



Resistance Status of Malaria Vector *An. sundaicus* and *An. subpictus* to Insecticide and Detection of Genotype Resistance using *Polymerase Chain Reaction (PCR)* in Sungai Nyamuk Village, Sebatik Island, Nunukan District, North Kalimantan

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ABSTRACT

Malaria remains as one of the serious health problems given priority by the Ministry of Health (MoH) of Indonesia. Vector resistance to insecticides is one of the problems in malaria vector control program in Indonesia. This study aimed to analyze the status of vector resistance Anopheles spp. to insecticides that have not and were already used in malaria vector control programs. The research was conducted in Sungai Nyamuk village, Sebatik Island, Nunukan District, North Kalimantan from January to December 2011. The results showed that An. sundaicus still vulnerable to the pyrethroid class insecticides (Deltamethrin of 0.05% and Permethrin 0.75%), organophosphates (Fenitrothion 1%), organochlorin (DDT 4%) and Carbamat (Bendiocarb 0.1%). The results also showed that An. subpictus were still vulnerable to the pyrethroid class insecticides (Lambdacyhalothrin 0.05%). In genotyping, An. sundaicus mosquito indication amounted to 75.8% resistant to DDT 4%, 29.4% to Permethrin 0.75%, 10% to Bendiocarb 0.1%. Whilst the Deltamethrin 0.05%, Fenitrothion 1% and Lambdacyhalothrin 0.05% showed no indication has been resistant. Indications of vector resistance status is a step of early awareness and is one of the critical success factors of malaria vector control programs. Re-use of DDT should be considered further as there are indications of a fairly high-resistance gene.

Keywords- Susceptibility test, DDT, Permethrin, *An. sundaicus*, *An. Subpictus*.

INTRODUCTION

Malaria remains as one of the serious health problems given priority by the Ministry of Health (MoH) of Indonesia. In Indonesia high transmission of malaria often occurs in areas of outside Java, Madura, and Bali. The MoH reported that from the total Indonesian population 255,881,112 in 2015, 66,529,089 (26% of population) lived in the malaria epidemic region.⁽¹⁾

Sebatik Island, Nunukan District, North Kalimantan Province, lies on the border of Indonesia and Malaysia. Sebatik Island is one of the malaria endemic regions in Nunukan District. The Health Agency of Nunukan District (2009) reported that the annual malaria incidence [AMI] was 12.53 per 1000 population in 2009, an increase from 10.58 per 1000 population in 2008.⁽²⁾ In 2013, 61 people in Sungai Nyamuk Village were tested positive for

Plasmodium falciparum from a population of 7,525 people with API 8.11 per 1,000 population.⁽¹⁾

Malaria vector control programs in Indonesia using on Long Lasting Insecticides Nets and Indoor Residual Spray (IRS). The focus of the IRS program is the density reduction of malaria vector *Anopheles* spp. within a short time period and spraying the unsustainable residual insecticides. Community who lives in malaria endemic areas preferred the chemical methods in malaria vector control efforts. The use of insecticide for a constantly and relatively long time will result in the emergence of resistant strains of mosquitoes to insecticides.

Vector resistance to insecticides is one of the problems in malaria vector control program in Indonesia. The success of malaria vector control programs in the Sungai Nyamuk Village depends on the resistance status of *Anopheles* spp. insecticides used. Data on the status of resistance of *Anopheles* spp. is a very important inputs for malaria vector control programs. This study aims to determine the resistance status of *Anopheles* spp. to the four classes of insecticides that have been used in malaria vector control programs. This study also aims to detect gene vector resistance to insecticides using *Polymerase Chain Reaction* (PCR) di Sungai Nyamuk Village, Sebatik Island, Nunukan District, North Kalimantan Province. The results are expected as the evidence base malaria vector control policy effectively, efficiently and on target in the area.

MATERIALS AND METHODS

Study Area. The topography of Nunukan District is dominated by coastal area (altitude <25 m above sea level). Mosquitoes were collected from Sungai Nyamuk Village, Sebatik Island. Sungai Nyamuk Village located in the border area of Sabah State of Eastern Malaysia. This area is characterized by tropical weather with average temperature 31°C, minimum temperature 22°C in January and Sept. and the maximum temperature 32.1°C in May.

