UNIVERSITAS INDONESIA

CYCLODIENE INSECTICIDE RESISTANCE : EXISTENCE OF *Rdl* ALLELE AND SUSCEPTIBILITY TEST TO ENDOSULFAN INSECTICIDE AMONG THE *Anopheles* MALARIA VECTOR IN INDONESIA

TESIS

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FAKULTAS MATEMATIKA DAN ILMU PENGETAHUAN ALAM
PROGRAM STUDI BIOLOGI
PROGRAM PASCASARJANA
DEPOK
JANUARI 2012

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Diajukan sebagai salah satu syarat untuk memperoleh gelar Magister Sains

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Judul : CYCLODIENE INSECTICIDE RESISTANCE: EXISTENCE OF *RDL* ALLELE AND SUSCEPTIBILITY TEST TO ENDOSULFAN INSECTICIDE AMONG THE *Anopheles* MALARIA VECTOR IN INDONESIA

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FOREWORDS

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The author realized that this thesis would be far from perfect but still hoped that it could be useful to all that read it.

Author

2012

CONTENTS

ABSTRACT

Name : Lepa S.
Program Study : Biology
Title : Existence of *Rdl* Allele and Susceptibility Test to Endosulfan among the *Anopheles* Malaria Vector in Indonesia

The gamma-aminobutyric acid (GABA) receptor-chloride channel complex is known to be the target site of dieldrin and endosulfan, a cyclodiene insecticide. Mutation in the gene encoding the GABA-receptors, resistance to dieldrin (*Rdl*), which renders amino acid substitutions at codon A302G/S in the putative ion-channel lining region. The mutation has been found in a wide range of insect including anopheline mosquitoes and confers resistance to cyclodiene insecticide, such as dieldrin and picrotoxin. The present study aims to explore the existence and frequency distribution of the *Rdl* mutant alleles among the *Anopheles* species in Indonesia. Molecular analyses have been performed on *Anopheles* mosquito samples collected from several areas across Indonesia (Aceh, North Sumatra, Bangka Belitung, Lampung, Central Java, East Nusa Tenggara, West Nusa Tenggara, West Sulawesi, Molucca and North Molucca) and the *Rdl* gene was Polymerase-Chain Reaction (PCR) amplified and sequenced to detect the existence of the *Rdl* mutant alleles. The results indicated that 11 % of the total 154 *Anopheles* samples examined carried the mutant *Rdl* alleles. The A302S allele was observed in *An. vagus* (from Central Java, Lampung and West Nusa Tenggara), *An. aconitus* (from Central Java), *An. barbirostris* (from Central Java and Lampung), *An. sundaicus* (from North Sumatra and Lampung), *An. nigerrimus* (from North Sumatra), whereas the A302G allele was only found in *An. farauti* from Molucca. Susceptibility test were carried out using World Health Organization (WHO), Centers Disease Control and Prevention (CDC) and previously publish method with tight modification standard procedures. The test using 0-0.4% (w/v) endosulfan concentrations (Akodan 35 EC trademark) with two replicates and 20-30 larvae samples from the field of Katibung and Rajabasa

sub-district, Lampung Province and followed by molecular analyses of the gene encoding the GABA subunit. The LC_{50} of the larvae were 0.00893 (0.00332-0.01697) and 0.00904 (0.00401-0.01586) from Katibung and Rajabasa and all of the larvae carried A302 *Rdl* allele. The existence of the *Rdl* mutant allele indicates that, either insecticide pressure on the *Anopheles* population in these area might still ongoing (though not directly associated with malaria control program) 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*: Anopheles*, dieldrin, endosulfan, GABA receptor, *Rdl*.

ABSTRAK

Nama : Lepa S.

Program Studi : Biologi

Judul : Resistansi Insektisida Siklodien: Keberadaan Alel *Rdl* dan Uji Suseptibilitas Endosulfan Terhadap Nyamuk *Anopheles* Sebagai Vektor Malaria di Indonesia.

Gamma aminobutyric acid (GABA) reseptor merupakan situs target insektisida dieldrin dan endosulfan, kelompok insektisida siklodien. Mutasi pada gen pengkode reseptor GABA menyebabkan resistansi terhadap dieldrin (*Rdl*). Resistansi ditandai dengan perubahan asam amino pada kodon A302G/S saluran ion reseptor GABA. Mutasi tersebut telah ditemukan terhadap beberapa jenis serangga, termasuk nyamuk anopheline dan dikaitkan dengan resistansi terhadap insektisida siklodien. Penelitian ini bertujuan untuk mengeksplorasi keberadaan mutan alel *Rdl* pada spesies *Anopheles* di Indonesia. Analisis molekuler dilakukan pada sampel nyamuk *Anopheles* dari beberapa daerah di Indonesia (Aceh, Sumatera Utara, Bangka Belitung, Lampung, Jawa Tengah, Nusa Tenggara Timur, Nusa Tenggara Barat, Sulawesi Barat, Maluku dan Maluku Utara) untuk mendeteksi keberadaan alel *Rdl*. Hasil penelitian menunjukkan bahwa 11% dari 154 total sampel *Anopheles* yang dianalisis mengalami mutasi. Mutasi A302S alel *Rdl* ditemukan pada *An. vagus* (dari Jawa Tengah, Lampung dan Nusa Tenggara Barat), *An. aconitus* (dari Jawa Tengah), *An. barbirostris* (dari Jawa Tengah dan Lampung), *An. sundaicus* (dari Sumatera Utara dan Lampung), *An. nigerrimus* (dari Sumatera Utara), sedangkan mutasi alel A302G hanya ditemukan pada *An. farauti* dari Maluku. Uji Kerentanan dilakukan dengan menggunakan prosedur standar dari WHO, CDC dan modifikasi dari penelitian sebelumnya. Uji tersebut menggunakan endosulfan (merk dagang Akodan 35 EC) dengan konsentrasi 0-0.4% (g/L), dua kali ulangan terhadap 20-30 sampel larva dari Kecamatan Katibung dan Rajabasa, Provinsi Lampung. Setelah bioasay dilanjutkan analisis molekuler pengkodean subunit GABA. Nilai LC_{50} larva adalah 0.00893 (0.00332-

0.01697) dan 0.00904 (0.00401-0.01586) dari Kecamatan Katibung dan Rajabasa. Analisis molekuler menunjukkan bahwa seluruh larva membawa alel *Rdl* A302, tipe normal. Adanya mutasi pada alel *Rdl* menunjukkan bahwa paparan insektisida pada populasi *Anopheles* di daerah ini mungkin masih berlangsung (meskipun tidak secara langsung terkait dengan program pengendalian malaria) atau spesies yang membawa alel resistan dapat bersaing dengan spesies normal pada populasi *Anopheles* sehingga bentuk mutan dari alel *Rdl* relatif stabil dalam ketiadaan insektisida dieldrin yang sudah tidak digunakan lagi. Meskipun demikian, hasil penelitian tersebut menunjukkan bahwa manajemen hama terpadu diperlukan pada daerah endemik malaria di mana insektisida juga digunakan untuk keperluan lain seperti pertanian.

Kata kunci*: Anopheles*, dieldrin, endosulfan, GABA reseptor*, Rdl.*

Name : Lepa S. Date: Januari 2012

Title : Cyclodiene Insecticide Resistance: Existence of *Rdl* Allele and Susceptibility Test to Endosulfan Insecticide among the *Anopheles* Malaria Vector in Indonesia

Thesis Supervisors : dr. Syafruddin, Ph.D.; Dr. Wibowo Mangunwardoyo, M.Sc.

