20% of the PCR product from each sample was electrophoresed on a 2% agarose gel and stained with ethidium bromide to confirm amplification (Ramzy et al. 1997).

RESULTS AND DISCUSSION

In total, a total of 1,678 mosquitoes were obtained in two capture periods, 1,197 and 581 respectively, with a proportion of female sex and parity of 66.25 and 86.00% and 63.37 and 70.95%, respectively. The majority (61.49 and 65.72%) of mosquitoes showed ≥ 4 ovarian dilatations or a lifespan of 12-16 days, which is an estimate based on the understanding that one gonotropic cycle is approximately 3-4 days (Fereda 2022). This study applied the mosquito-catching method with HLC and Resting Catch and obtained a high composition of female mosquitoes and parosity. Gravid female mosquitoes suck blood to meet the protein needs for the development of embryos in eggs. Blood-feeding behavior among mosquitoes in tropical regions occurs throughout the year, whereas in temperate regions gravid mosquitoes only appear in spring (Siperstein et al. 2023). This fact shows that air temperature influences mosquitoes in their mating and pregnancy behavior. A total of five mosquito species were found in the first fishing period, and only three mosquito species were found in the second period with the proportion for each species being Cx. quinquefasciatus (69.59 and 65.40%), *Cx. tritaeniorhyncus* (5.76 and 0.00%), Cx. vishnui (5.85 and 27.54%), An. Vagus (0.58 and 0.00%), and Ae. aegypti (18.21 and 7.06%). The proportion of female mosquitoes in the first and second captures based on species is *Cx. quinquefasciatus* (60.78 and 59.78%), *Cx. tritaeniorhyncus* (8.70 and 0.00%), *Cx. vishnui* (8.83 and 32.06%), An. Vagus (0.88 and 0.00%), and *Ae. aegypti* (20.81 and 8.22%) (**Tables 1 and 3**). These data indicate that *Cx quinquefasciatus* is the dominant mosquito species in both study locations, while *Cx. tritaeniorhyncus* and An. vagus was not found on the second arrest. The dominance of *Cx. quinquefasciatus* in LF endemic areas was also reported in several studies such as in Tanzania (Derua et al. 2017, Lupenza et al. 2021), and Gamapaha, Sri Lanka (Pilagolla and Amarasinghe 2021). Similar findings were also reported from several studies in Indonesia, such as in Bogor Regency, West Java (Nirwan et al. 2022), Pekalongan Regency, Central Java (Nurjazuli et al. 2022), and Tangerang (Prasetyowati et al. 2019). However, several studies report different dominant vector species of LF in endemic areas such as *Armigeres subalbatus* in Musi Rawas, South Sumatra (Mulyaningsih et al. 2019), *Ae. scutellaris* and *Ae. kochi* in the South Pacific while *An. gambiae*, *A. funestus*, and *An. punctulatus* in rural Asia and Africa (Bhuvaneswari et al. 2023). Differences in species dominance are influenced by habitat conditions, especially the presence of aquatic plants (Pratiwi et al. 2019), chloride content, and water temperature (Amini et al. 2020).

The results of catching mosquitoes showed different results, wherein the first period they produced greater numbers. This indicates that mosquito abundance varies over time. However, mosquito abundance did not correlate with microfilaria findings. The surgery and PCR examination results of mosquito samples from the first capture period did not reveal microfilariae (**Table 2**). In contrast, in the second period of capture, fewer numbers and species of mosquitoes were obtained, namely 581 mosquitoes from three species (**Table 3**), but the results of dissection of female mosquitoes after holding for 12 days found that 4 out of 298 (1.34%) mosquitoes contained L3 microfilariae, with species *W. bancrofti* (**Fig.** 1). All microfilariae were detected from mosquito samples aged 12-20 days (ovarian dilatation 4 and 5) with proportions of 25% and 75%. This fact shows that microfilariae are found in mosquitoes that have gone through 4-5 periods of sucking blood. Detection of microfilariae in mosquito vectors in Indonesia is still limited. This study adds to the fact that there is still an infectious vector of LF in Pekalongan City, Central Java, Indonesia, even with a higher percentage compared to findings in other areas (Derua et al. 2017, Lupenza et al. 2021), including in nearby areas (Nujazuli et al. 2022). This finding confirms that this area still has a high potential for LF transmission with the main vector being the *Cx. quinquefasciatus*. Apart from adding data on the number of microfilaria infections in mosquito vectors, these findings also confirm the role of *Cx. quinquefascitus* as the main vector of LF in Pekalongan. *W. bancrofti* is the microfilariae most frequently found in mosquito vectors (Kinyatta et al 2023), especially *Cx. quinquefasciatus*. This finding is also following similar reports in Tanzania where this species is also the main vector of LF although with a much smaller infection rate (Lupenza et al. 2021). The variation in species and proportion of mosquitoes found in this study is also similar to other reported studies where Cx. quinquefasciatus is the dominant species in LF endemic areas (Derua et al. 2017, Lupenza et al. 2021). In the local context, this study found more mosquito species than previous findings at other nearby locations where only three mosquito species were found, namely *Cx. quinquefasciatus, Cx. vishnui*, and *Ae. aegypti*, although the proportion is not stated (Nurjazuli et al. 2022). The findings of this study illustrate the high potential for LF transmission in the region. This is supported by the fact that the majority of *Cx. quinquefasciatus* is a female with a high proportion of parity and an estimated lifespan of more than 12 days. These findings indicate a higher proportion of mosquitoes with ovarian dilatations ≥ 4 than the number of dilatations below. This data illustrates that the majority of female Culex mosquitoes in this population have a high potential to become competent vectors if in the first or second gonotrophic cycle they succeed in obtaining microfilariae from the blood of parasitemia sufferers. This possibility is strengthened by the fact that all L3 microfilariae identified in this study came from mosquito samples with an ovarian dilation range of 4-5 times or 12-20 days old. This finding follows other reports which state that the incubation period for extrinsic microfilariae in the mosquito's body to reach the L3 stage and enter the salivary glands takes 13 days (Xu et al. 2018, Dharmarajan et al. 2019). The potential for LF transmission is increasing along with climate change, where increasing the average daily air temperature can shorten the extrinsic incubation period for microfilariae (Simon et al. 2017).