Collection, mosquito maintenance, *Anopheles* species identification. Larvae of *Anopheles* spp. collection result of potential breeding habitat in

Sungai Nyamuk Village, Sebatik Sub District, Nunukan District, North Kalimantan Province from January to December 2011. Data of breeding places characteristics and density of *Anopheles* spp larvae. were recorded. The larvae of *Anopheles* spp. accommodated in the tray and maintained in the laboratory field. The coordinates of breeding habitats were recorded using a Global Positioning System (GPS) Garmin 76 CSX. Larvae survey results were maintained and used to identify mosquito species morphologically following the identification keys of O'Connor and Soepanto.⁽³⁾

Resistance Test of *Anopheles* spp. Malaria vector resistance test method was used a reference from WHO, namely WHO Susceptibility Test.⁽⁴⁾ This method used *Anopheles* spp. mosquito species, results of rearing larvae natural collection numbered 80-100 non-blood feed females aged 3-5 days. The test method of resistance of *Anopheles* spp. that were used as follows: (1) Aspirated 20-25 *Anopheles* spp. using aspirator into a tube with a green dot as many as four tubes, (2) Prepared 3 tubes with red dot then inserted to special impregnated paper for *Anopheles* spp. which was DDT 4%, Fenitrothion 1%, Deltamethrin 0.05%, Bendiocarb 0.1%, Permethrin 0.75%, Lambdacyhalothrin 0.05% (WHO 2013), (3) Prepared one tube with a green dot and then put the non-insecticide paper as a control, (4) mosquitoes in the tube dot green color was contacted to the red dot tube for 1 hour (except Lambdacyhalothrin 0.05% for 2 hours), the same treatment also for mosquito control, (5) Upon contact, the mosquitoes was transferred back into the green dot tube by blowing, then mortality mosquito test was observed and recorded at 30 minutes, 1 hour and 24 hours after contact, (6) After 24 hours observation, all mosquitoes were inserted into the ependorf 1.5 ml tube with silica gel, (7) During the observations temperature and humidity of the room were also recorded.

The observation result of mortality mosquito test after 24 hours using resistance criteria by the WHO (2013) were as follows: mortality mosquitoes test > 98% = susceptible; mortality mosquitoes test 80-97% = tolerance (resistance suspected); mortality

mosquitoes test <80% = resistant (resistant individuals present).

Test of Gene Resistance Vector Detection through Insecticides using Polymerase Chain Reaction (PCR). The *Anopheles* spp. mosquito during the WHO Susceptibility Test, test of detection of gene vector resistance test to insecticides was continued using PCR, with several stages which were (a) DNA extraction of *Anopheles* spp. Mosquitoes to be tested were placed one by one in 1.5 mL ependorf tubes, PBS 7.4 mL of 100 was added, then crushed the mosquito using an electric grinder. Once the mosquito destroyed, then centrifuged 12,000 rpm for 1 minute, then the supernatant was discarded. Wash using aquabidest for 1 times. Add Instagene Matrix 100 mL kit, and then incubated with a temperature of 56 ° C for 45 minutes. Afterwards, the tube was centrifuged 12,000 rpm for 10 seconds. Place the tube into the hot block (temperature of 100 ° C) for 45 minutes. Tube was then centrifuged 12000 rpm for 1 minute. Mosquito homogenate was stored at -20 ° C until it is time to be tested. (b) The process of making molds agarose. Scale 2 grams of agarose. Dissolve in Buffer TBE 0.5, stir for 1 minute or until agarose completely dissolved. Then added ethidium bromide 0.5 µg/ml with a concentration of 10 mg/ml. Pour into agarose mold to make the plate, allow ± 20 minutes, so that agarose solidifies. (c) DNA electrophoresis process

of *Anopheles* spp. In the agarose mold, put 10 µl of sample 3 µl standard 100 bp DNA Ladder. Gel works at 100 V for 30 minutes. Check the DNA under ultraviolet light by comparing DNA Ladder.