SUMMARY

Control of anopheline mosquitoes as malaria vectors has long been done using a group of organochlorine insecticides such as dieldrin and dichloro diphenyl trichloroethane (DDT) with the main strategy to kill the mosquitoes. Exposures of insecticides at high frequency over long periods without adequate supervision could select for resistant strains of mosquitoes. The resistance causes a decrease in target susceptibility in the mosquito population and therefore reduction in the efficacy of the vector control program. Dieldrin, a cyclodiene insecticide was first introduced in health program in Indonesia in1950 and proved to be highly effective and its use was then promoted in agriculture. Some species of *Anopheles* have demonstrated resistance to DDT and dieldrin insecticides in recent years following the constant use of ths insecticide. In Indonesia, double resistant to DDT and dieldrin respectiely have been reported through biochemical tests of *An. aconitus* and *An. sundaicus* in Central Java.

Resistance to dieldrin involves a subunit of the gamma aminobutyric acid (GABA) receptor, particularly associated with the single nucleotide polymorphisms in the M2 transmembrane domain of the GABA-gated chloride ion channel. The encoded *Rdl* subunit assembles with other GABA receptor subunits to form the target site of the cyclodiene insecticides. Dieldrin resistance is associated with the replacement of a single amino acid, alanine at position 302 to serine or glycine.

The present study aims to explore the allelic distribution of the *Rdl* gene and to determine the susceptibility status of cyclodiene insecticide among the anopheline malaria vectors from different malaria endemic areas of Indonesia.

This study was conducted from August 2010 until July 2011 at the Laboratory of Malaria 1, Eijkman Institute for Molecular Biology, Central of Jakarta, Indonesia.

This study analysed several *Anopheles* species collected from different provinces of Indonesia. After a proper morphological identification, Deoxyribonucleic acid (DNA) mosquitoes were extracted using chelex-100 ion exchanger. Gene fragment encompassing the mutational site at codon A302 was amplified using seminested-polymerase chain reaction (PCR). The oligos were designed based on the published sequence of *Rdl* gene of *An.gambiae, An. stephensi, Culex quinquifasciatus* and *An. sundaicus* from Indonesia (GenBank). The amplicons were then prepared for DNA sequencing and the DNA sequences obtained were analyzed using Bioedit computer program.

Insecticide susceptibility test was conducted using previously published method with tight modification. Briefly, the susceptibility test was performed on 20-30 *Anopheles* larvae using a wide concentration ranges of endosulfan (0-0.4%). The LC_{50} values were calculated using probit analysis SPSS computer program, followed by chi square test and molecular analyses were performed to confirm their *Rdl* allele.

A total of 154 samples were collected from 10 provinces in Indonesia: Aceh, North Sumatra, Bangka Belitung, Lampung, Central Java, West Sulawesi, West Nusa Tenggara, East Nusa Tenggara, North Molucca and Molucca. Molecular analysis indicated that 11 % of the total samples carried either A302S and A302G *Rdl* mutant alleles in homozygous form. The A302S *Rdl* allele was observed in *An. vagus* (from Central Java, Lampung and West Nusa Tenggara), *An. aconitus* (from Central Java), *An. barbirostris* (Central Java and Lampung), *An. sundaicus* (from North Sumatra and Lampung), *An. nigerrimus* (from North Sumatra) and allele A302G was only found in *An. farauti* (from Molucca).

Susceptibility test of *Anopheles* larvae from Katibung and Rajabasa (Lampung Province) revealed different mosquitoes larvae mortality in each concentrations used. The LC_{50} of endosulfan in both location were 0.00893 (0.00332-0.01697) and 0.00904 (0.00401-0.01586) in Katibung and Rajabasa sub-districts, respectively. Molecular analyses of the 18 *Anopheles* larvae selected

from the total larvae examined revealed that they all carried the wildtype, A302 *Rdl* allele.

The existence of the *Rdl* mutant alleles in six *Anopheles* species examined across a wide geographic region of Indonesia implies that; first, cyclodiene insecticides pressures might still occur in many malaria endemic areas of Indonesia. Cyclodiene insecticides were used in malaria control program during the decade of 1950 following the spread of mosquito resistance to DDT and so far, dieldrin was the only cyclodiene insecticides that had been used in health program in Indonesia. This insecticide was only used for a short period since1950 until 1960 decade. Doubly-resistant *An. aconitus* to DDT and dieldrin were discovered in Central Java through biochemical assay. The use of dieldrin in malaria control was terminated in 1960. However, in agricultural areas, several cyclodiene insecticides are currently still used such as endosulfan and fipronil and resistance of these insecticides to various agricultural insect have been documented in several areas. Second, the mutant *Rdl* alleles might be relatively fit in comparison to wildtype allele so that it could be passed to the offsprings in the absence of relevant insecticide pressures. In our findings, as most of the *Anopheles* species that carried the *Rdl* mutant alleles use the agricultural land as their breeding sites, therefore their existence might be associated with the use of cyclodiene in agricultural area.

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INTRODUCTION

Malaria is one of the major public health problem and the most parasitic disease in the world, particularly in the Southeast Asia region. Amount 10 out of 11 countries of the region are malaria endemic (WHO 2010). In Southeast Asia region, during 2000-2010, the reported malaria incidences were around 2.30 - 3.08 million case and deaths between 2 423 - 6 978 annually (SEARO 2011). Based on malaria report in 2010, the highest number of confirmed cases were reported from India (1 495 817) followed by Myanmar (420 808) and Indonesia (229 819). In Indonesia, report in 2009 indicated that population in malaria areas are 107.96 million, with 544 470 cases were confirmed as malaria cases, 46% cases of them are caused by *Plasmodium falciparum.* Based on the WHO report in 2008, in the world there are 243 million cases, approximately 2.5-3 million clinical cases of malaria in Indonesia in 2007 and increased to 15 million in 2009, 42 000 of them leading to death (WHO 2009; Ministry of Health 2011; SEARO 2011).

Malaria in human is caused by infection with a single-cell protozoan of the genus *Plasmodium* that transmitted by female anopheline mosquitoes as a vector of malaria. Male mosquitoes feed only on sugar-rich fluids, females may as well survive on these nutrients, but egg maturation requires the consumption of a blood meal. Female *Anopheles* proboscis structures are sharper than male *Anopheles*,it could be more easily penetrate to the skin tissue so that *Plasmodium* can be sucked into the lead female *Anopheles* mosquito gut to act more as a vector of malaria (CDC 2004). In Indonesia, approximately 80 species of *Anopheles* mosquitoes have been documented, 22 of which have been incriminated are potential vectors of malaria disease (Sukowati, unpublished data).

Malaria control strategies in Indonesia have been done through several ways including vector control and reduction of morbidity and mortality in human using anti malarial drugs (PP & PL 2008: WHO 2011). Vector control is an important part of the global malaria control strategy. The primary objective of

vector control is to reduce the malaria morbidity and mortality by through reduction the levels of transmission. The main vector control methods include the use of insecticide-treated nets (ITNs) (Gunawan 1999; Harijanto 2008) and indoor residual spraying (IRS), its can prevent malaria transmission inside the houses (Roberts et al. 1997). Control measures directed towards adult mosquitoes, such as IRS and ITNs are more broadly applicable (WHO 2011).

