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Original Research

The Polymorphism of Voltage-Gated Sodium Channel Gene in Culex Mosquitoes in Banyuwangi District, Indonesia

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ABSTRACT

Culex mosquito is able to act as transmission vector of filariasis and Japanese encephalitis. Both diseases occur in Indonesia and filariasis exists in Banyuwangi district. The *Voltage-Gated Sodium Channel* (*VGSC)* gene mutations are related to pyrethroid insecticide resistance, especially L1014F/S mutations have not yet been fully studied in Indonesia. This study aimed to investigate the polymorphism of *VGSC* gene mutation in *Culex* mosquitoes in Banyuwangi District of East Java Province, Indonesia. *Culex* mosquitoes were collected using mosquito trap in a two-day collection from Banyuwangi Sub-district and two sub-villages in Cluring Sub-district of Banyuwangi District in August 2023. *Culex* mosquitoes were identified by morphological examination and *Culex* DNA was extracted by Chelex100 from each *Culex* mosquito. The polymorphism of *VGSC* gene mutations of L1014F/S were determined by Allele Specific-Polymerase Chain Reaction (AS-PCR). A total of 62 *Culex* mosquitoes were analyzed. The wild type *Culex* mosquitoes were found predominant (77%, 48/62). Two types of homozygous mutations were identified as 1014F and 1014 S (16%, 10/62 and 2%, 1/62, respectively). The heterozygous mutation was only 1014F/S type with frequency of 5% (3/62). The predominance of wild type *Culex* mosquitoes indicated that *Culex* population in the study areas is still susceptible to pyrethroid insecticide. However, the mutant types of *Culex* are already occurred, so insecticide resistance among *Culex* mosquitoes is required to be regular monitoring.

Keywords: *Culex, Voltage-Gated Sodium Channel* gene, Polymorphism, Banyuwangi, Indonesia

INTRODUCTION

Culex mosquitoes are among the most common mosquito species in the world. Mosquito-borne diseases transmitted through *Culex*, for instance, are lymphatic filariasis and Japanese encephalitis (Wang and Zhu, 2023). Both diseases occur in Indonesia and lymphatic filariasis is endemic in 236 districts/cities within 28 provinces. Although East Java is one of the provinces where filariasis is not considered endemic, chronic filariasis cases have been found in many districts/cities (Kementerian Kesehatan RI, 2021). In 2022, there are 180 documented cases of chronic filariasis in 30 districts/cities, 12 of them are in Banyuwangi district[3] (Dinas Kesehatan Provinsi Jawa Timur, 2022). Mosquitoes from the genus *Culex* act as the major vectors for the filarial helminth *Wuchereria bancrofti*, which is one of the 3 filarial helminth species found in Indonesia along with *Brugia malayi* and *Brugia timori* (Ai and Vythilingam, 2014; Bhattacharya and Basu, 2016; Nasution et al, 2018)

Because of the non-endemic status of East Java province, there is no Mass Drug Administration (MDA) program in this area for lymphatic filariasis[3]. Furthermore, the lack of vaccine as a preventive measure against many mosquito-borne diseases including filariasis, makes management of vector population the main strategy of disease control. Long term use of chemical insecticides such as DDT and pyrethroids to combat mosquito vectors has raised concern for insecticide resistance (Liu et al, 2006) Although no specific programs targeting filariasis vectors exists in this area, pyrethroids insecticides are vastly used to control dengue vectors. Thus, development of insecticide resistance in non-targeted species like *Culex* is of concern.

There are several known mechanisms leading to this resistance. One of the main mechanisms is target site insensitivity resulting from mutation at the target receptor where the insecticide binds (Khan et al, 2020) Target site insensitivity against DDT and pyrethroids happens through *knockdown resistance (kdr)* mutations of the *voltage-gated sodium channel (VGSC)* gene. This mutation reduces the sensitivity of the nervous system to DDT and pyrethroids through structural modification of the sodium channel (Liu et al, 2006; Scott et al, 2015). Mutation in the 1014 position of *VGSC* gene from Leucine to Phenylalanine (L1014F) is the most frequently reported *kdr* mutation found in *Culex*. The L1014S mutation (Leucine to Serine) has been found in *Culex pallens*, *Culex pipiens*, and *Culex quinquefasciatus* (Scott et al, 2015) These mutations have not yet been fully studied in Indonesia. This study aimed to investigate the polymorphism of *VGSC* gene mutations of *Culex* mosquitoes in Banyuwangi, East Java, Indonesia.