Data analysis. Data were analyzed by descriptive and analytic based on the characteristics. Descriptive analysis with frequency distribution table, numerical descriptions, graphics and images; while the analytic analysis using appropriate statistical tests.

RESULTS AND DISCUSSION

Examination of vector vulnerability test (*Anopheles* spp.) against six types of insecticides can be seen in Table 1. These results indicate that the population of *An. sundaicus* mosquito is still vulnerable (susceptible) to the pyrethroid class insecticides (Deltamethrin of 0.05% and Permethrin 0.75%), organophosphate (Fenitroton 1%), organochlorin (DDT 4%) and Carbamat (Bendiocarb 0.1%). This is shown by the number of mortalities (mortality) 24 hours was 100%. Similar results were shown by *An. subpictus* which is still vulnerable (susceptible) to the pyrethroid class insecticides (Lambdacyhalotrin of 0.05%). WHO (2013) stipulates that the mosquito population is said to be vulnerable to insecticides if mortality reaches 98% or more, tolerant if mortality between 80-97%, and resistant if mortality is less than 80%.

Table 1 Data percentage mortality of mosquitoes in Sungai Nyamuk Village, Sebatik Island, Nunukan Distric, North Kalimantan, Januari - Desember 2011

Species tested	Mortality in 24 hours (%)						Indicator
	Deltamethrin 0.05%	Bendiocarb 0.1%	Permethrine 0.75%	DDT 4%	Fenitroton 1%	Lambdacyhalotrin 0,05%	
<i>An. sundaicus</i>	100						Mortality > 98% = susceptible; Mortality 80–97% = tolerance Mortality < 80% = resisten
<i>An. sundaicus</i>		100					
<i>An. sundaicus</i>			100				
<i>An. sundaicus</i>				100			
<i>An. sundaicus</i>					100		
<i>An. subpictus</i>						100	

Mosquito samples were tested by PCR vector resistance from Sungai Nyamuk Village were about 151 mosquitos with details as many as 127 *An. sundaicus* mosquitos species and 24 *An. subpictus*

mosquitos species. PCR test results can be seen in Table 2. Table 2 shows that the mosquitoes *An. sundaicus* were exposed to DDT 4% showed indications of resistance by 75.8% and were

exposed to permethrin 0.75% showed indications of resistance by 29.4%. *An. sundaicus* exposed Bendiocarb 0.1% only 10% is an indication of resistance. While *An. sundaicus* mosquitoes that were exposed to Deltamethrin 0.05% and Fenitrotrion 1% did not show resistant indications. Similar results were shown by *An. subpictus* mosquitoes which were exposed to Lambdacyhalotrin 0.05%, shows the nonresistant result. An incidence of mosquito resistance of *An. sundaicus* and *An. subpictus* in Sungai Nyamuk village against the four classes of insecticides (organochlorine, organophosphore, carbamate and pyrethroid) has not been seen, with the mortality of 100% mosquitoes test at 24 hours observation. Vulnerability test of *Anopheles* spp. was used the standard methods of impregnated paper-based WHO. However, based on the resistance test vector by PCR seen that *An. sundaicus* mosquitoes which were exposed to DDT 4% showed indications of resistance by 75.8% and were exposed to Permethrin 0.75% showed indications of resistance by 29.4%. *An. sundaicus* mosquito which were exposed to Bendiocarb 0.1% was only 10% which shows an indication of resistance. While *An. sundaicus* mosquitoes which were exposed to Deltamethrin 0.05% and Fenitrotrion 1% did not show resistant indications. Similar results were prohibited. The nature of persistence and tendency to accumulate in the environment, has led to the banning of the insecticide.⁽⁶⁾ The use of DDT in

shown by *An. subpictus* mosquitoes exposed to Lambdacyhalotrin 0.05%, shows the nonresistant result.