The primary insecticides used against malaria vector are DDT and dieldrin. DDT is an insecticides recommended by WHO for IRS, the most long-lasting and cost-effective. DDT was first used in India for the control of malaria and vector borne disease in 1944. In Indonesia, since 1950 dieldrin was first introduced into the public health programs (Zavon et al. 1958). The problems that arise following the continuous use of insecticides over a long time period at high frequency with lack of supervision is a matter of resistance. The use of insecticides can cause a decrease in mosquito susceptibility targets, or in other words, mosquito resistance to insecticides. More than 125 species of mosquitoes, including the genus *Anopheles* have been recorded to be resistant to one or more insecticides (CDC 2004).

Mosquito resistance to insecticides has been well documented in recent years after inconstant exposure to insecticide use. Some species of *Anopheles* have been resistant to dieldrin, among other *An. albimanus* in El Salvador reportedly resistant to DDT and dieldrin (Davidson 1963), *An. gambiae* (Haridi 1974), *An. sacharovi* in Turkey (Zulueta et al. 1959). Khan (1961) reported cross resistance to dieldrin and DDT in *Aedes aegypti* in Puerto Rico (Khan and Brown 1961). In Indonesia, through biochemical tests have been reported that dieldrin-DDT resistant to *An. aconitus* in Central Java (Soerono et al. 1965[1]).

Resistance monitoring should be an integral part of vector or public health pest control program. Resistance monitoring will be used for better on formulating evidence base vector control strategies and most importantly for management of insecticide resistance in malaria vectors. Detecting and monitoring resistance could be performed through bioassay, biochemical assay or molecular assay (Brogdon & McAllister 1998). Bioassay or susceptibility test or vulnerability test is a test to determine the level of susceptibility or toxicity of insects, against a

poison or insecticide. Biochemical and molecular methods can detect resistance mechanisms in individual insects; therefore, it can confirm resistance with the use of only a small number of insects. Molecular information on resistance mechanisms will increasingly be incorporated into resistance diagnostic procedures. One type of mechanism that will become much easier to detect will be the point mutations that cause target-site resistance or changes in detoxification enzyme specificity.

Dieldrin acts through interference of the mosquitos nervous system, particularly subunit of the insect GABA receptor that is encoded by *Rdl* gene. The GABA subunit assembles with other GABA receptor subunits to form the target site of the cyclodiene insecticides (ffrench-Constant et al. 2000). Dieldrin resistance was associated with replacement of a single amino acid (alanine at position 302) in the *Drosophila melanogaster Rdl* allele (ffrench-Constant et al. 1993). Target-site mechanisms have been detected by polymerase chain reaction (PCR) amplification of specific alleles (Wilkins et al. 2006; Brunet et al. 2009).

The aims of this thesis are to explore the existence of mutant *Rdl* allele and the susceptibility of endosulfan insecticide among *Anopheles* mosquitoes in Indonesia. This study will try to adress some questions, such as: does cyclodiene resistance exist in *Anopheles* species, does *Anopheles* species in Indonesia carry the mutant allele of the *Rdl* gene, and are the larvae of *Anopheles* species in Indonesia still susceptible to endosulfan. The results of this work would be divided in two papers, the first paper would be entitled: “Existence of *Rdl* allele among the *Anopheles* malaria vector in Indonesia and the second paper would be entitled: “Susceptibility of *Anopheles* larvae to endosulfan insecticide in Katibung and Rajabasa districts, Lampung Province”. The first paper was accepted in Malaria Journal (In Press Malaria Journal, February 2012).

Paper I

EXISTENCE OF *RDL* ALLELE AMONG THE *Anopheles* MALARIA VECTOR IN INDONESIA

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ABSTRACT

The gamma aminobutyric acid (GABA) receptor-chloride channel complex is known to be the target site of dieldrin, a cyclodiene insecticide and lindane. *Rdl* (resistance to dieldrin) GABA-receptors, with a naturally occurring amino acid substitution, A302S/G in the putative ion channel lining region, confer resistance to dieldrin and picrotoxinin. 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 that used for analyses using sequencing method. Molecular analysis indicated 11 % of the total 154 *Anopheles* samples examined carried *Rdl* mutant alleles. All of the alleles were found in homozygous form. *Rdl* A302S mutation was observed in *An. vagus* (from Central Java, Lampung and West Nusa Tenggara), *An. aconitus* (from Central Java), *An. barbirostris* (from Central Java and Lampung), *An. sundaicus* (from North Sumatra and Lampung), *An. nigerrimus* (from North Sumatra) and whereas the A302G mutation was only found in *An. farauti* from Molucca. The existence of the *Rdl* mutant allele indicates that, either insecticide pressure on the *Anopheles* population in these areas might still ongoing (though not directly associated with malaria control program) 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 : *Anopheles,* dieldrin, GABA receptor, malaria, *Rdl.*

I. INTRODUCTION

Malaria parasites in Indonesia are transmitted by 22 species of *Anopheles* mosquitoes (Sukowati unpublished data) 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- and

larval habitats. Such variation will impact the effectiveness of insecticide-treated nets (ITNs), indoor residual spraying (IRS) and larval habitat treatments or modifications (Bangs et al. 1993). Malaria control strategies in Indonesia have been done through several ways including the eradication of malaria and rapid treatment in patients (Ministry of Health-PP & PL 2008; WHO 2011). Combating malaria vectors has been done using several insecticides (Gunawan 1999; Harijanto 2008).

Vector control using a group of organochlorine insecticides, organophosphates, pyrethroids and carbamates are the basic principles to kill mosquitoes (ffrench-Constant et al. 1998). However, continuous use of insecticides in a long term, high frequency and lack of supervision selects up resistant strain of mosquito. The use of insecticides can cause a decrease in target susceptibility in mosquito and therefore resistance to insecticides. Currently, a total 125 species of mosquitoes, including the genus *Anopheles* have been recorded to be resistant to one or more insecticides (CDC 2004).

Organochlorine insecticides are grouped into three; DDT, cyclodiene (aldrin, dieldrin and endosulfan) and chlorinated benzene and cyclohexane (HCB and Lindane). DDT and dieldrin were used in malaria eradication program. Dieldrin (cyclodiene) was introduced to Indonesia since 1950 (Sudaryanto et al. 2007). The use of dieldrin in health program proved a high effectiveness and promoted its use in agriculture (Zavon et al. 1961). Until 1958, 982 481 million kg of dieldrin have been used in Indonesia. The number does not include the use of dieldrin in agriculture. In agriculture, a group of organochlorine insecticides such dieldrin, aldrin and endrin has been used since 1958.

Mosquito resistance to insecticides emerged in recent years after insecticide use. Some species of *Anopheles* have been resistant to dieldrin, among others; *An. albimanus* in El Salvador reportedly resistant to DDT and dieldrin (Davidson 1963), *An. gambiae* (Haridi 1974), *An. sacharovi* in Turkey (Zulueta et al. 1959). Khan and Brown (1961) reported cross resistance to dieldrin and DDT in *Ae. aegypti* in Puerto Rico. In Indonesia, double resistant to DDT and dieldrin have been reported through biochemical tests of *An. aconitus* and *An. sundaicus* in Central Java (Soerono et al. 1965[1]).