Results of nucleotide substitution analysis of the ace1 and *Cx. quinquefasciatus* VGSC genes as an indication of the insecticide resistance status of the mosquito population. Part (A) in Figure 2 shows the results of nucleotide alignment at codon 119 of the ace1 gene where all DNA samples show the same wildtype base arrangement, namely GGC (Valine amino acid). Part (B) is a chromatogram display of the base arrangement. Part (C) shows the results of aligning the nucleotide sequence in codon 1014 of the VGSC gene where there are three forms of nucleotide substitution, namely from T to C in the first base, T to G in the second base, and A to T in the third base. There are three nucleotide substitution variations in codon 1014 of the VGSC gene of *Cx. quinquefasciatus* from the formation of wild-type leucine (TTA) to phenylalanine (TTT) and Cysteine (TGT), and one silent mutation from TTA to CTA (Table 4). The first change is a form of silent mutation where the base substitution (TTA to CTA) does not change the amino acid, namely Leucine. The second change results in an amino acid change from Leucine (TTA) to Cysteine (TGT) and the third change results in a change in the amino acid Phenylalanine (TTT) (Fig. 2). The L1014F and L1014C mutations (Fig. 3) show homozygous and heterozygous forms. Parts A and B are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Phenylalanine (TTT) and Cysteine (TGT), while C and D are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Cysteine (TGT). Image B is a heterozygous form where the T and G bases are at the same locus, although T is more dominant. The opposite condition occurs in image D. The overall results of mutation analysis of the VGSC codon 1014 gene show that the proportion of mosquitoes resistant to insecticides in this population is 98- 100%. This finding is supported by the fact that the activity of using insecticides by the community is also very high with a range of 85-92.5%, even 63.3-65% for more than two years, with a daily intensity of 40-53.3%, especially the pyrethroid group (80-81.7%) and 58-60% in burnt coil formulations (**Table 5**). Reports of resistance to Culex mosquito species, especially *Cx. quinquefasciatus* in Indonesia is still limited. This finding complements previous data where *Cx. quinquefasciatus* in Central Java, Indonesia has been resistant to pyrethroid insecticides (Chakim et al. 2017), but a study in Jakarta reported that this species is still susceptible to pyrethroids and organophosphates (Subahar et al. 2022). Recent data show that *Cx. quinquefasciatus* in Surabaya has been resistant to both groups of insecticides, and it has even been proven that there are mutations in the VGSC and ace-1 genes (Panjinegara et al. 2022). This condition proves that Culex mosquito resistance to insecticides has become increasingly widespread. The phenomenon of expanding the resistance area of Cx mosquitoes. quinquefasciatus was also reported in Bengal, India (Anju-Viswan et al. 2020, Rai et al. 2019). Cameroon (Talipouo et al. 2021), Brazil (Lopes et al. 2019), Nigeria (Omotayo et al. 2023), and Korea (Jeon et al. 2024). Molecular analysis of mosquito samples only showed four genotypes of the VGSC gene, namely wild type, silent mutation, and mutant (Leu-Phe and Leu-Cis). The Leu-Phe mutation is the most frequently reported mutant form in Indonesia (Panjinegara et al. 2024) and other countries (Talipouo et al. 2021). This study did not find any mutations in the ace-1 gene. This is different from the findings in Surabaya which found wildtype, mutant-heterozygous, and mutant-homozygous genetic variations (Panjinegara et al. 2024). A more serious genetic mutation in this species was reported from Brazil involving esterase, ace-1, and VGSC (Lopes et al. 2019). Studies in Korea also did not find the G119S mutation but did find heterozygous forms of AGC/GGC, L1014F, and L1014S (Jeon et al. 2024). This genetic mutation phenomenon indicates a serious problem that can hamper vector control efforts in LF-endemic areas, so the search for other effective methods is necessary.

In conclusion, *Cx. quinquefasciatus* is the dominant species among the five vector mosquito species in the LF endemic area of Pekalongan Regency, and 1.34% of them were proven to carry *W. bancrofti* microfilariae. This fact shows that the area is still vulnerable to LF transmission. This condition is exacerbated by *Cx. quinquefasciatus* resistance, both against pyrethroid and organophosphate class insecticides. Molecular analysis of the VGSC gene found the wild-type allele and three mutant alleles, namely silent mutation (TTA to CTA), TTA to TTT mutation, and TTA to TGT. Further investigation is needed to detect the susceptibility of microfilariae to various antiparasitic drugs and educate the public to implement methods of self-protection from exposure to mosquitoes accompanied by environmental cleaning movements to eradicate the habitat of LF vector mosquitoes.

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CONFLICT OF INTEREST

All of the authors state that there was no conflict of interest.

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Table 1. Distribution of Mosquitos Based on Characteristics and Study Sites (1st Period of Mosquito Capture)

No.	Variables	Study Sites: Medono n	%	Jenggot n	%
1	Mosquito species and sex				
	Cx. quinquefasciatus	**280**	**33.61**	**553**	**67.39**
	Male	124	44.39	227	41.09
	Female	156	55.61	326	58.91
	Cx. tritaeniorhyncus	**31**	**44.93**	**38**	**55.07**
	Male	0	0.00	0	0.00
	Female	31	100.00	38	100.00
	Cx. vishnui	**29**	**41.43**	**41**	**58.57**
	Male	0	0.00	0	0.00
	Female	29	100.00	41	100.00
	An. vagus	**0**	**0.00**	**7**	**100.00**
	Male	0	0.00	0	0.00
	Female	0	0.00	7	100.00
	Ae. aegypti	**83**	**38.07**	**135**	**61.93**
	Male	17	20.62	36	26.67
	Female	66	79.38	99	73.33
2	**Parity**	**103**	**33.99**	**200**	**66.01**
	Nulliparous	23	22.33	88	44.00
	Parous	80	77.67	112	56.00
3	**Number of Ovary Dilatation (age in days)**	**80**	**41.67**	**112**	**58.33**
	1 (4)	0	0.00	8	7.14
	2 (8)	5	6.25	14	12.50
	3 (12)	18	22.50	29	25.89
	4 (16)	14	20.48	7	6.25
	5 (20)	18	22.50	10	8.93
	6 (24)	6	7.50	26	23.21
	7 (28)	19	23.75	18	16.07

Table 2. Results of Microscopic Microfilariae Detection of Female Mosquitoes (1st Period of Mosquito Capture)

No.	Species	Study Sites: Medono n	%	Jenggot n	%
1	***Cx. quinquefasciatus***	**50**	**50.00**	**50**	**50.00**
	Positive	0	0.00	0	0.00
	Negative	50	100.00	50	100.00
2	***Cx. tritaeniorhyncus***	**10**	**50.00**	**10**	**50.00**
	Positive	0	0.00	0	0.00
	Negative	10	100.00	10	100.00
3	***Cx. vishnui***	**10**	**50.00**	**10**	**50.00**
	Positive	0	0.00	0	0.00
	Negative	10	100.00	10	100.00
4	***An. vagus***	**-**	**-**	**7**	**100.00**
	Positive	-	-	0	00.00
	Negative	-	-	7	100.00
5	***Ae. aegypti***	**10**	**50.00**	**10**	**50.00**
	Positive	0	0.00	0	00.00
	Negative	10	100.00	10	100.00

Figure 1. *Wuchereria bancrofti* larvae were detected from four mosquito samples of *Cx quinquefasciatus* collected from Jenggot village, Pekalongan City.

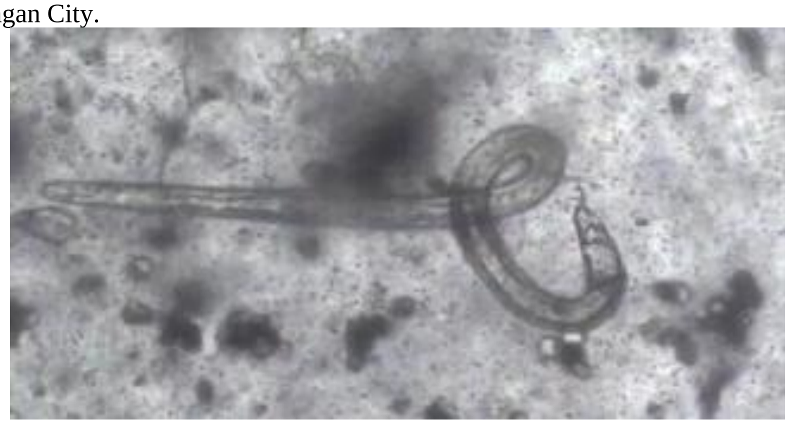

Table 3. Distribution of Mosquito Characteristics from Jenggot Village (2nd Period of Mosquito Capture)

No.	Characteristic of Mosquitoes	n	%
1	Mosquito species and sex		
	Cx. quinquefasciatus	**380**	**65.40**
	Male	82	21.58
	Female	298	78.42
	Cx. vishnui	**160**	**27.54**
	Male	0	0.00
	Female	160	100.00
	Ae. aegypti	**41**	**7.06**
	Male	0	0.00
	Female	41	100.00
	Total	**581**	**100.00**
2	**Parity**		
	Nulliparous	86	29.05
	Parous	210	70.95
	Total	**296**	**33.99**
3	**Number of Ovary Dilatation (Age of Mosquito; days)**		
	1 (4)	7	3.33
	2 (8)	18	8.57
	3 (12)	47	22.38
	4 (16)	43	20.48
	5 (20)	47	22.38
	6 (24)	23	10/95
	7 (28)	25	11.90
	Total	**210**	**100.00**
4	**Microscopic detection of microfilariae in female mosquitoes**		
	Cx. quinquefasciatus	**298**	**59.72**
	Positive	4	1.34
	Negative	294	98.66
	Cx. vishnui	**160**	**32.06**
	Positive	0	0.00
	Negative	160	100.00
	Ae. aegypti	**41**	**8.22**
	Positive	0	0.00
	Negative	41	100.00
	Total	**499**	**100.00**