Based on the data above shows that mosquito test *Anopheles* spp. which came from Sungai Nyamuk Village were phenotypically not show resistance to DDT, thus, based on insecticide resistance with the PCR test seen that *An. sundaicus* mosquitoes were exposed to DDT 4% showed indications of resistance by 75.8%. This is occurred due to the effects of the use of DDT in the past. Trigger factor of the resistance was the use of the same/similar resistance insecticide continuously for long periods of time. DDT is persistent and these compounds are still active for years. DDT is stable so that it can last a long time in the soil because it can be bound with organic matter in the soil particles. DDT is included in the persistent organic pollutants. Based on the research result conducted by Andina (2015) showed that Siam Unus rice originated from Banjarbaru South Kalimantan, after being analyzed using the standard methods of gas chromatography, showed Limit of Detection (LOD) pesticide residue analysis for endosulfan < 0,0074 mg.Kg-1, endrin < 0,0166 mg.Kg-1, dieldrin < 0,0078 mg.Kg-1, aldrin < 0,0025 mg.Kg-1, p,p-DDT < 0,0094 mg.Kg-1, dan heptaklor < 0,002 mg.Kg-1.⁽⁵⁾ Since the 1970s, the use of the DDT pesticide in Indonesia otherwise Indonesia in the field of malaria control was done in the period 1959-1968.⁽¹⁾

Table 2 Detection genotype vector resistance of *Anopheles* spp. to insecticide used Polymerase Chain Reaction (PCR) methode in Sungai Nyamuk Village, Sebatik Island, Nunukan Distric, North Kalimantan, Januari - Desember 2011

Species tested	Insecticide tested	No.tested	Result of PCR (+/-)			
			Positive		Negative	
			n	%	n	%
<i>An. sundaicus</i>	Deltamethrin 0.05%	18	0	0,0	18	100,0
<i>An. sundaicus</i>	Bendiocarb 0.1%	20	2	10,0	18	90,0
<i>An. sundaicus</i>	Permethrin 0.75%	17	5	29,4	12	70,6
<i>An. sundaicus</i>	DDT 4%	33	25	75,8	8	24,2
<i>An. sundaicus</i>	Fenitrotrion 1%	39	0	0,0	39	100,0
<i>An. subpictus</i>	Lambdacyhalotrin 0,05%	24	0	0,0	24	100,0

The results of the PCR test vector resistance seen that *An. sundaicus* mosquitoes were exposed to

permethrin 0.75% showed indications of resistance by 29.4%. Indications genotype resistance vector to

permethrin must be considered as permethrin is one of the active ingredients currently used in Long Lasting Insecticides Nets/LN in Indonesia. One of the factors behind the use of insecticide resistance was the same for all stages of growth vectors (eggs, larvae, pupae, nymphs and adults). Resistance is the ability of vector populations to survive against a dose of insecticide in normal circumstances which can kill the vector species.⁽⁷⁾

An. sundaicus mosquito testing results which were exposed to Bendiocarb 0.1% showed indications of resistance results as much as 10%. Insecticide active ingredient Bendiocarb 0.1% was used for Indoor Residual Spray (IRS) in malaria vector control. IRS provide greater opportunities to create a generation of resistant compared to the way of other applications, because the chance of contact between the vector with the active ingredient to be larger. IRS usage policy in Indonesia is done when there is an malaria outbreak. Since 2009, the IRS activities in Sungai Nyamuk village were started to reduce because the community have been using Long Lasting Insecticides Nets/LN. The occurrence of resistance can be quickly or slowly depending on the frequency of insecticides that were used.⁽⁸⁾

