Existence of the \( rdl \) mutant alleles among the \textit{anoph eles} malaria vector in Indonesia

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Abstract

Background: The \gamma\textendash\textit{aminobutyric acid (GABA) receptor-chloride channel complex is known to be the target site of dieldrin, a cyclodiene insecticide. GABA-receptors, with a naturally occurring amino acid substitution, A302S/G in the putative ion-channel lining region, confer resistance to cyclodiene insecticides that includes aldrin, chlordane, dieldrin, heptachlor, endrin and endosulphan.}

Methods: A total of 154 mosquito samples from 10 provinces of malaria-endemic areas across Indonesia (Aceh, North Sumatra, Bangka Belitung, Lampung, Central Java, East Nusa Tenggara, West Nusa Tenggara, West Sulawesi, Molucca and North Molucca) were obtained and identified by species, using morphological characteristic. The DNA was individually extracted using chelex-ion exchanger and the DNA obtained was used for analyses using sequencing method.

Results: Molecular analysis indicated 11\% of the total 154 \textit{Anopheles} samples examined, carried \( rdl \) mutant alleles. All of the alleles were found in homozygous form. \( rdl \) 302S allele was observed in \textit{Anopheles vagus} (from Central Java, Lampung, and West Nusa Tenggara), \textit{Anopheles aconitus} (from Central Java), \textit{Anopheles barbirostris} (from Central Java and Lampung), \textit{Anopheles sundaicus} (from North Sumatra and Lampung), \textit{Anopheles nigerrimus} (from North Sumatra), whereas the 302 G allele was only found in \textit{Anopheles farauti} from Molucca.

Conclusion: The existence of the \( rdl \) mutant allele indicates that, either insecticide pressure on the \textit{Anopheles} population in these areas might still be ongoing (though not directly associated with the malaria control programme) or that the mutant form of the \( rdl \) allele is relatively stable in the absence of insecticide. Nonetheless, the finding suggests that integrated pest management is warranted in malaria-endemic areas where insecticides are widely used for other purposes.

Keywords: \textit{Anopheles}, Dieldrin, GABA, Receptor, Malaria, \( rdl \)

Background

Malaria parasites in Indonesia are transmitted by 24 species of \textit{Anopheles} mosquitoes [1] that vary markedly in biological attributes, including patterns of blood feeding, response to volatile insecticides, and larval habitats. Such variation will impact the effectiveness of insecticide-treated nets (ITNs), indoor residual spraying (IRS) and larval habitat treatments or modifications [2]. Malaria control strategies in Indonesia are aimed at the \textit{Anopheles} malaria vector and rapid treatment of patients [3,4]. Control of malaria vectors has been done using insecticides that target the immatures and adult stage [5,6].

       Vector control uses a group of organochlorine insecticides, organophosphates, pyrethroids, and carbamates to kill mosquitoes [7]. However, continuous use of insecticides at high frequency and over long periods without inadequate supervision selects for resistant strains of mosquitoes. This resistance causes a decrease in target susceptibility in the mosquito population with a reduction in the efficacy of the vector control programme. Currently, a total 125 species of mosquitoes, including...
the genus *Anopheles* have been recorded to be resistant to one or more insecticides [8].

Organochlorine insecticides are classified into three groups; dichlororodiphenyl trichlor ethane (DDT), hexachlorhexana (HCH) and cyclodiene (aldrin, chlordane, heptachlor, dieldrin, endrin and endosulfan) [9]. DDT was used in malaria eradication programmes in Indonesia in the early 1950s but was subsequently banned in the 1970s as resistance to DDT emerged and spread rapidly. Dieldrin (cyclodiene) was introduced to malaria control programme in Indonesia since 1955 [10,11]. The rapid development of mosquito resistance to this insecticide later prompted the national malaria control program to terminate the use of dieldrin in 1965. However, several cyclodiene compounds, such as endosulfan and endrin are currently still used as pesticide in Indonesia.

Mosquito resistance to insecticides has been detected in recent years following insecticide use. Some species of *Anopheles* have demonstrated resistance to dieldrin. *Anopheles albimanus* in El Salvador, *Anopheles gambiae* [13] and *Anopheles sacharovi* in Turkey [14] have shown resistance to DDT and dieldrin [15]. Khan (1961) reported multiple resistance to dieldrin and DDT in *Aedes aegypti* in Puerto Rico [16]. In Indonesia, double resistance to DDT and dieldrin has been reported through biochemical tests of *Anopheles aconitus* in Central Java [17].