Table 4. Frequency of VGSC gene mutations among *Cx. quinquefasciatus* mosquito

No.	Nucleotide Substitution	Type of Mutation			
		Homozygous	**%**	**Heterozygous**	**%**
1	TTA to TTT	33	55.00	16	26.67
2	TTA to CTA	1	1.67	0	0.00
3	TTA to TGT	4	6.67	6	10.00

Table 5. The Insecticide Resistance Status and History of Insecticide Use in Study Sites

No	Variabel	Study Sites			
		Medono		**Jenggot**	
		n	**%**	**n**	**%**
1	**Resistance Status**				
	Sensitive	0	0	1	2
	Resistance	20	100	59	98
2	**The Use of Household Insecticide**				
	Yes	37	92.50	51	85
	No	3	7.50	9	15
3.	**Insecticide Group**				
	Organophosphate	8	20	11	18.33
	Pyrethroid	32	80	49	81.67
4.	**History of Insecticide Use (years)**				
	< 2	14	35	22	36.67
	≥ 2	26	65	38	63.33
5.	**Insecticide Use Intensity**				
	Not everyday	24	60	28	46.67
	Everyday	16	40	32	53.33
6.	**Insecticide Formulation**				
	Coil	24	60	35	58.33
	Spray	6	15	10	16.67
	Electric	2	5	4	6.67
	Repellent	8	20	11	18.33

Figure 2. Molecular analysis of codon 119 of ace-1 gene of *Cx. quinquefasciatus*

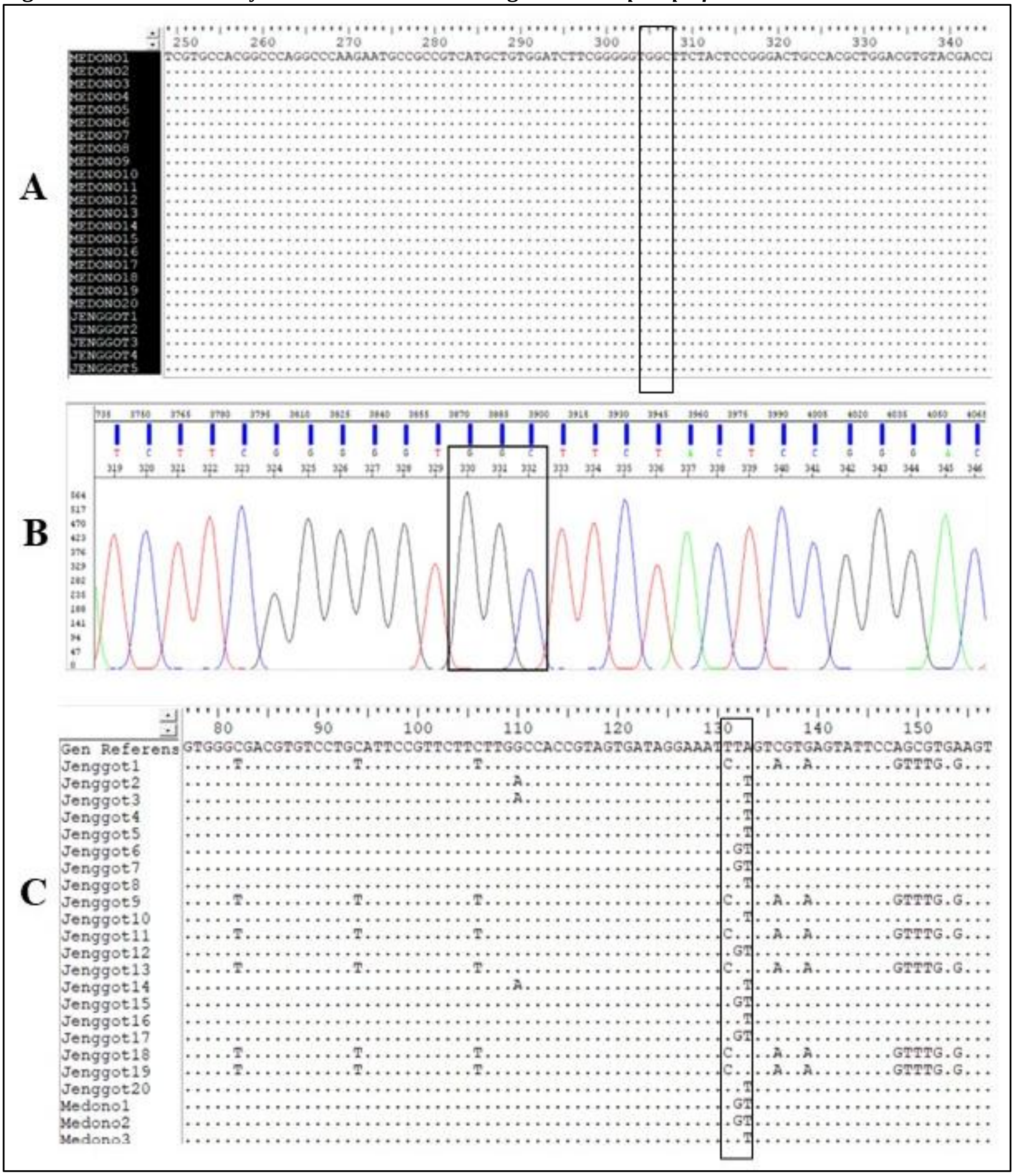

Fig. 2. Results of nucleotide substitution analysis of the ace1 and VGSC gene of *Cx. quinquefasciatus* as an indication of the insecticide resistance status of the mosquito population. No nucleotide substitutions were found in codon 119 of the ace1 gene (A) and the chromatogram shape of codon 119 (B). Part C is a variation of nucleotide substitution in codon 1014 of the VGSC gene which shows three variant changes, namely TTA-CTA (silent mutation), TTA-TTT, and TTA-TGT.

Figure 3. Homozygous and heterozygous nucleotide substitution in codon 1014 VGSC gene of *Cx. quinquefasciatus*