An. Sundaicus mosquitoes using WHO Susceptibility Test against Deltamethrin 0.05% shows the results of vulnerable/susceptible, which is consistent with the results of the PCR test that showed indication of non resistance. This can occur due to the occurrence of resistance can take place quickly or slowly depending on the frequency of insecticides used. The use of insecticide active ingredient Deltamethrin in the field of malaria vector control is still limited to Long Lasting Insecticides Nets/LN. The use of insecticides alone actually do not cause resistance, if there is no resistance genes in the population. Resistance occurs when genetic mutations occur naturally from the population. The population of resistant in nature is ± 1 in 100,000 individuals. This population is able to survive and stay alive due to insecticide. If this happens continuously using the same insecticides, insect-resistant will be reproduce and genetic changes will occur which decrease the descent (resistant). These conditions in turn will increase the

proportion of resistant vector populations. The selection process occurs due to the use of insecticides similar to other evolutionary changes, and the process will happen much longer if the frequency of resistance transporter gene is low.⁽⁸⁾ Results are aligned between WHO Susceptibility Test and PCR tests which can also be seen from the test of *An. Sundaicus* against Fenitrothion 1% and *An. Subpictus* were exposed to Lambdacyhalotrin 0.05%, which shows the results are not resistant. This occurs because the two types of active materials was rarely used insecticides in malaria vector control programs especially in Sebatik Island. However, both types of these insecticides should also be a concern as it is also used for agricultural pesticides. The results of this study differs from that has been done by Widiarti et al. (2009) which states that *An. Subpictus* of Labuhan Haji Village, Nusa Tenggara Barat showed resistant result to Deltamethrin 0.05% and Fenitrothion 1.0% with mortality 77.0% and 78.0%.⁽⁹⁾ The triggering factors of resistance in the area was because the insecticide has been used in the field of Agriculture. The pace of the resistance depends on the level of selection pressure received by the insect population.⁽⁴⁾

Data percentage of mosquitos' knockdown time based on the contact time and class of insecticides can be seen in Figure 1. Result showed that DDT 4% (organokhlorin) was a class of insecticide active ingredients that deliver excellent knockdown effect. The percentage of 100% knockdown of mosquitoes tested was at the 30th minute after contact. Carbamate insecticides and organophosphorus group had a pretty good knockdown effect after organochlorin. The percentage of 100% knockdown of mosquitoes tested on organophosphorus seen in the 30th minute and the percentage of 100% knockdown of mosquitoes tested for carbamate seen in the 50th minute after contact. It is not owned by the active ingredient from the class of pyrethroid (Deltamethrin 0.05%, Permethrin 0.75% and Lambdacyhalothrin 0.05%) with a knockdown percentage rate of 85% on 60th minute after observation.

Mosquito knockdown time against the four classes of insecticide indicate that organochlorin has a knockdown effect which was best compared with the other three classes of insecticide. DDT has an excellent effect of knockdown because DDT destroy the balance of ions Na and K on the axon, causing muscle spasms (DDT jitter) to convulsions and mortality in insects. Pyrethroids have an unfavorable knockdown effect compared to the other three classes of insecticide. Piretorid have slower knockdown speeds because it has the effect of excellent insecticide repellency.⁽⁶⁾ However, the knockdown effect is not an indication of the vulnerability of populations of *Anopheles* spp. Mosquito. Vector vulnerability status determined from mosquito mortality percentage on a 24-hour observation.

CONCLUSIONS

An. sudaicus mosquitoes was still vulnerable to the pyrethroid class insecticides (Deltamethrin 0.05% dan Permethrin 0.75%), organofosfat (Fenitrothion 1%), organochlorin (DDT 4%) and Carbamat (Bendiocarb 0.1%); and *An. subpictus* mosquito was still vulnerable to the pyrethroid class insecticides (Lambdacyhalothrin 0.05%). In genotyping, *An. sudaicus* mosquito indication amounted to 75.8% resistant to DDT 4%, Permethrin 0.75%, 10% against Bendiocarb 0.1%. Whilst for Deltamethrin 0.05%, Fenitrothion 1% and Lambdacyhalotrin 0.05% showed no resistant indication. Organochlorin is the type of insecticide that has the fastest time of knockdown when compared to other groups. Indications of genotypic resistance is a step of early awareness on the incidence of vector resistance. Indications of genotypic resistance to permethrin vector is something that must be considered because permethrin is one of the active ingredients currently used in Long Lasting Insecticides Nets/LN. Reuse of the DDT active ingredient insecticide should be considered as there are indications of a fairly high-resistance gene.

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