Earlier, it was demonstrated that resistant traits depend on major genetic factors and that the nervous system of the resistant insect is more tolerant to the action of cyclodiene [18]. Ghiasuddin and Matsumura (1982) [19] first proposed that the GABA receptor is the target of these cyclodiene insecticides and this was later confirmed by others [20-22]. Resistance to dieldrin involves a subunit of the insect gamma aminobutyric acid (GABA) receptor, The encoded *Rdl* subunit assembles with other GABA receptor subunits to form the target site of the cyclodiene insecticides and this was later confirmed by others [21]. Resistance to dieldrin involves a subunit of the insect gamma aminobutyric acid (GABA) receptor, The encoded *Rdl* subunit assembles with other GABA receptor subunits to form the target site of the cyclodiene insecticides and this was later confirmed by others [22]. Resistance to dieldrin involves a subunit of the insect gamma aminobutyric acid (GABA) receptor, The encoded *Rdl* subunit assembles with other GABA receptor subunits to form the target site of the cyclodiene insecticides and this was later confirmed by others [23].

**Methods**

**Study area of mosquito collection**

Female *anopheline* mosquitoes were collected from 10 provinces across Indonesia with different malaria endemicities - Aceh, North Sumatra, Bangka Belitung, Lampung, Central Java, East Nusa Tenggara, West Nusa Tenggara, West Sulawesi, Molucca and North Molucca (Figure 1). After morphological identification to species, mosquitoes were stored individually in a 1.5 ml Eppendorf microtube containing cotton flap and silica gel and kept at 4°C until use.

**Extraction of mosquito DNA**

Mosquitoes were ground with teflon pestles in 50 μl blocking buffer (BB), containing 5.0 g Casein; 0.01 g/L Phenol Red; 900 ml phosphate buffered saline (PBS), pH 7.4; 100 ml of 0.1 N NaOH; with additional IGEPAL (5 μl IGEPAL: 1 ml BB). The teflon pestles were subsequently rinsed with additional 200 μl volume of blocking buffer. Mosquito DNA from 50 μl homogenate was extracted using chelex-100 ion exchanger (Biorad Laboratories, Hercules, CA) essentially according to the procedure described previously [27]. The remaining 200 μl homogenate was used for other analysis. The DNA was either used immediately for a polymerase chain reaction (PCR) or stored at -20°C for later analysis.

**Gene amplification with the seminested-PCR**

Semi-nested PCRs were performed on the *Rdl* gene. All reactions were carried out in 25 μl reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl2, 200 mM dNTP, 1 U Taq Polymerase and a

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**Figure 1 Study area of mosquito collection**

*Anopheles* species were collected from several provinces in Indonesia: 1. Aceh 2. North Sumatra 3. Bangka Belitung 4. Lampung 5. Central Java 6. West Sulawesi 7. West Nusa Tenggara 8. East Nusa Tenggara 9. North Molucca 10. Molucca.
pair of primers (20 pM each). 1.5 μlo fD N Aw a su s e d
as template in the first reaction and 1-2 μlo ft h ef i r s
to PCR. Secondary PCR products were resolved by
electrophoresis on 2% agarose gel and visualized by
staining with ethidium bromide. The Rdl gene was
amplified using primers RDLF F10 (5′ SAG TTT TCG
ATG CGT GTA TAT GGT WW 3′), F11 RDLF (5′
AGC ATG TGA AAT TTK ASA G 3′), and R12 RDLF
(5′ CCA CAA ATA GCA TGG GAC CCA RGA 3′).
The initial nucleotide S is C/G, W is T/A, K is T/G, and
R is A/G. Cycling conditions for first PCR using oligos
F11 × R12 RDLF was denaturation at 94°C, annealing at
50°C, extension at 72°C and final polymerization
at 72°C, each phase lasts for 30 s, 30 s, 1 min and 30
s, and 5 min to 30 cycles. The second round PCR condi-
tions used oligos F10 × F12 RDLF for the stages of dena-
turation, annealing, extension, and final polymerization
are 94°C, 50°C, 72°C, and 72°C, each phase lasts for 30 s,
30 s, 45 s and 45 s (40 cycles). The final PCR products of
approximately 250 bp in size were sequenced in all indi-
vidual mosquitoes. The PCR products were purified
using PCR clean up system (PROMEGA Corporation,
Madison, WI, USA). The purified amplicons were
sequenced using an ABI Prism™ Dye BigDye Terminator
Cycle Sequencing Ready Kit (Applied Biosystem, Foster
City, USA) in automatic sequencer fluorescent DNA
capillary electrophoresis (ABI 3130 × l) at the Eijkman
Institute, Jakarta, Indonesia.