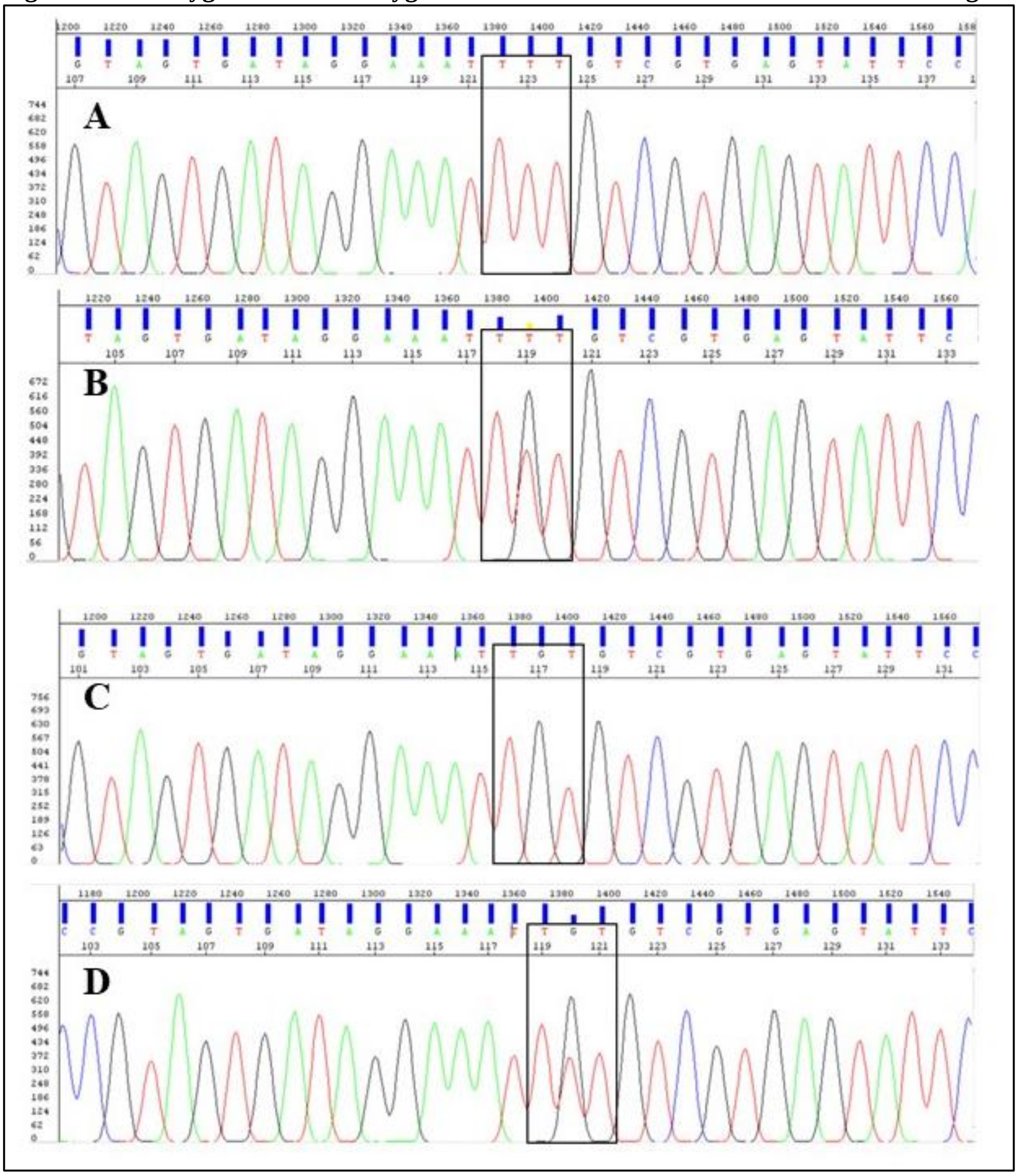

Fig.3. Homozygous and heterozygous nucleotide substitution in codon 1014 of VGSC gene of *Cx. quinquefasciatus*. A and B are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Phenylalanine (TTT) and Cysteine (TGT), while C and D are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Cysteine (TGT). Image B is a heterozygous form where the bases T and G are at the same locus, although T is more dominant. The opposite condition occurs in image D.

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Detection of microfilaria L3 and insecticide resistance among wild-caught mosquito vectors in endemic areas of lymphatic filariasis

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Abstract. *Ghofur A, Hadisaputro S, Sayono S. 2024. Detection of microfilaria L3 and insecticide resistance among wild-caught mosquito vectors in endemic areas of lymphatic filariasis. Biodiversitas 25: xxxx*. The decline in the global prevalence of Lymphatic Filariasis (LF) is evident in the persistent endemic foci in Africa and Southeast Asia. In this context, the presence of infectious and insecticide-resistant mosquitoes and the annual biting rate in the area are key to microfilaria transmission. Therefore, this research aimed to determine insecticide resistance and microfilariae infection in mosquito vectors in endemic areas. In Jenggot and Medono Villages, twice entomological surveys were conducted based on six LF cases, where indoor and outdoor mosquito capturing was carried out in ten houses within a 100m radius of each case as well as household interviews about insecticide use. In addition, laboratory works were performed for species identification and detection of ovarian dilatation, microfilariae infection, and knockdown resistance mutations. A total of 1,197 and 581 mosquitoes were distributed to five species and their proportions, namely *Culex quinquefasciatus* (69.59 and 65.40%), *Cx. tritaeniorhyncus* (5.76 and 0.00%), *Cx. vishnui* (5.85 and 27.54%), *Anopheles vagus* (0.58 and 0.00%), and *Aedes aegypti* (18.21 and 7.06%). The percentage of female mosquito infection, parity, and microfilaria were 66.25 and 86.00%, 63.37 and 70.95%, as well as 0.00 and 1.34%, respectively. Furthermore, TTA-TTT, TTA-CTA, and TTA-TGT base substitutions were reported in codon 1014 of *Cx. quinquefasciatus* VGSC gene with proportions of 81.66%, 1.67%, and 26.67%, respectively. The result showed that the vulnerability of the research location to transmission emphasized the necessity for early detection, vector control, and further analyses of the susceptibility of microfilariae to antiparasitic drugs.

Keywords: *Culex quinquefasciatus*, microscopic, molecular detection, mosquito vectors, *Wuchereria bancrofti*

INTRODUCTION

The decline in the global prevalence of Lymphatic Filariasis (LF) is evident in the persistent endemic foci in Africa and Southeast Asia (NTD Collaborators 2020). The prevalence of LF in Asia is stable at 3%, and only four countries are free of the disease, namely China, Japan, Vietnam, and South Korea (Bizhani et al. 2021). Despite six rounds of mass drug administration (MDA), the persistence of microfilaria infections continues, a situation exemplified by Myanmar (Dickson et al. 2018). This low decrease in LF prevalence after Preventive chemotherapy, Operations, Monitoring, and Participation (POMP) in endemic areas is reported to be due to a lack of community understanding and participation in the efforts (Aboagye and Addison 2022). Other research reports that the transmission is influenced by high mosquito vector density due to favorable rainfall, temperature, and air humidity (Sinha et al. 2023), resulting in the proportion of infectious vectors (Dharmarajan et al. 2019; Davis et al. 2019).

Data and information on competent vectors and the circulation of microfilariae in mosquitoes are needed to complete MDA in endemic areas by prioritizing vector mosquito control efforts. However, this data and information is very limited in Indonesia, specifically in Central Java. The condition requires further research to understand the interaction of pathogens with mosquitoes as input. This strengthens methods and strategies for controlling LF vector mosquitoes (Famakinde 2018), including developing predictive models of risk factors for LF transmission and public health interventions (Zerbo et al. 2021). Furthermore, the results of entomological surveys in several countries report varying results. Research in Mafia Island, Tanzania resulted in three mosquito species of LF vector, namely *Cx. quinquefasciatus*, *Anopheles gambia*e, and *An. funestus*, and 0.25% of the examined mosquitoes were infected with *W. bancrofti* (Derua et al. 2017). In Masasi District, Tanzania, the research found the same three species with a higher *W. bancrofti* infection rate for *Cx. quinquefasciatus* (Lupenza et al. 2021). Research in Bogor District (West Java) reported five mosquito genera, namely *Culex*, *Mansonia*, *Aedes*, and *Armigeres*, without detecting microfilariae (Nirwan et al. 2022). Another research in Pekalongan District (Central Java) captured three mosquito species, namely *Cx. quinquefasciatus*, *Cx. vishnui*, and *Ae. aegypti*, and 0.43% of samples were positive for microfilaria (Nurjazuli et al. 2022). The results showed a variation in the vectors and the infection rates of microfilaria in different regions.

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Local Burden of Disease 2019 Neglected Tropical Diseases Collaborators. 2020. The global distribution of lymphatic filariasis, 2000-18: a geospatial analysis. Lancet Glob Health. 8(9):e1186-e1194. doi: 10.1016/S2214-109X(20)30286-2.