Most resistance mechanisms can be divided into two groups, metabolic encompassed alterations in the levels or activities of detoxification proteins; target sites encompassed mutations in the sodium channel, acetylcholinesterase and GABA (ffrench-Constant et al. 1993). GABA as a neurotransmitter throughout the central and peripheral nervous systems and now believed to be responsible for at least 40% of all inhibitory synaptic events which occur in the mammalian and insect brain. It is synthesis is largely controlled by the activity of glutamate decarboxylase which converts the amino acid glutamate directly into GABA (Kanner 1996). At an early stage it has been shown that such a resistant trait depends on a major genetic factor and that the nervous system of the resistant strains of insect are more tolerant to the action of cyclodiene (Brown 1960). The idea that the GABA receptor is the target of cyclodiene insecticides was initially proposed by Ghiasuddin and Matsumura (1982) and later confirmed by others (Lawrence and Casida 1983; Tanaka and Matsumura 1986; and Thompson et al. 1993).

GABA receptors can be readily sub-divided into ionotropic ($GABA_A$) and metabotropic ($GABA_B$) class although further divisions within the ionotropic group isoform including $GABA_C$ sites have been suggested. In *Drosophila*, at least three genes are thought to encoded $GABA_A$ receptors: *Rdl*, GABA and glycine-like receptor (Grd) and ligand-gated chloride channel homologue 3 (Lcch3). $GABA_A$ receptor is part of super family of fast ion channel receptor and it is provide distinct and specific drug target sites. *Rdl* is by far the best characterized and functional expression experiments and also has an important role in insecticide resistance. The receptor comprises a pentametric structure with each protein subunit linking to form a central pore through which Cl^- selectively. GABA activates the receptor by attaching to the recognition site, barbiturates, benzodiazepines, alcohol, neurosteroids and fixed general anesthetics facilitate (Hosie et al. 1997; ffrench-Constant et al. 2004; Buckingham et al. 2005).

Resistance to dieldrin involves a gene that encodes a subunit of the insect GABA receptor and the encoded *Rdl* subunit assemble with other GABA receptor subunits to form the target site of the cyclodiene insecticides (ffrench-Constant et al. 2000). Dieldrin resistance was associated with replacement of a single amino

acid (alanine at position 302) in the *D. melanogaster Rdl* allele (ffrench-Constant et al.1993), and it has been particularly associated with the single nucleotide polymorphisms in the M2 transmembrane domain of the GABA-gated chloride ion channel (*Rdl* allele) (Li et al. 2006). A homologous mutation has also been indicated to confer dieldrin resistance in a wide variety of insect species such as *Ae. aegypti*, *D. simulans, Musca domestica, Lucilia cuprina, Blattella germanica*, *Tribolium castaneum*, *Hypothenemus hampei, Bemisia* and *Myzus persicae*. The present study aims to explore the *Rdl* allelic existence of GABA receptor mutations among the malaria vectors from different malaria endemic areas in Indonesia.

II. MATERIALS AND METHODS

2.1 Materials

The equipments that used in this study are tips, pipette micro, 1.5 and 0.2 ml microcentrifuge tube, vortex machine, thermal cycler machine (Gene AMP® PCR system 9700), digital scale (Sartorius), microwave, electrophoresis (Maxi cell ™ EC360M), Gel-Doc 1000 (Bio-RAD), program quantity one Analysis Software computer program, SV minicolumn tube, Integrated Speed-Vac System machine (DNA 110, Savant), ABI PRISM sequence machine, Basic Local Alignment Search Tool Nucleotide computer program (BLASTN), Bioedit computer program, freezer, grinder and glass equipments that commonly used in genetic laboratories.

Materials that we use in this study are *Anopheles* samples, from Malaria Laboratory, Eijkman Institute collection, forward and reverse primer; F10 RDLF (5' SAG TTT GTA CGT TCG ATG GGT TAT WW 3'), F11 RDLF (5' TTK AGC ATG TGA AAT ASA G 3'), and R12 RDLF (5' CCA GCA GAC TGG CAA ATA CCA RGA 3'), with S for C/G, W for T/A, K for T/G, and R for A/G, grinding buffer solution, ethanol 70%, TAE buffer, PCR buffer (Invitrogen), $MgCl_2$ (Invitrogen), dNTP (NE Biolabs), DNA polymerase enzyme (Invitrogen-platinum, agarose powder (SEAKEM), EDTA, loading dye (NE Biolabs),

ethidium bromida, ladder marker (NE Biolabs), Wizard SV gel kit and PCR clean–up system (Promega).

2.2 Methods

2.2.1 Study Area of Mosquito Collection

Female anopheline mosquitoes were collected from 10 provinces across Indonesia with different malaria area endemicities - Aceh, North Sumatra, Bangka Belitung, Lampung, Central Java, East Nusa Tenggara, West Nusa Tenggara, West Sulawesi, Molucca and North Molucca (Figure 2.2.1.1). After morphological identification to species, mosquitoes were stored individually in a 1.5 ml Eppendorf microtube that already containing cotton flap, silica gel and kept at 4^{o}C until used.

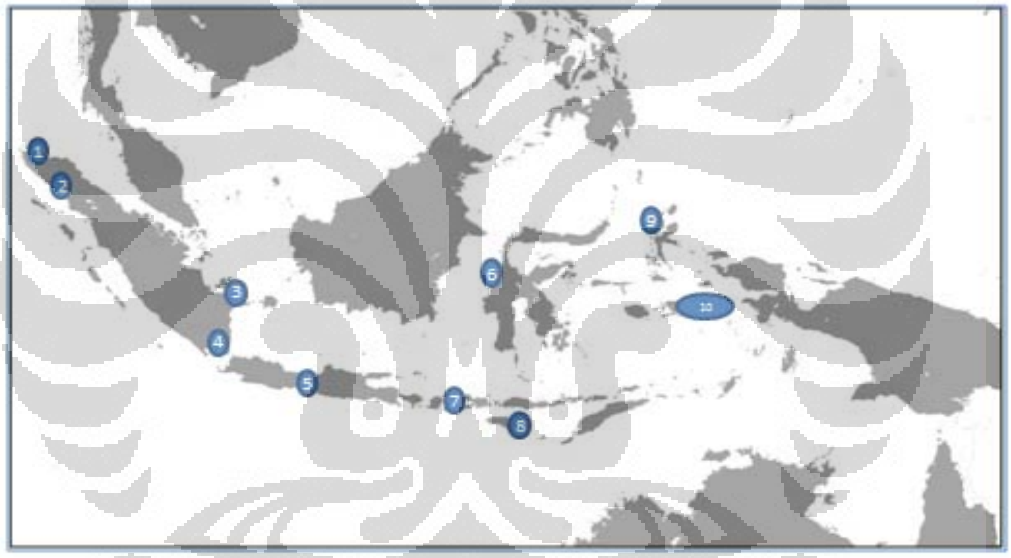

Figure 2.2.1.1 Study area of mosquito collection. *Anopheles* species were collected from the several provinces in Indonesia. Blue circle shown the sampling location: 1. Aceh 2. North Sumatra 3. Bangka Belitung 4. Lampung 5. Central Java 6. West Sulawesi 7. West Nusa Tenggara 8. East Nusa Tenggara 9. North Molucca 10. Molucca.

2.2.2 Extraction of Mosquito DNA

Mosquitoes were ground with 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 ul IGEPAL : 1 ml BB). The 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 (Wooden et al. 1993). The remaining 200 µl homogenate was used for other analysis. The DNA was either used immediately for a PCR or stored at -20 ºC for later analysis.