METHODS

Study sites

Culex populations were collected from Banyuwangi Sub-district (BWI) and two sub-villages in Cluring Sub-districts of Banyuwangi District. The two sub-villages in Cluring Sub-district are Purwosari and Pancursari. Both sub-villages will be called B1 and B2, respectively. Banyuwangi Sub-district (population density 3,958/km2) resides in a more urban area compared to Cluring (population density 805/km2) (BPS Kabupaten Banyuwangi, 2015, 2023). As of 2021, there were 6 reported cases of chronic filariasis in Banyuwangi sub-district and 0 cases in Cluring. Banyuwangi District has a tropical climate with two seasons, alternating between dry season (April-September) and rainy season (October-March) (Dinas Kesehatan Kabupaten Banyuwangi, 2021). The molecular analysis was done in Institute of Tropical Disease Universitas Airlangga.

Mosquito collection

Adult mosquitoes were obtained in a 2-day collection from each site using SBT (Simple Build Trap) in August 2023. The traps were put indoor and outdoor. The collection was done twice a day, day trap was set from 8 a.m.-8 p.m. and night trap was set from 8 p.m.-8 a.m. Samples from each site were grouped according to the time of collection (day/night) and location (indoor/outdoor).

Culex mosquito identification and DNA extraction

Culex mosquitoes were examined morphologically and identified using dissecting microscope at the field. They were separated into male and female *Culex* group, then from each mosquito, the abdomen was separated from the rest of the body. DNA was extracted from each *Culex* mosquito abdomen using Chelex-100 5% (w/v).

Detection of kdr mutation and genotyping

Detection of two *kdr* mutations (L1014F and L1014S) was achieved by performing Allele-Specific Polymerase Chain Reaction (AS-PCR) assay using the extracted DNA individually following the established protocol (Martinez-Torres et al, 1999; Sarkar et al, 2009; Rai and Saha, 2022). Five primers were selected for the assay, two of them [Cgd1 (Forward), 5-GTGGAACTTCACCGACTTC-3 and Cgd2 (Reverse), 5-GCAAGGCTAAGAAAAGGT

TAAG-3] were common primers used as internal control for the PCR reaction and to amplify the fragment of the *VGSC* gene. The other three primers [Cgd3 (Forward), 5-CCACCGTAGTGATAGGAAATT

TA-3; Cgd4 (Forward), 5-CCACCGTAGTGATAGGA

AATTTT-3; and Cgd5 (Forward), 5-CCACCGTAGTGATAGGAAATTC-3] were allele specific primers used in genotyping *kdr* mutations. These allele specific primers were identical except for the last codon at 3’-OH end where ‘TTA’ in Cgd3 (wild type) was replaced by ‘TTT’ in Cgd4 (L1014F mutation) and ‘TTC’ in Cgd5 (L1014S mutation). Four PCR reactions which contain the combination between primer Cgd1 and Cgd2, Cgd2 and Cgd3, Cgd2 and Cgd4, then Cgd2 and Cgd5 were run in parallel for each DNA specimen. The DNA fragments were separated by gel electrophoresis on agarose gel and visualized by ethidium bromide staining under UV light. The genotypes were identified by the characteristic 380 bp band corresponding to the susceptible (Cgd3) and resistant (Cgd4 & Cgd5) specific primers. The presence of this band only in susceptible primer (Cgd3) indicated homozygous susceptible/wild type (LL) and in resistant specific primers (Cgd4 only or Cgd5 only) indicated homozygous resistant (FF or SS). This 380 bp PCR product in both susceptible and resistant primer or two different resistant primers of an individual mosquito indicated heterozygous resistant.

Data analysis

Culex mosquito distributions in Banyuwangi were described based on sex, time and location of collection. The frequencies for the susceptible/wild type (L) and resistant alleles for each of the point mutations (F or S) were described and analyzed with Hardy-Weinberg Equilibrium (HWE) test.

RESULTS

Culex mosquito distribution

A total of 62 *Culex* adult mosquitoes were collected from three sites of the two sub-districts in Banyuwangi district. The collected *Culex* population were mostly found indoor (73%, 45/62) and during night-trapping (65%, 40/62). Female *Culex* mosquitoes were predominant (61%, 38/62). More *Culex* mosquitoes were found in rural areas [B1 (45%, 28/62) and B2 (40%, 25/62)] compared to urban area [BWI (15%, 9/62)]. Details of the sample distributions were provided in Table 1.

Detection of kdr mutations, genotyping and allele frequencies

The AS-PCR assay displayed the presence of kdr mutations (leucine-phenylalanine and leucine-serine) in the studied population of Culex. The majority of the Culex population consisted of homozygous susceptible/wild genotype mosquitoes (77%, 48/62) as seen on Table 2. Two types of homozygous mutations were observed, FF (16%, 10/62) and SS (2%, 1/62). L1014F mutation with homozygous (FF) genotype was found to occur in all study sites and L1014S mutation with homozygous (SS) genotype was only found in B2. Of the three possible heterozygous genotypes, only FS was observed in the population (5%, 3/62) at two study sites (BWI and B1) (Figure 1).