Commented [MSI3]: Please add reference of this citation

Commented [A4R3]: Nirwan M, Hadi UK, Soviana S, Satrija F, Setiyaningsih S. 2022. Diversity, domination and behavior of mosquitoes in filariasis endemic area of Bogor District, West Java, Indonesia. Biodiversitas 23(4):2093-2100. DOI: 10.13057/biodiv/d230444

The burden of LF vector control efforts is compounded by the resistance of *Cx. quinquefasciatus* to insecticides as reported in Bengal, India (Rai et al. 2019) and Uganda (Silva-Martins et al. 2019). Resistance of *Cx. quinquefasciatus* mosquitoes to insecticide classes, deltamethrine 0.05% and temephos 0.75% occur widely in Sri Lanka accompanied by the L1014F knockdown (kdr) mutation (Chandrasiri et al. 2020). The L1014S and L1014F mutations were also reported in *Cx. pipiens pallens* mosquito in China (Liu et al. 2019). Resistance to *Cx. quinquefasciatus* was also reported from Grobogan and Pekalongan in Central Java to 0.75% permethrin (Chakim et al. 2017) based on bioassay tests. Therefore, this research aimed to determine the biological characteristics, insecticide resistance, and the presence of microfilariae in mosquito vectors in LF endemic areas of Pekalongan City, Central Java Province, Indonesia.

MATERIAL AND METHODS

Study sites

As many as 20 sites in Jenggot and 9 sites in Medono Villages were selected as research locations based on filariasis case surveillance data from the Pekalongan City Health Service in 2020 (Figure 1). Furthermore, Jenggot Sub-district possessed the highest microfilaria rate (5.4%) in Pekalongan City and five cases were detected in 2020. Medono Village was not an endemic area for LF, but two new filariasis sufferers were found in a finger blood survey in 2020 (Anonymus 2021). This cross-sectional research included six cases and 10 houses around the case within a radius of 100 m.

Ethical clearance, data collection and analysis

Data collection was conducted after the ethical review was achieved. This research has received a letter of recommendation with the issuance of ethical clearance number: 419/EA/KEPK-FKM/2021 from the health ethics committee KEPK FKM Universitas Diponegoro Semarang. Furthermore, twice entomological and household survey was conducted in Jenggot and Medono Villages. The data collected were analyzed to describe each research variable in the form of tables, pictures, and maps.

Mosquito catching procedures

Mosquito catching was carried out at night from 18.00-06.00 at the house of the new LF case and 9-10 neighboring houses within a 100m radius according to standard procedures (WHO 2013). As many as 6 people were trained to apply the Human Landing Catch (HLC) and Resting Collection (RC) methods. In general, the HLC method involved the mosquito catcher carrying an aspirator and sitting at the capture location with one leg up to the calf open. Mosquitoes that land on their feet are sucked in using an aspirator. In contrast to HLC, the target of the RC method is mosquitoes that land on the walls of the house. The team was divided into two to catch mosquitoes inside and outside the house for 40 and 10 minutes to change the paper cup. Every 1 hour, the mosquitoes are put in a paper cup with a code and time of capture. RC was carried out from 06.00-07.30 to catch samples resting on walls, window curtains, mosquito nets, and hanging clothes, using an aspirator and net. The mosquitoes caught are put into a paper cup filled with cotton filled with sugar solution. The paper cup is placed in a container covered with banana stems and a wet towel to maintain optimum temperature and humidity of 27±2°C and 80±10%, respectively. The captured mosquito samples were held for twelve days and fed a 10% sugar solution before examination.

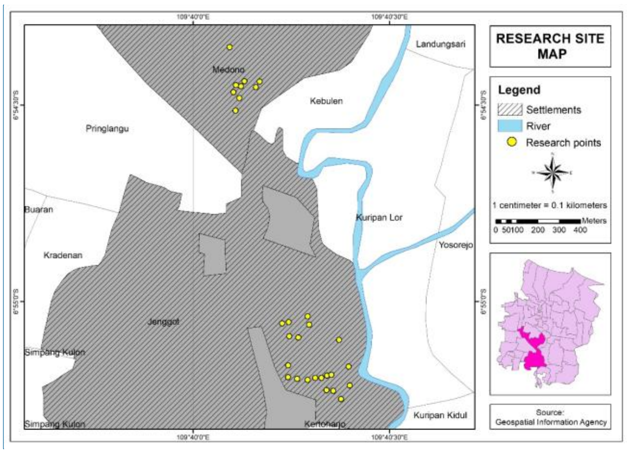

Figure 1. The Map of Research Sites

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Chakim I. Sayono, Astuti R. 2017. High Levels of Resistance in a *Culex quinquefasciatus* Population to the Insecticide Permethrin in Filariasis Endemic Areas in Central Java. Makara Journal of Science. 21(4):149-154. DOI: 10.7454/mss.v21i4.5664

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So that the map of the research location can be customised

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Identification of mosquito species and ovary dilatation

Mosquito species were identified using previously published identification keys (Rattanarithikul et al. 2005; Nugroho and Mujiyono 2021). The parosity was determined using a surgical method to determine the amount of ovarian dilatation. The identified mosquito is carefully positioned within a petri dish, where the wings and legs are separated from the body. The specimen is delicately positioned onto a glass apparatus, where physiological NaCl solution is precisely administered. Additionally, surgery is performed using a surgical needle and carried out using a stereo microscope. The surgical needle in the left and right hands presses the chest and the 7th segment. The ovaries were placed on a glass object given distilled water to view the tracheoles using a stereo microscope with a magnification of 40x10.

Microfilaria detection

The wings of the mosquito slated for dissection were incised with precision to prevent the dispersion of scales, ensuring the integrity of the microscope's field of view. Mosquitoes were placed on glass slides dripped with physiological saline. The thorax and abdomen were cut into pieces with a dissecting needle and observed under a microscope at 40X magnification. Meanwhile, the presence of filarial worms appears to move depending on the stage. In this context, stages 1-2 are short, fat, and slow-moving, while stage 3 appears long and fast-moving (Laney et al. 2010). Microfilaria detection also uses molecular methods with the stages of DNA extraction, amplification, electrophoresis, and imaging.

DNA extraction

A total of 10-20 female mosquitoes from each location were pooled based on species. Each pool was homogenized with a pestle in a microcentrifuge tube containing 180 µl of ATL buffer (pH 7.2; PBS) and ground, 20 µl of proteinase K, vortexed, and incubated for 24 hours. In the subsequent step, the specimen was vortexed for 15 seconds, and 200 µl AL buffer was added. A total of 600 µl of extract samples were put into a mini-column and centrifuged for 1 minute at a speed of 8000 rpm. Approximately 500 µl of Aw1 was added to the specimen, and centrifuged for 1 minute, before adding 500 µl of AW2 buffer and centrifuged at 14,000 rpm for 3 minutes. The sample was transferred to a 1.5 ml tube and 60 µl AE buffer was added, incubated for 1 minute, and centrifuged at 8000 rpm for 1 minute.

DNA amplification with polymerase-chained reaction

The extracted mosquito DNA was amplified using a thermal cycler (Perkin-Elmer Cetus, Norwalk, Connecticut, USA) with 2 oligonucleotide primers, NV-l: 5' CGTGATGGCATCAAAGTAGCG 3' (21-mer) and NV-2: 5'CCCTCACTTACCATAAGACAAC 3' (22- mer). Each amplification reaction was carried out in a final volume of 50 and contained 10 ~ I-IMT ri-HCl pH 9.2, 1.5 mM MgCl, 75 mM KCl, 1.25 mM each deoxy-nucleotide triphosphate, 10 pmol each primer NV-l and NV -2, and 2 units of Taq polymerase. Furthermore, the temperature program for PCR was 5 minutes at 95°C, followed by 35 cycles of 1 minute each at 94°C, 55°C, and 72°C, and an elongation of 10 minutes at 72°C. A total of 20% of the PCR product from each sample was electrophoresed on a 2% agarose gel and stained with ethidium bromide to confirm amplification (Ramzy et al. 1997).