2.2.3 Gene Amplification with the Semi-nested PCR

Semi-nested PCR was performed on the *Rdl* allele. All reactions were carried out in 25 µl reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 200 mM dNTP, 1U taq polymerase and a pair of primers (20 pM each). 1-5 µl of DNA was used as template in the first reaction and 1-2 µl of the first round PCR product was used as template for the secondary 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 primer that has been designed. Cycling conditions for first PCR using oligos F11 x R12 RDLF, denaturation at 94°C, annealing at 50°C, extension at 72°and final polymerization at 72°C, each phase lasts for 30 seconds, 30 seconds, 1 minute 30 seconds, and 5 minutes to 30 cycles. The second round PCR conditions used oligos F10 x F12 RDLF for the stages of denaturation, annealing, extension, and final polymerization are 94°C, 50°C, 72°C, and 72°C, each phase lasts for 30 seconds, 30 seconds, 45 seconds and 45 seconds (40 cycles). The final PCR products of approximately 250 bp in size were sequenced in all individual mosquitoes.

2.2.4 Electrophoresis

Amount 1g agarose powder dissolved in 50 ml of 1x TAE buffer and boiled in a microwave oven for two minutes, added 5µl EtBr. A total of 5 µL sample of the second round PCR product put in the electrophoresis buffer aparatus and mixed with 2 µl loading dye then inserted into the gel wells. A total of 5µl marker ladder added to the wells gel as markers. Gel electrophoresis performed at a voltage 80 volts for ± 1 hour. The gel was documented using the Gel-Doc 1000.

2.2.5 Purification of DNA

Purification of DNA was performed using a Wizard ® SV Gel and PCR Clean-Up System protocol (Promega) kit according to procedures the recommended by the manufactures. Briefly, PCR product was transferred into a 1.5 mL microcentrifuge tube and an equal volume of membrane binding solution was added. The tubes were then incubated at 25°C for 1 minute, then transferred into a SV mini column tube and centrifuged at 12 000 rpm for 1 minute. The formed supernatant was discarded and 700 µL of membrane wash solution was added into the tube. The tube was further spun for 5 minutes at 12 000 g, and the supernatant was discard and 500 µL membrane wash solution was added, spun for 1 minute at 12 000 rpm. The SV mini column tube was then transferred into new 1.5 ml Eppendorf tube, added with 30 µL nuclease-free water. Sample tubes were incubated at room temperature for 2 minutes and centrifuged at a speed of 12 000 rpm for 2 minutes. Supernatant containing the purified DNA was stored at 4°C or -20°C.

2.2.6 Sequencing DNA

2.2.6.1 Cycle Sequencing

DNA sequencing was carried out in accordance with the BigDye ® Terminator v3.1 Cycle Sequencing protocol (Applied Biosystem) kit according to procedures the recommended by the manufactures. The components of the reagent, consisting 6 µL of big-dye, 1 µL forward or reverse oligos primer F10 or F12 RDLF (4 pmol) ddH_20 and purified DNA were mixed in a 0.2 ml microcentrifuge tube. The amount of purified DNA measurements at a wavelength of 260/280 nannodrop then added ddH_20 until the total volume to 15 µL. Conditions used in thermal Cycler machine was 96°C for pre-denaturation for 3 minutes and for the denaturation step, annealing and polymerization were 96°C, 50°C, 60° C, each phase lasts for 10 seconds, 5 minutes and 4 minutes for 25 cycles. Cycle sequencing product was stored at 4°C.

2.2.6.2 DNA Precipitation

DNA precipitation was carried out with BigDye ® Terminator v3.1 Cycle Sequencing protocol (Applied Biosystem). Briefly, a total of 15 µL cycle sequencing product was transferred into 1.5 mL Eppendorf tube and then added 37.5 µL 100% of ethanol, 1.5 mL of sodium acetate 3M pH 5.2 and 1.5 µL of EDTA 125 Mm. Vortex a tube and incubated at room temperature for 15 minutes, centrifuged at 4°C at 12 000 rpm for 20 minutes. Supernatant was discarded and the pellet formed that remains in the tube was dissolved with 250 µL 70% ethanol. The tube was further spun at 12 000 rpm at 4°C for 20 minutes. The supernatant was discard and the tube was dried in a Speed-Vac ® machine at 6 000 rpm for 15 minutes. The tube was wrapped with aluminum foil paper and stored at -20°C.

2.2.6.3 Automated DNA Sequencing

The process of automated DNA sequencing performed by the laboratory staff of the Eijkman Institute sequencing services with reference to automated DNA sequencing protocol (Applied Biosystem). The results of DNA precipitation was mixed with 12 mL deionized formamide high, then vortex for 30 seconds. The tube was inserted into the plate and was heated in a thermal cycler machine at a temperature of 95°C for 3 minutes and cooled the tube in ice (snap cooling). Plate containing the samples was mounted on a tray and placed in the machine sequencing ABI PRISM ® 3130xl Genetic Analyzer 16-capillary (Applied Biosystem). Sequencing process takes 45 minutes. Nucleotide sequence of readings was recorded by a computer and converted into graphs showing by electroferogram form and arrangement of the sequence DNA.

2.3 Data Analysis

Electroferogram of GABA gene sequences were analyzed using a Bioedit computer program and performed alignment of DNA sequences with one GABA sequence of *An. gambiae* (accession number XM316070.4). Sites of base mutation can be seen in the amino acid alanine 302. Changing of amino acid at this codon indicated the mutations occured at Ala302 to glycine or serine, the

sample carried mutant *Rdl* allele. The samples were wildtype if *Anopheles* species samples carried Ala302.

III. RESULTS AND DISCUSSION

A. Results

3.1 Sample Collection

The amount of *Anopheles* species collected in each site varied markedly in quantity and species distribution (Table 3.1.1).

Table 3.1.1 Number of *Anopheles* species samples obtained in each province.

No	Species	n	AC	NS	BB	LM	CJ	WN	EN	WS	MO	NM
1	*An. punctulatus*	6									4	2
2	*An. vagus*	22		4		5	4	5	1			3
3	*An. sundaicus*	47		8	10	23		4	2			
4	*An. aconitus*	11					11					
5	*An. subpictus*	10						5	4			1
6	*An. barbirostris*	13				1			1	3		
7	*An. farauti*	11									10	1
8	*An. tessellatus*	2							1			1
9	*An. lesteri*	2									1	1
10	*An. flavirostris*	2							2			
11	*An. indefinitus*	3							3			
12	*An. kochi*	4							1			3
13	*An. balabacensis*	6					6					
14	*An. letifer*	3			3							
15	*An. maculatus*	4	2				1		1			
16	*An. sulawesi*	1								1		
17	*An. barbumbrosus*	1										1
18	*An. peditaeniatus*	2		1						1		
19	*An. nigerrimus*	4		3						1		
	Total	154	2	16	13	29	30	14	16	6	15	13

Note: AC: Aceh; NS: North Sumatera; BB: Bangka Belitung; LM: Lampung; CJV: Central Java; WN: West Nusa Tenggara; EN: East Nusa Tenggara; WS: West Sulawesi; MO: Molucca; NM: North Molucca.