Results
PCR amplification and DNA sequencing of the fragment of Rdl gene of various anopheles species from Indonesia
Using primers that has been designed based on the pub-
lished sequence of Rdl gene from An. gambiae (GenBank
acc no. AF470112 and AF470116), Anopheles stephensi
(GenBank acc no. EU883213), Aedes aegypti (GenBank
acc no. AAU28803), and Culex quinquefasciatus (Gen-
Bank acc no. XM001850045) and sequences of
Anopheles sundaicus from Indonesia (GenBank acc no. JN675907-
JN675923), encompassing the Rdl gene mutation site
was successfully amplified and amplicons of approxi-
mately 250 bp in size in 19 Anopheles species were
obtained. Amplicons were then sequenced and submitted
to GenBank acc no. JN690008 - JN690025. Alignment of the
207 bp DNA sequencing results of each species is
shown in Figure 2. The DNA sequences of the Rdl gene
showed 12 variable nucleotide sites among the Anopheles
species analyzed but the deduced amino acid sequences
indicated a high sequence conservation.

Existence of rdl allele
Analysis of DNA sequences of 154 amplicons represent-
ing 19 Anopheles species indicated that the majority of
the Anopheles carried the wildtype 302A allele. The
302S polymorphism of the Rdl gene, popularly known as Rdl allele was detected in four provinces: North
Sumatra, Central Java, Lampung and West Nusa Teng-
gara while the 302 G allele was detected in Molucca
Province (Table 1). The Rdl allele was detected in Ano-
opheles vagus, An. aconitus, Anopheles barbirostris, Ano-
opheles sundaicus and Anopheles nigerrimus whereas the
302 G allele was only detected in Anopheles farauti
from Molucca (Figure 3). All of the alleles were found
in homozygous form. The Rdl 302S/G allele was not
found in any of the Anopheles species examined from
Aceh, Bangka Belitung, West Sulawesi, East Nusa Teng-
gara and North Molucca.

Frequency distribution of the rdl allele
In this report, the frequency distribution of the Rdl allele in each species examined could not be determined
as many of them were represented by an individual sam-
ple. In Central Java and Lampung Provinces, however, it
was evident that An.s vagus, An. aconitus and An.s bar-
birostris represent the species with the higher Rdl
mutant allele frequency, respectively. The three species
are well known to closely associate with agriculture area
as mostly use rice field or stream in inland area as their
breeding sites.
| Study site | Species                  | N  | Genotype frequency | Allele frequency (%) |
|------------|--------------------------|----|--------------------|-----------------------|
|            |                          | Σ=154 | AA | SS | GG | A | S | G |
| North Molluca | *An. punctulatus*         | 2   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. subpictus*          | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. tesselatus*         | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. kochi*              | 3   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. barbumbrosus*       | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. farauti*            | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. lesteri*            | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. vagus*              | 3   | 100  | 0  | 0  | 100 | 0 | 0 |
| Molluca     | *An. punctulatus*        | 4   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. lesteri*            | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. farauti*            | 10  | 90   | 10 | 0  | 90  | 0 | 10 |
| North Sumatra | *An. vagus*             | 4   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. peditaeniatus*      | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. nigerimus*          | 3   | 67   | 33 | 0  | 67  | 33 | 0 |
|            | *An. sundacius*          | 8   | 87.5 | 12.5 | 0  | 87.5 | 12.5 | 0 |
| Central Java | *An. vagus*             | 4   | 50   | 50 | 0  | 50  | 50 | 0 |
|            | *An. aconitus*           | 11  | 55.5 | 45.5 | 0  | 55.5 | 45.5 | 0 |
|            | *An. barbirostris*       | 8   | 75   | 25 | 0  | 75  | 25 | 0 |
|            | *An. Maculatus*          | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. balabacensis*       | 6   | 100  | 0  | 0  | 100 | 0 | 0 |
| Lampung    | *An. vagus*              | 5   | 60   | 40 | 0  | 60  | 40 | 0 |
|            | *An. sundacius*          | 23  | 96   | 4  | 0  | 96  | 4 | 0 |
|            | *An. barbirostris*       | 1   | 0    | 100 | 0 | 0  | 100 | 0 |
| Bangka Belitung | *An. sundacius*   | 10  | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. lesteri*            | 3   | 100  | 0  | 0  | 100 | 0 | 0 |
| East Nusa tenggara | *An. vagus*     | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. sundacius*          | 2   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. subpictus*          | 4   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. flavirostris*       | 2   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. indefinitus*        | 3   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. barbirostris*       | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. tesselatus*         | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. kochi*              | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. maculatus*          | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
| West Nusa  | *An. vagus*              | 5   | 80   | 20 | 0  | 80  | 20 | 0 |
| Tenggara   | *An. subpictus*          | 5   | 100  | 0  | 0  | 100 | 0 | 0 |
| Aceh       | *An. maculatus*          | 2   | 100  | 0  | 0  | 100 | 0 | 0 |
| West Sulawesi | *An. barbirostris*    | 3   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. sulawesi*           | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. peditaeniatus*      | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
|            | *An. nigerimus*          | 1   | 100  | 0  | 0  | 100 | 0 | 0 |
Discussion