RESULTS AND DISCUSSION

A total of 1,678 mosquitoes were obtained in two capture shift, namely 1,197 and 581, with a proportion of female sex and parity of 66.25 and 86.00% as well as 63.37 and 70.95%. The majority (61.49 and 65.72%) of mosquitoes showed ≥ 4 ovarian dilatations or a lifespan of 12-16 days, which is an estimate based on the understanding that a gonotropic cycle is approximately 3-4 days (Fereda 2022). This research applied the mosquito-catching method with HLC and resting catch to obtain a high composition of female mosquitoes and parosity. Gravid female mosquitoes suck blood to meet the protein needs for the development of embryos in eggs. In tropical regions, blood-feeding behavior persists year-round, but in temperate regions, gravid mosquitoes are breeded only during the spring season (Siperstein et al. 2023). Therefore, air temperature influences mosquitoes in their mating and pregnancy behavior. A total of five species were found in the first fishing period, and only three were reported in the second period with the proportion being *Cx. quinquefasciatus* (69.59 and 65.40%), *Cx. tritaeniorhyncus* (5.76 and 0.00%), *Cx. vishnui* (5.85 and 27.54%), *An. vagus* (0.58 and 0.00%), and *Ae. aegypti* (18.21 and 7.06%). The proportion of female mosquitoes in the first and second captures based on species is *Cx. quinquefasciatus* (60.78 and 59.78%), *Cx. tritaeniorhyncus* (8.70 and 0.00%), *Cx. vishnui* (8.83 and 32.06%), *An. Vagus* (0.88 and 0.00%), and *Ae. aegypti* (20.81 and 8.22%) (Table 1 and Table 3). These data show that *Cx quinquefasciatus* is the dominant species in the locations, while *Cx. tritaeniorhyncus* and *An. vagus* was found on the second shift of mosquito catching. The dominance of *Cx. quinquefasciatus* in LF endemic areas was also reported in several research such as in Tanzania (Derua et al. 2017, Lupenza et al. 2021), and Gamapaha, Sri Lanka (Pilagolla and Amarasinghe 2021). Similar findings were reported in Bogor Regency, West Java (Nirwan et al. 2022), Pekalongan Regency, Central Java (Nurjazuli et al. 2022), and Tangerang (Prasetyowati et al. 2019). However, several research report different dominant vector species of LF in endemic areas such as *Armigeres subalbatus* in Musi Rawas, South Sumatra (Mulyaningsih et al. 2019), *Ae. scutellaris* and *Ae. kochi* in the South Pacific, as well as *An. gambiae*, *A. funestus*, and *An. punctulatus* in rural Asia and Africa (Bhuvaneswari et al. 2023). Differences in species dominance are influenced by habitat conditions, specifically the presence of aquatic plants (Pratiwi et al. 2019), chloride content, and water temperature (Amini et al. 2020).

Table 1. Distribution of mosquitos based on characteristics and research sites (1st period of mosquito capture)

Variables	Research Sites			
	Medono		Jenggot	
	n	%	n	%
Mosquito species and sex				
Cx. quinquefasciatus	280	33.61	553	67.39
Male	124	44.39	227	41.09
Female	156	55.61	326	58.91
Cx. tritaeniorhyncus	31	44.93	38	55.07
Male	0	0.00	0	0.00
Female	31	100.00	38	100.00
Cx. vishnui	29	41.43	41	58.57
Male	0	0.00	0	0.00
Female	29	100.00	41	100.00
An. vagus	0	0.00	7	100.00
Male	0	0.00	0	0.00
Female	0	0.00	7	100.00
Ae. aegypti	83	38.07	135	61.93
Male	17	20.62	36	26.67
Female	66	79.38	99	73.33
Parity	103	33.99	200	66.01
Nulliparous	23	22.33	88	44.00
Parous	80	77.67	112	56.00
Number of ovary dilatation (age in days)	80	41.67	112	58.33
1 (4)	0	0.00	8	7.14
2 (8)	5	6.25	14	12.50
3 (12)	18	22.50	29	25.89
4 (16)	14	20.48	7	6.25
5 (20)	18	22.50	10	8.93
6 (24)	6	7.50	26	23.21
7 (28)	19	23.75	18	16.07

Table 2. Results of microscopic microfilariae detection of female mosquitoes (1st period of mosquito capture)

No.	Species	Research Sites			
		Medono		Jenggot	
		n	%	n	%
1	*Cx. quinquefasciatus*	50	50.00	50	50.00
	Positive	0	0.00	0	0.00
	Negative	50	100.00	50	100.00
2	*Cx. tritaeniorhyncus*	10	50.00	10	50.00
	Positive	0	0.00	0	0.00
	Negative	10	100.00	10	100.00
3	*Cx. vishnui*	10	50.00	10	50.00
	Positive	0	0.00	0	0.00
	Negative	10	100.00	10	100.00
4	*An. vagus*	-	-	7	100.00
	Positive	-	-	0	00.00
	Negative	-	-	7	100.00
5	*Ae. aegypti*	10	50.00	10	50.00
	Positive	0	0.00	0	00.00
	Negative	10	100.00	10	100.00

Figure 2. *Wuchereria bancrofti* larvae were detected from four mosquito samples of *Cx. quinquefasciatus* collected from Jenggot village, Pekalongan City

The results of catching mosquitoes showed different results, where the first period produced greater numbers. This shows that mosquito abundance varies over time but does not correlate with microfilaria findings. The surgery and PCR examination results of mosquito samples from the first capture period did not report microfilariae (Table 2). In the second period of capture, fewer numbers and species were obtained, namely 581 mosquitoes (Table 3). The results of dissection after holding for 12 days found that 4 out of 298 (1.34%) mosquitoes contained L3 microfilariae, with species *W. bancrofti* (Figure 2). The microfilariae were detected from mosquito samples aged 12-20 days (ovarian dilatation 4 and 5) with proportions of 25% and 75%. Therefore, microfilariae are found in mosquitoes subjected to 4-5 periods of sucking blood. Detection of microfilariae in mosquito vectors in Indonesia is still limited. There is still an infectious vector of LF in Pekalongan City, Central Java, even with a higher percentage compared to findings in other areas (Derua et al. 2017, Lupenza et al. 2021) (Nurjazuli et al. 2022). In addition, the area still has a potential for LF transmission with the main vector being the *Cx. quinquefasciatus*. Apart from additional data on the number of microfilaria infections in mosquito vectors, the findings also confirm the role of *Cx. quinquefascitus* as the dominance vector of LF in Pekalongan. *W. bancrofti* is the microfilariae most frequently found in mosquito vectors (Kinyatta et al. 2023), specifically *Cx. quinquefasciatus*. The finding also follows similar reports in Tanzania where the species is the main vector of LF (Lupenza et al. 2021). The variation in species and proportion of mosquitoes is also similar to other reports where *Cx. quinquefasciatus* is the dominant species in LF endemic areas (Derua et al. 2017, Lupenza et al. 2021). In the local context, this research found more mosquito species than previous findings at other nearby locations (Nurjazuli et al. 2022). The findings show the high potential for LF transmission in the region. The majority of *Cx. quinquefasciatus* is a female with a high proportion of parity and an estimated lifespan of more than 12 days. These findings show a higher proportion of mosquitoes with ovarian dilatations ≥ 4 than the number. The data reports that the majority of female Culex mosquitoes have a high potential to become competent vectors when in the first or second gonotrophic cycle to obtain microfilariae from the blood of parasitemia sufferers. The probability is

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supported by the observation that all L3 microfilariae originated from mosquito samples showing an ovarian dilation range of 4-5 times or 12-20 days old. This discovery is consistent with previous findings showing an extrinsic incubation period of 13 days for ~~extrinsic~~ microfilariae within the body to transition to the L3 stage and migrate to the salivary glands (Xu et al. 2018, Dharmarajan et al. 2019). The potential for LF transmission is increasing with climate change, where the average daily air temperature shortens the extrinsic incubation period for microfilariae (Simon et al. 2017).