Therefore, the study could only provide a descriptive information of the *Rdl* allele among the collected species such as; Aceh (*An. maculatus* 2), North Sumatera (*An. vagus* 4, *An. sundaicus* 8, *An. peditaeniatus* 1 and *An. nigerrimus* 3), Bangka Belitung (*An. sundaicus* 10 and *An. letifer* 3), Lampung (*An. vagus* 5. *An.sundaicus* 23, *An. barbirostris* 1), Central Java (*An. vagus* 4, *An.aconitus* 11,

An. barbirostris 8, *An.balabacensis* 6 and *An. maculatus* 1), West Nusa Tenggara (*An. vagus* 5, *An. sundaicus* 4 and *An.subpictus* 5), East Nusa Tenggara(*An. vagus* 1, *An. sundaicus* 2, *An. subpictus* 4, *An. barbirostris* 1, *An. flavirostris* 2, *An. indefinitus* 3, *An. kochi* 1 and *An. maculatus*), West Sulawesi (*An. barbirostris* 3, *An. sulawesi* 1, *An. peditaeniatus* 1and *An. nigerrimus* 1), Molucca (*An. punctulatus* 4, *An. farauti* 10 and *An. lesteri* 1), and North Molucca (*An. punctulatus* 2, *An. vagus* 3, *An. subpictus* 1, *An. tesselatus* 1, *An. farauti* 1, *An. lesteri* 1, *An. kochi* 3 and *An. barbumbrosus* 1).

3.2 PCR Amplification and DNA Sequencing of the *Rdl* Gene Fragment

Using oligos that have been designed based on the published sequences of *Rdl* gene and sequences of *An. sundaicus* from Indonesia to amplify fragment of *Rdl* gene, the PCR succesfully amplified DNA of 19 species of *Anopheles* from Indonesia. Alignment of DNA sequencing results of each species is shown in Figure 3.2.1.

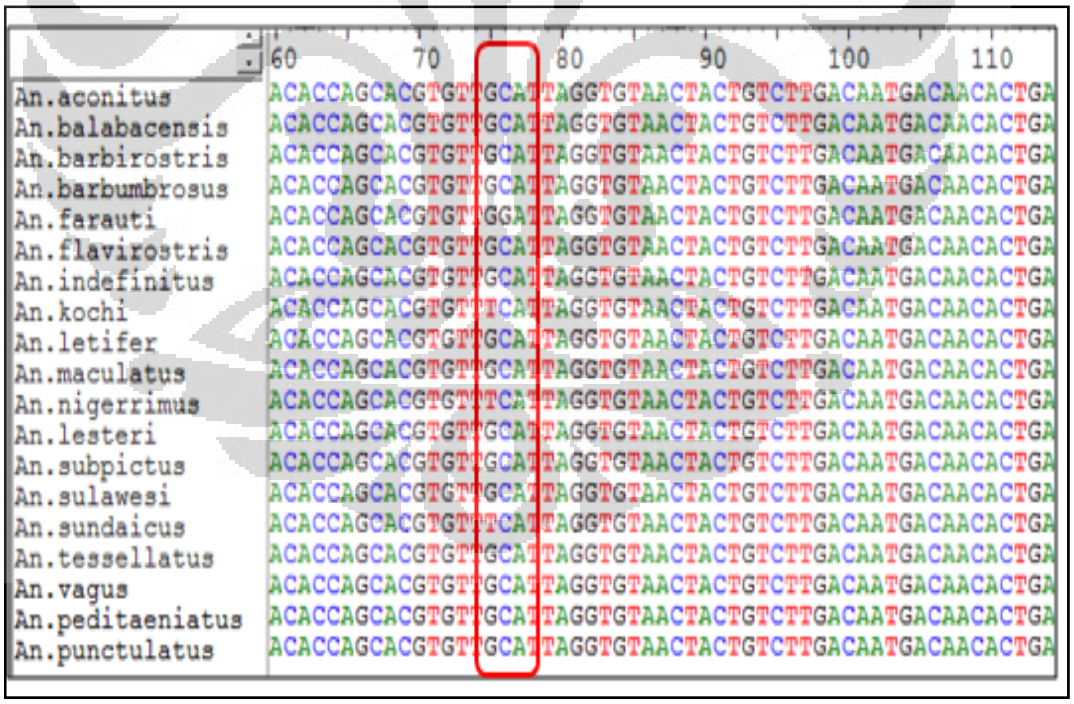

Figure 3.2.1 DNA sequence aligment of the *Rdl* allele fragment in 19 various *Anopheles* species from Indonesia. A red-rectangle are showing the site mutation of *Rdl* allele at 302 codon position.

The DNA sequences have been deposited in the GenBank (Appendix 1): *An. aconitus* (GenBank JN690008.1), *An. balabacensis* (GenBank: JN690009.1), *An. barbirostris* (GenBank: JN690010.1), *An. barbumbrosus* (GenBank: JN690011.1), *An. flavirostris* (GenBank: JN690013.1), *An. indefinitus* (GenBank: JN690014.1), *An. letifer* (GenBank: JN690016.1), *An. maculatus* (GenBank: JN690017.1), *An. lesteri, An. nigerrimus* (GenBank: JN690019.1), *An. punctulatus* (GenBank: JN690020.1), *An. subpictus* (GenBank: JN690021.1), *An. sulawesi* (GenBank: JN690022.1), *An. sundaicus* (GenBank: JN690023.1), *An. tesselatus* (GenBank: JN690024.1), *An. vagus* (GenBank: JN690025.1), *An. peditaeniatus* and *An. kochi* were carried A302S; and *An. farauti* (GenBank: JN690012.1) was carried A302G.

As the oligos failed to amplify the *Rdl* gene in certain species, degenerate oligos was further designed. The degenerate oligos successfully yielded a single band of DNA fragment of approximately 250 bp in size that was further processed for DNA sequencing (Appendix 2). The sequences obtained were compared by pair wise alignment to check their homology or to confirm the base substitution at the mutational site. As expected, the DNA sequence of the GABA gene varied significantly among the *Anopheles* species analyzed but the deduced amino acid sequences indicated a high sequence conservation.

3.3 Existence of *Rdl* Allele

Analysis of DNA sequences of 154 amplicons representing 19 *Anopheles* species indicated that the majority of the *Anopheles* carried the wild type A302 allele. The A302S allele was detected in 4 Provinces: North Sumatra, Central Java, Lampung and West Nusa Tenggara whereas the A302G allele was only detected in Molucca Province. The A302S *Rdl* allele was detected in *An. vagus, An. aconitus, An. barbirostris, An. sundaicus* and *An. nigerrimus* whereas the A302G allele was only detected in *An. farauti*. The *Rdl* A302S/G allele were not found in any of the *Anopheles* species examined from Aceh, Bangka Belitung, West Sulawesi, East Nusa Tenggara and North Molucca. All of the *Rdl* mutant alleles were found in homozygous form (Figure 3.3.2).