The Rdl gene fragment encompassing the mutational sites associated with dieldrin resistance has been successfully amplified and sequenced in 19 species of Anopheline mosquitoes in Indonesia and the DNA sequences have been deposited in the GenBank. Molecular analyses of the Rdl gene of the Anopheles malaria vectors collected in 10 endemic areas of Indonesia: Aceh, North Sumatra, Bangka Belitung, Lampung, Central Java, East Nusa Tenggara, West Nusa Tenggara, West Sulawesi, Molucca and North Molucca indicated a high sequence conservation at the protein levels to the previously published Rdl sequence [25]. The existence of the resistant Rdl allele in five provinces - the Rdl 302S in four provinces: North Sumatra, Lampung, Central Java and West Nusa Tenggara, among An. vagus, An. aconitus, An. barbirostris, An. sundaicus and An. nigerrimus and the Rdl302G in the An. farauti in Molucca Province, was revealed. This finding indicates that cyclodiene insecticides pressures along this specific target in anopheline mosquitoes are still in place in many malaria-endemic areas of Indonesia. Cyclodiene insecticides were used in malaria control programmes during the 1955 following the spread of mosquito resistance to DDT, and so far, dieldrin was the only cyclodiene insecticide that had been used in the programme in Indonesia. This insecticide was used only for a short period following the discovery of doubly-resistant An. aconitus to DDT and dieldrin in Central Java in 1965 [16]. However, in agricultural areas, several cyclodiene insecticides are currently still in use such as, endosulfan, aldrin and heptachlor, and resistance of these insecticides by various agricultural insects has been documented in several areas [28].

Although the resistance to dieldrin had been first documented in An. aconitus, in Central Java in 1965, this finding is the first report of the existence of the Rdl dieldrin-resistant alleles, A302A/G in Indonesian Anopheline vectors. As dieldrin is no longer used in Indonesia, the existence of Rdl mutant allele in many Anopheles species in Indonesia might be associated with either the use of cyclodiene insecticides in agriculture or the relative high fitness of the mutant alleles in comparison to the wildtype.

In conclusion, this study reports the the existence of the Rdl mutant alleles among the major malaria vectors in Indonesia and their existence might be associated with insecticide use in agricultural area. Further biochemical study to assess the sensitivity of the Anopheles that carries the Rdl allele to the cyclodiene insecticides used in agriculture are now in progress.

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Authors’ contributions

DS, LS, PBSA, IEPR, NRP, SSM, S, WM and DSa performed molecular assays, data analysis, and the manuscript writing. PBSA and LS have equal contribution for this study. SS, S collected field samples and performed data analysis. DS, SS, FL, NFL, and WH designed the study and manuscripts writing were responsible for management and fund raising for this study. All authors read and approved the manuscript. This study is part of the thesis for Master of Science Programme at the University of Indonesia for LS.

Competing interests

The authors declare that they have no competing interests.

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Figure 3 Electropherogram of the DNA sequencing of GABA-Rdl gene. a. indicated the wildtype allele and b. indicated the resistance allele GCA. (alanin) replacement by TCA (serine) and c. GCA replacement by GGA (glycine).
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