The results of nucleotide substitution analysis conducted on the ace1 and *Cx. quinquefasciatus* VGSC genes serve as indicative markers for assessing the insecticide resistance status within the mosquito population. Part (A) in Figure 3 shows the results of nucleotide consistency at codon 119 of the ace1 gene where DNA samples show the same wildtype base arrangement, namely GGC (Valine amino acid). Meanwhile, part (B) is a chromatogram analysis of the base arrangement. Part (C) is the results of consistency in the nucleotide sequence in codon 1014 of the VGSC gene where there are three forms of nucleotide substitution from T to C, T to G, and A to T in the first, second base, and third bases, respectively. There are three nucleotide substitution variations in codon 1014 of the VGSC gene of *Cx. quinquefasciatus* from the formation of wild-type leucine (TTA) to phenylalanine (TTT) and Cysteine (TGT), as well as a silent mutation from TTA to CTA (Table 4). The first change is a form of silent mutation where TTA to CTA does not change the amino acid, namely Leucine. The second change results in an amino acid change from Leucine (TTA) to Cysteine (TGT) while the third leads to a change in Phenylalanine (TTT) (Figure 2). The L1014F and L1014C mutations (Figure 4) show homozygous and heterozygous forms. Parts A and B are homozygous and heterozygous forms of changing Leucine (TTA) to Phenylalanine (TTT) and Cysteine (TGT), while C and D are homozygous and heterozygous forms of changing Leucine (TTA) to Cysteine (TGT). Image B is a heterozygous form where T and G bases are at the same locus but T is more dominant. The mutation analysis of the VGSC codon 1014 gene shows that the proportion of mosquitoes resistant to insecticides in the population is 98-100%. The utilization of insecticides remains prevalent, with rates ranging from 85% to 92.5%, and some families (63.3-65.0%) still use it for more than two years. Moreover, daily usage intensity ranges from 40% to 53.3%, with particular emphasis on the pyrethroid group at 80% to 81.7% as well as burnt coil formulations, which account for 58% to 60% of usage (Table 5). Reports of resistance to Culex mosquito species, specifically *Cx. quinquefasciatus* is still limited. This finding complements previous data where *Cx. quinquefasciatus* in Central Java has been resistant to pyrethroid insecticides (Chakim et al. 2017). However, research in Jakarta reported that the species was still susceptible to pyrethroids and organophosphates (Subahar et al. 2022). According to recent data, *Cx. quinquefasciatus* in Surabaya has been resistant to both groups of insecticides with mutations in the VGSC and ace-1 genes (Panjinegara et al. 2022). Therefore, Culex mosquito resistance to insecticides has become increasingly widespread. The phenomenon of expanding the resistance area of *Cx quinquefasciatus* was also reported in Bengal, India (Viswan et al. 2020, Rai et al. 2019), Cameroon (Talipouo et al. 2021), Brazil (Lopes et al. 2019), Nigeria (Omotayo et al. 2023), and Korea (Jeon et al. 2024). Molecular analysis of mosquito samples showed four genotypes of the VGSC gene, namely wild type, silent mutation, and mutant (Leu-Phe and Leu-Cis). The Leu-Phe mutation is the most frequently reported mutant form in Indonesia (Panjinegara et al. 2024) and other countries (Talipouo et al. 2021). This is different from the findings in Surabaya which reports wildtype, mutant-heterozygous, and mutant-homozygous genetic variations (Panjinegara et al. 2024). A serious genetic mutation was reported in Brazil including esterase, ace-1, and VGSC (Lopes et al. 2019). Research in Korea did not obtain the G119S mutation but reported heterozygous forms of AGC/GGC, L1014F, and L1014S (Jeon et al. 2024). This genetic mutation phenomenon shows a serious problem affecting vector control efforts in LF-endemic areas.

Table 3. Distribution of Mosquito Characteristics from Jenggot Village (2nd Period of Mosquito Capture)

Characteristics of mosquitoes	n	%
Mosquito species and sex		
Cx. quinquefasciatus	380	65.40
Male	82	21.58
Female	298	78.42
Cx. vishnui	160	27.54
Male	0	0.00
Female	160	100.00
Ae. aegypti	41	7.06
Male	0	0.00
Female	41	100.00
Total	581	100.00
Parity		
Nulliparous	86	29.05
Parous	210	70.95
Total	296	33.99
Number of Ovary Dilatation (Age of Mosquito; days)		
1 (4)	7	3.33
2 (8)	18	8.57
3 (12)	47	22.38
4 (16)	43	20.48
5 (20)	47	22.38
6 (24)	23	10/95
7 (28)	25	11.90
Total	210	100.00
Microscopic detection of microfilariae in female mosquitoes		
Cx. quinquefasciatus	298	59.72
Positive	4	1.34
Negative	294	98.66
Cx. vishnui	160	32.06
Positive	0	0.00
Negative	160	100.00
Ae. aegypti	41	8.22
Positive	0	0.00
Negative	41	100.00
Total	499	100.00

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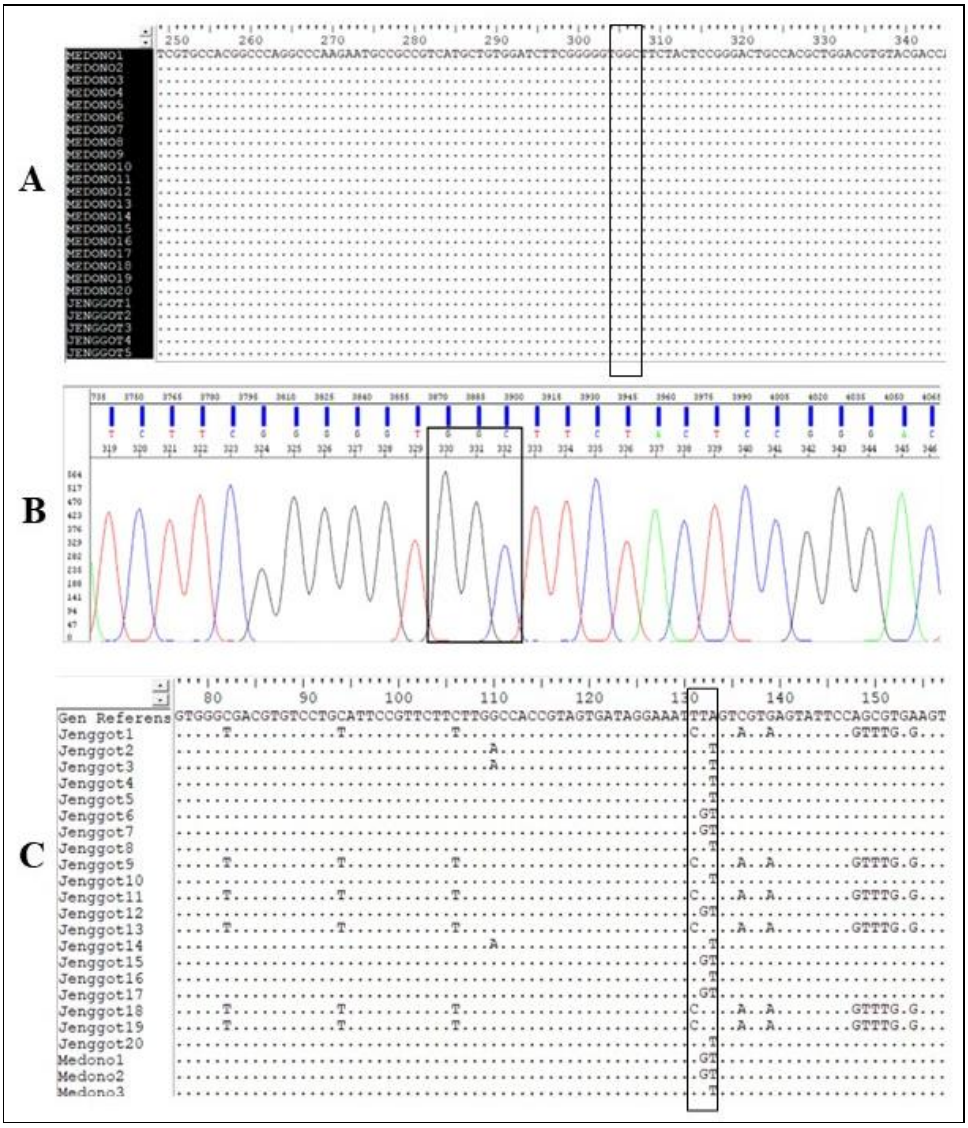