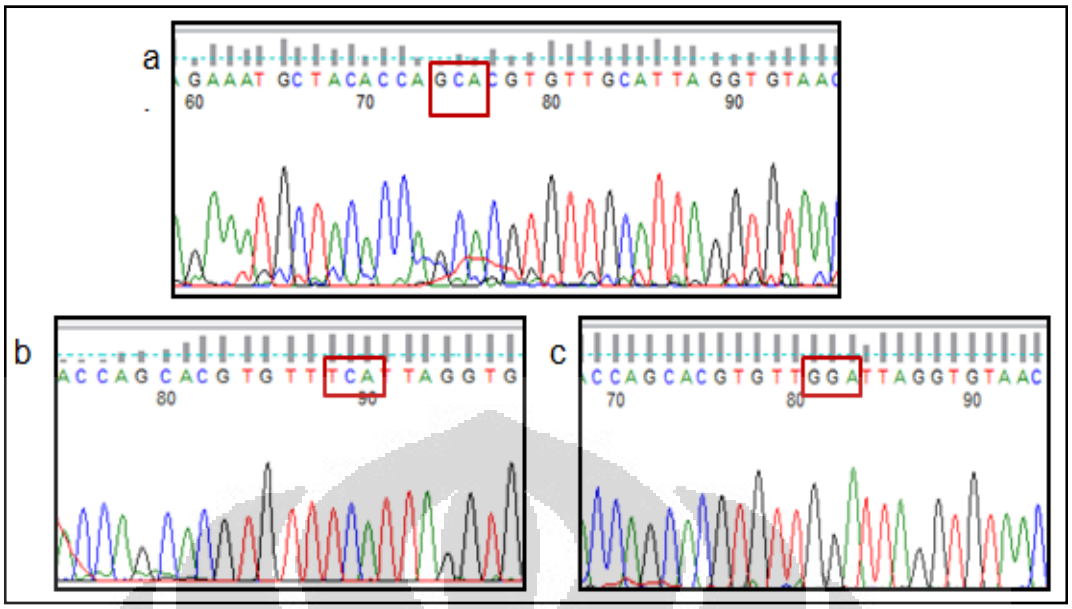

Figure 3.3.2 a. Indicated the wildtype allele A302 (alanine) and b. indicated the resistance allele A302S, GCA (alanin) replacement by TCA (serine) and c. Indicated the resistance allele A302G, GCA replacement by GGA (serine). All of *Rdl* allele found in single peak, homozygous form.

To confirm the species of the *Anopheles* examined, the ITS2 fragment was PCR amplified and sequenced. ITS2 sequences confirmed only 50% of the morphological identification (Unpublished data). Several ITS2 and *CO1* gene sequences were shown in Appendix 3-5. The Genotype and allele frequency of *Rdl* gene were shown in Table 3.3.1. Central Java is province with highest Rdl mutant allele frequency and and the highest anopheles species affected, namely *An. aconitus*, *An. vagus* and *An. barbirostris.*

Table 3.3.1 Frequency of *Rdl* allele in each *Anopheles* species examined at each study site

Study Site Provinces	Species	N ∑ 154	Genotype frequency %			Allele frequency (%)		
			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	0	10	90	0	10
North Sumatra	*An. vagus*	4	100	0	0	100	0	0
	An. peditaeniatus	1	100	0	0	100	0	0
	An. nigerrimus	3	67	33	0	67	33	0
	An. sundaicus	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. sundaicus	23	96	4	0	96	4	0
	An. barbirostris	1	0	100	0	0	100	0
Bangka Belitung	*An. sundaicus*	10	100	0	0	100	0	0
	An. letifer	3	100	0	0	100	0	0
East Nusa Tenggara	*An. vagus*	1	100	0	0	100	0	0
	An. sundaicus	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 Tenggara	*An. vagus*	5	80	20	0	80	20	0
	An. subpictus	5	100	0	0	100	0	0
	An. sundaicus	4	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. nigerrimus	1	100	0	0	100	0	0

Note. Genotypic frequency is the frequency of a genotype, percentage calculation done by observe homozygous or heterozygous in a population. AA = alanine, wildtype; SS = serine, mutant; GG = glycine, mutant. Allele frequency is the type of frequency that observed. A = alanine, wildtype; S = serine, mutant; G = glycine, mutant.

B. DISCUSSION

Molecular analyses of the *Rdl* gene of 154 *Anopheles*, representing 19 species from different geographic region of Indonesia revealed the existence of *Rdl* mutant alleles in Anopheles species from Aceh, North Sumatera, Central Java, Lampung and West Nusa Tenggara, respectively. Central Java is the province with the highest *Rdl* mutant allele frequency. The finding indicates that the *Rdl* mutant allele is widely distributed in Indonesia.

The *Rdl* mutant alleles have been associated with the mosquito resistance to cyclodiene insecticide, such as dieldrin and endosulfan. Dieldrin was used in malaria control program in Indonesia in the early 1959s when the resistance to DDT emerged but this insecticide was only used for a brief period as the biochemical assay confirmed the resistance of *An. aconitus* in Central Java (Soerono et al. 1965[2]). Our finding on the existence of the *Rdl* mutant allele in *An. aconitus* and other species in Central Java today corroborates the previous report that malaria vector in Central Java have been simultaneously resistant to dieldrin and DDT. Dieldrin has only been used for a short period and has long been retracted from the malaria control, curiosity on cause of its existence in various region in Indonesia have arisen. In this regard, it might be suggested that the existence of *Rdl* mutant allele among the anopheles species was either the result of continuous exposure to cyclodiene insecticides or that the *Rdl* mutant alleles are relatively stable or fit in comparison to the wildtype allele in the absence of the insecticide.

Of the seven Anopheles species (11%) that were found to carry the *Rdl* mutant alleles, *An. vagus* and *An. aconitus* were the most frequent species and both are closely associated with agricultural lands. *Anopheles vagus* and *An. aconitus* commonly use rice field and stream in low land area as its breeding sites. Likewise, *An. sundaicus* and *An. barbirostris* in Lampung and Central Java were also found to carry the *Rdl* mutant allele. Generally *An. sundaicus* and *An. barbirostris* are major malaria vectors in coastal or low land areas in Indonesia that are commonly used as agricultural land, such as rice field in the island setting (Ndoen et al. 2010). According to Sundararaman (1957), WHO and Idram et al. (2009) studies, *An. sundaicus* is exophagic and also endophilic and exophilic

mosquito. The remaining species that was found to carry the *Rdl* mutant alleles was *An. nigerrimus* and *An. farauti*. Anopheles *nigerrimus* is one of the few almost entirely exophilic species that breeds in large numbers in agriculture areas (Hemingway et al. 1986), whereas *An. farauti* usually use swamps and waterways as its breeding site.

In Indonesia, several cyclodiene insecticides such as endosulfan and fipronil are currently still in use in agricultural areas. Resistance of these insecticides to various agricultural insect has been documented in several areas (McCaffery et al. 1991; Udiarto and Setiawati 2007; Hadiyani et al. 2008). Although endosulfan and fipronil are mainly used to eliminate the agricultural pest, it is very likely that they also affect the anopheles population that commonly breed in agricultural area and that select for the *Rdl* allele. Further biochemical study to assess the sensitivity of the anopheles that carries the *Rdl* allele to the cyclodiene insecticides used in the agriculture is now in progress to ensure the association.

According to DNA sequences, 100% *Rdl* wildtype allele were detected in Sumba, East Nusa Tenggara; Halmahera, North Molucca; Bangka Belitung, Aceh and Mamuju, West Sulawesi. WHO in 2009 reported that during the three years 2007-2009 has reduced insecticide use associated with economic limitations. Based on these assumptions can be assumed that *Rdl* existence of wildtype allele may be caused by reduced use of insecticides, so the selection pressure of insecticides to be low. WHO in 2009 reported that the use of insecticide application in Halmahera, North Molucca district implemented a new last year and is only done through the use of ITNs or nets. *Anopheles* mosquitoes in Halmahera, North Molucca is exophilic which means more outside the home like environment, making it less for the mosquito to contact with a mosquito net that is in the house. As a result of low-intensity exposure to insecticides and resistant alleles did not appear.

Resistance alleles were not found in North Molucca, Bangka Belitung, Aceh and West Sulawesi. It is supported by the use of insecticides and the ecological conditions of *Anopheles* mosquitoes in those areas. Insecticides commonly used in agricultural areas, so that areas do not have the agricultural

areas, rarely use insecticides. In addition, due to the use of organochlorine insecticides has been replaced by a class of synthetic pyrethroid insecticides. Another factor that led to the lack of a resistant allele is due to small sample size (Reimer 2008) and the possibility of individuals carrying allele *Rdl* not elected to the studied sample.