Table 1. Distribution of *Culex* in the study area

Mosquito collection		**BWI**		**B1**		**B2**			**N (%)**
		F	**M**	**F**	**M**	**F**	**M**		
Day-trap	In-door		5	2	5	7	2	21	22 (35%)
	Out-door			1				1	
Night-trap	In-door	1	3	12	4	4		24	40 (65%)
	Out-door			1	3	10	2	16	
		1	8	16	12	21	4		62 (100%)
		9 (15%)		28 (45%)		25 (40%)			

BWI is Banyuwangi sub-district, B1 is Purwosari sub-village, B2 is Pancursari sub-village. F: female. M: Male

Allele frequencies were closely similar in all study sites with wild allele (L) predominating, between 76-78%. Leucine-Phenylalanine mutation allele (F) was the second highest ranging from 17-20%, and Leucine-Serine mutation allele (S) was the rarest (4-6%). The allele frequencies for BWI individuals followed HWE, but for B1 and B2 individuals they deviated significantly (P ≤ 0.05).

DISCUSSION

This study was undertaken as an initial investigation on the polymorphism of *VGSC* gene mutations of *Culex* mosquitoes in Banyuwangi. A total of 62 *Culex* mosquitoes were collected from the study sites (BWI, B1, and B2). The collected *Culex* population were mostly found indoor (73%, 45/62), this might correlate with the indoor resting habit of these species (Kaliwal et al., 2010; Seang-arwut et al., 2023).

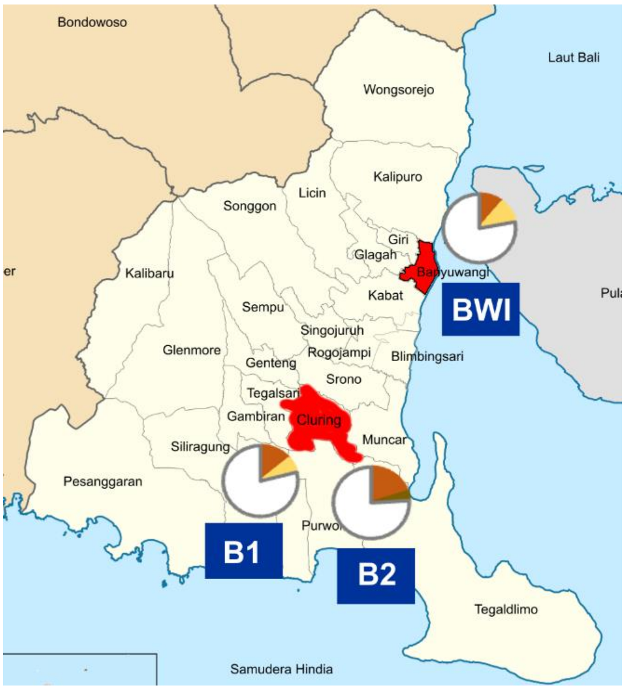

Figure 1. Distribution of *Culex* carrying the mutation in codon 1014 of *VGSC* gene at the study sites in Banyuwangi district. Brown circle indicates L1014F, the yellow is L1014F/S, the dark green is L1014S, and the white circle is L1014L. B1 = Purwosari sub-village, B2 = Pancursari sub-village, BWI = Banyuwangi sub-district.

Table 2. Allele frequencies of L1014 L/F/S *kdr* in *Culex* population

Location	**N**	***kdr* genotypes**						**Allele frequencies**			**P_{HWE}**
		L 1014						**L**	**F**	**S**	**L1014**
		LL	**LF**	**LS**	**FS**	**FF**	**SS**				
BWI	9	7	0	0	1	1	0	0.77	0.17	0.06	0.07
B1	28	22	0	0	2	4	0	0.78	0.18	0.04	0.00
B2	25	19	0	0	0	5	1	0.76	0.20	0.04	0.00
Total	62	48	0	0	3	10	1	0.77	0.19	0.04	0.00

LL = *wildtype*, LF, LS, FS = heterozygote, FF, SS = homozygote, P_{HWE} = *Hardy-Weinberg Equilibrium (P ≤0.05: significant)*

The majority of the population was also caught during night-trapping (65%, 40/62) corresponding to the *Culex* mosquito nocturnal activity (Anderson et al, 2007). The host-seeking behavior of female mosquitoes might explain the reason female mosquitoes were predominant compared to male (61%, 38/62 and 39%, 24/62, respectively). It is known that female mosquitoes of various species including *Culex* use multiple cues, both chemical and physical, such as body odor, CO_2, vision, heat and humidity to orient towards hosts (Coutinho-Abreu et al., 2022). More *Culex* mosquitoes were found in rural areas [B1 (45%, 28/62) and B2 (40%, 25/62)] compared to urban area [BWI (15%, 9/62)]. Although BWI is more densely populated, it is suspected that breeding sites were more prevalent in rural areas, such as ponds, puddles, containers and other stagnant bodies of water (Amara Korba et al., 2016; Liu et al., 2019).