Figure 3. Results of nucleotide substitution analysis of the ace1 and VGSC gene of *Cx. quinquefasciatus* as an indication of the insecticide resistance status of the mosquito population. No nucleotide substitutions were found in codon 119 of the ace1 gene (A) and the chromatogram shape of codon 119 (B). Part C is a variation of nucleotide substitution in codon 1014 of the VGSC gene which shows three variant changes, namely TTA-CTA (silent mutation), TTA-TTT, and TTA-TGT

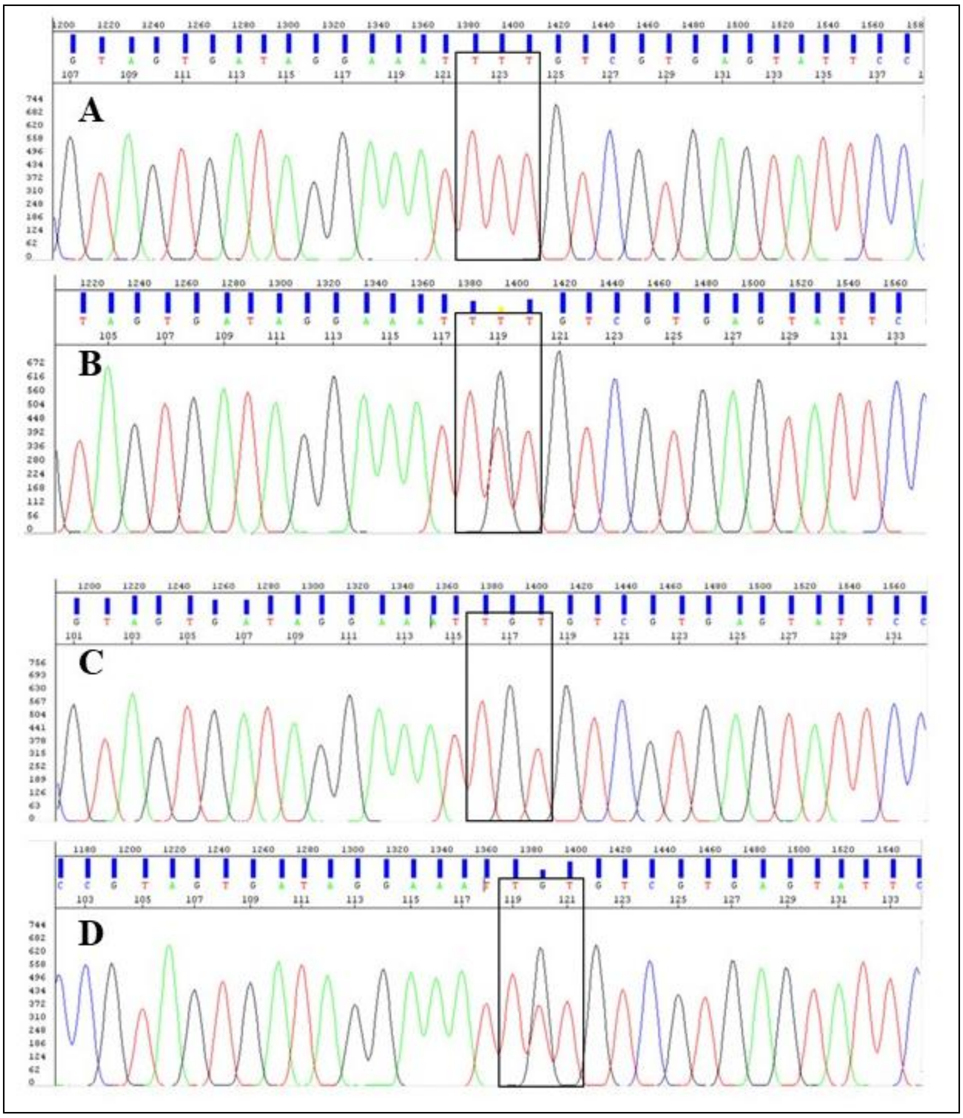

Figure 4. Homozygous and heterozygous nucleotide substitution in codon 1014 of VGSC gene of *Cx. quinquefasciatus*. A and B are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Phenylalanine (TTT) and Cysteine (TGT), while C and D are homozygous and heterozygous forms of changing the amino acid Leucine (TTA) to Cysteine (TGT). Image B is a heterozygous form where the bases T and G are at the same locus, although T is more dominant. The opposite condition occurs in image D

Table 4. Frequency of VGSC gene mutations among *Cx. quinquefasciatus* mosquito

Nucleotide substitution	Type of mutation			
	Homozygous	%	Heterozygous	%
TTA to TTT	33	55.00	16	26.67
TTA to CTA	1	1.67	0	0.00
TTA to TGT	4	6.67	6	10.00

Table 5. The insecticide resistance status and history of insecticide use in research sites

Variabel	Research sites			
	Medono		Jenggot	
	n	%	n	%
Resistance Status				
Sensitive	0	0	1	2
Resistance	20	100	59	98
The Use of Household Insecticide				
Yes	37	92.50	51	85
No	3	7.50	9	15
Insecticide Group				
Organophosphate	8	20	11	18.33
Pyrethroid	32	80	49	81.67
History of Insecticide Use (years)				
< 2	14	35	22	36.67
≥ 2	26	65	38	63.33
Insecticide Use Intensity				
Not everyday	24	60	28	46.67
Everyday	16	40	32	53.33
Insecticide Formulation				
Coil	24	60	35	58.33
Spray	6	15	10	16.67
Electric	2	5	4	6.67
Repellent	8	20	11	18.33

In conclusion, *Cx. quinquefasciatus* was the dominant species among the five vector mosquitos in the LF endemic area of Pekalongan District, and 1.34% were proven to carry *W. bancrofti* microfilariae. Therefore, the area was vulnerable to LF transmission and the condition was increased by *Cx. quinquefasciatus* resistance against pyrethroid and organophosphate class insecticides. Molecular analysis of the VGSC gene found the wild-type allele and three mutant alleles, namely TTA to CTA, TTA to TTT, and TTA to TGT mutations. Further investigation should be conducted to detect the susceptibility of microfilariae to various antiparasitic drugs and educate the public in implementing methods of self-protection from exposure to mosquitoes accompanied by environmental cleaning movements.

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Link: https://smujo.id/biodiv/authorDashboard/submission/17511

Ahmad Dwi Setyawan

[Kutipan teks disembunyikan]

Sayono Sayono <say.epid@gmail.com> 10 Mei 2024 pukul 14.54
Kepada: Dianti <dianti@smujo.id>, Ahmad Dwi Setyawan <editors@smujo.id>

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Sayono Sayono <say.epid@gmail.com>

[biodiv] Editor Decision

1 pesan

Smujo Editors via SMUJO <support@smujo.com> 16 Mei 2024 pukul 20.39
Balas Ke: Smujo Editors <editors@smujo.id>
Kepada: ABDUL GHOFUR <omopung@gmail.com>, SUHARYO HADISAPUTRO <prof_haryo@yahoo.co.id>, SAYONO SAYONO <say.epid@gmail.com>

ABDUL GHOFUR, SUHARYO HADISAPUTRO, SAYONO SAYONO:

The editing of your submission, "Detection of microfilaria L3 and insecticide resistance among wild-caught mosquito vectors in endemic areas of lymphatic filariasis," is complete. We are now sending it to production.

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/17511

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