In the present study, the presence of the L1014F and L1014S mutations of *VGSC* gene in *Culex* mosquitoes was reported for the first time in Banyuwangi district. L1014F mutation is the most commonly observed and studied *kdr* mutation, although L1014S, L1014C, and V1016G mutation have also been reported in *Culex* (Scott et al., 2015). Two types of homozygous mutations were observed from the AS-PCR assay, FF (16%, 10/62) and SS (2%, 1/62). L1014F mutation with homozygous (FF) genotype was found to occur in all study sites and L1014S mutation with homozygous (SS) genotype was only found in B2. L1014F mutation has been associated with high level of resistance to DDT and pyrethroids in *Cx. Quinquefasciatus* (Liu et al., 2009; Wang et al., 2011) and L1014S is associated with high levels of resistance to DDT and a low level of resistance to pyrethroids (Martinez-Torres et al., 1999). However, both frequency of homozygous mutations on genotypes FF and SS were lower than wild type in *VGSC* gene indicating that *Culex* population in Banyuwangi is still sensitive to pyrethroid insecticides. This might correlate with the local habit because based on our interview with the locals in the study sites, most people prefer to use electric fans to drive away mosquitoes compared to household insecticides. In comparison, a study conducted in 3 sub-districts of Surabaya where most of the residents commonly utilize household insecticides (95%, 95%, and 90%) found that the frequency of *VGSC* gene mutations of *Culex* is higher than wild type (LL 9.8%, FS 62.6%, FF 26.2%, LS 0.9%, and SS 0.5%) (Panjinegara et al, 2024).

The present study has produced a baseline data on the allele frequencies of *Culex* in Banyuwangi. Both resistant alleles (F and S) were observed in all study sites, these results suggest the emergence of insecticide resistance in Banyuwangi. The results ranged from 17-20% for F allele and 4-6% for S allele. The allele frequencies for BWI individuals followed Hardy-Weinberg Equilibrium, but for B1 and B2 individuals they deviated significantly ($P \leq 0.05$). HWE model acts as null hypothesis where the population is assumed to be unchanging. A significant result might indicate gene migration, mutation, natural selection, inbreeding or even problems with genotyping (Wiggiton et al, 2005). However, the sample size and design of our study did not allow to appropriately conclude the genetic equilibrium or evolutionary pattern of L1014 alleles in the *Culex* population.

As a result of the studied correlation between *kdr* mutations and insecticide resistance, the detection of these mutations can become a proxy for the emergence and prevalence of insecticide resistance in *Culex* population. Although the results obtained from this study refer to relatively few *Culex* samples, they represent a first effort to analyze the overall distribution of the 1014F and 1014S mutations in Banyuwangi. Drastic changes in this distribution in the future could serve as an early warning of the emergence or development of resistance status (WHO, 2022).

STRENGTH AND LIMITATION

This study presents the initial investigation of *kdr* mutations in *VGSC* gene of *Culex* in Banyuwangi and Indonesia in general for the first time. Unfortunately, it was not conducted concurrently with susceptibility bioassay, hence the study could not provide the correlation between the different types of *kdr* mutations and the degrees of insecticide resistance. However, the presence of these mutations might correlate with the emergence of insecticide resistance in the study area which is important for us to be aware of.

CONCLUSION

This study identified the presence of *kdr* mutations (L1014F/S) in *VGSC* gene of *Culex* in Banyuwangi. However, our study was not adequate to confirm the presence of insecticide resistance. Wild type *Culex* mosquitoes was dominant in the population, suggesting that *Culex* population in Banyuwangi is still susceptible to pyrethroid insecticides. Further studies that include larger samples, more study sites, DNA sequencing and susceptibility bioassay may help lift the veil on the emergence of resistance. Nevertheless, regular monitoring of insecticide resistance in *Culex* population is needed.

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AUTHOR’S CONTRIBUTIONS

YAA, HD, CCC, SAA, WPV, MAP, FG, CM, WJ and MF contributed in sample and data collection. BA wrote and edited the manuscript. F performed molecular analysis. LR and SP conceived the experimental design and performed data analysis. SB conceived the experimental design, data analysis and review the manuscript. All authors approved the final version of the manuscript.

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