IV. CONCLUSIONS

This study reports that the existence of the *Rdl* mutant alleles among the major malaria vector in Indonesia and their existence associated with insecticide use in agricultural area. Although dieldrin insecticide has been banned since 1960, this findings shown amount 11% of mutant *Rdl* alleles has been detected in Indonesia, 17 samples of 154 total samples. From 19 *Anopheles* species, we obtained the A302S *Rdl* allele was detected in 4 Provinces: North Sumatera, Central Java, Lampung and West Nusa Tenggara. The *Rdl* allele was detected *An. vagus, An. aconitus, An. barbirostris, An. sundaicus* and *An. nigerrimus*. The A302G allele was only detected in *An. farauti* from Molucca. All of the alleles were found in homozygous form. The A302S and A302G allele was not found in any of the *Anopheles* species examined from Aceh, North Molucca, Bangka Belitung, West Sulawesi and East Nusa Tenggara. From those provinces, all *Anopheles* species carried A302 *Rdl* allele or wild type.

Most *Anopheles* species was detected carrying the mutant *Rdl* allele use rice field or stream in inland area as their breeding sites. Its closely associate with insecticide use in agriculture area encourages resistance to *Anopheles* species. Mutated *Rdl* allele was relatively stable in nature at the population level.

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Paper II

SUSCEPTIBILITY OF *Anopheles* LARVAE TO ENDOSULFAN IN KATIBUNG AND RAJABASA DISTRICTS, LAMPUNG PROVINCE

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ABSTRACT

Susceptibility of *Anopheles* larvae to endosulfan in Lampung Province was investigated. The objectives of this study are to determine the LC_{50} of *Anopheles* larvae to endosulfan and its association with the existence of the *Rdl* mutant alleles. The susceptibility test was performed on duplicate of 20-30 larvae samples originated from Katibung and Rajabasa sub-districts in the presence of a wide concentration range of endosulfan (0-0.4%). The existence of the *Rdl* mutant alleles on the larvae was confirmed using semi-nested PCR and DNA sequencing. The results indicated that the LC_{50} of larvae from Katibung and Rajabasa sub-districts were 0.00893 (0.00332-0.01697) and 0.00904 (0.00401-0.01586). The PCR and DNA sequencing results of 18 representative larvae taken from each concentration revealed that all samples carried the wildtype allele of the *Rdl* gene. Both of susceptibility and molecular test shown that larva *Anopheles* species in Lampung and Rajabasa are still susceptible to endosulfan, but need susceptibility test in adult mosquitoes to check their susceptibility status compare with WHO standard dosage.

Keywords: *Anopheles*, endosulfan, larvae, *Rdl alleles*, susceptibility.

I. INTRODUCTION

Lampung is one of malaria endemic areas located in the southern part of the island Sumatera, Indonesia. The annual malaria incidence (AMI) in Lampung Province was 4.47-7.27% in 2005-2007, respectively and based on the evidence, the province was classified as area with mesoendemic (medium) status for malaria (PP&PL 2008). The geographic condition of the Lampung province that consist of a vast coastal region and tidal marsh low land and tropical mountain environment provides a suitable breeding sites for most of the majors malaria vectors such as

An. aconitus, An. maculatus and *An. sundaicus* (Garjito et al. 2004; Setyaningrum et al. 2008).

Cyclodiene is one of the organochlorine insecticides that were developed principally in the 1940s with the synthesis of aldrin and dieldrin. Dieldrin was also widely used a public health control measure for vectors of tropical diseases such as malaria, yellow fever and filariasis (Omardeen 1961; Zavon et al. 1961). In early 1950, this insecticide was widely used as effective agents to control the soil-borne insects attacking the seeds of the plants. Dieldrin and chlordane were used for treating perennial crops such as sugar-cane and banana (NHMRC 2000). The existance of the insect resistance to the cyclodiene insecticides, such as: aldrin, dieldrin, chlordane, heptachlor and toxaphene an important problem in the planning and operation of vector control programmes, especially in malaria eradication. Dieldrin resistance in houseflies developed very rapidly in certain wild populations after selection by residual spraying (Sadasivaiah et al. 2007).

In 1973, the use of organochlorine insecticides began to be stopped and banned because of the nature toxic to the environment and long-term risk to human health (Kimball 2003). WHO in 2006 allow the use of organochlorine DDT and dieldrin to control mosquito vector. WHO declared that organochlorine does not cause health risks when used in adequate doses (Janeira et al. 2008). Therefore, since 2006, organochlorine was used again with certain restrictions such as use for indoor house spraying and at a sufficient dose (Reimer et al. 2008). In Indonesia organochlorine insecticide was never used again for malaria control since its ban in 1960. Instead, the currently used insecticides are class of organophosphate insecticides, carbamates and pyrethroids (Winarno & Hutajulu 2007).

Endosulfan is one of the cyclodiene group that commonly used as pesticide; organochlorine group that used to kill insects and mites on crops including tea, coffee, cotton, fruits, vegetables, rice and grains. The chemical is out of patent and it is marketed by many different companies and under a variety of names including: Agrosulfan; Aginarosulfan; Banagesulfan; Cyclodan; Endocel; Endoson; Endonit; Endomil; Endosol; Endostar; Endodaf; Endosulfer; E-sulfan; Endorifan; Hildan; Redsun; Seosulfan; and Thiodan (www.inchem.org).

After the ban of organochlorine insecticides in malaria control in Indonesia, many people in Lampung Provinces has been still used organochlorines insecticide such as endosulfan, akodan and thiodan in agricultural areas.

Agricultural demand for pesticides continues to be substantial and this is reflected in the continued increase in cases of pesticide resistance in crop pests. A total of 506 species of insects and mites have been reported to have developed resistance to one or more pesticides (WHO 1992). It was also noticed that some *Anopheles* species had developed resistance to insecticide used agricultural activities such as organochlorine and organophosphorus insecticides (Sharma 1996). Various insecticides products are used to control agricultural pests and the amount applied is generally higher than that consumed in public health against vector (Chandre et al. 1999). Endosulfan is both effective insecticide and economical, so it has been well accepted and used for several years (Sharif et al. 2007).

Resistance to cyclodiene insecticide results in a reduction in the sensitivity of an insect population. This is reflected in repeated failure of an insecticide to achieve the expected level of control of insects when used according to the product label recommendations and where problems of product storage, application and unusual climatic or environmental conditions can be eliminated as causes of the failure. Insecticide resistance may be due to one or several of the following mechanisms, such as target insensitivity (mutation in the sodium channel, achetylcolinesterase and GABA receptor), increased detoxification or excretion, decreased penetration, or behavioral avoidance of the insecticide (Pasteur & Raymond 1996; Hemingway et al. 2004). Resistance to dieldrin (*Rdl*) involves a GABA receptor, particularly associated with the single nucleotide polymorphisms in the M2 transmembrane domain of the GABA-gated chloride ion channel (*Rdl* gene) (Wilkins et al. 2006). The encoded *Rdl* subunit assembles with other GABA receptor subunits to form the target site of the cyclodiene insecticides. Dieldrin resistance is associated with the replacement of a single amino acid, alanine at position 302 to serine or glycine (Thompson et al. 1993).

Resistance to cyclodiene insecticide had been documented in several insects such as *Coleoptera* resistance in New Caledonia (Brun 1989),

Leptinotarsa decemlineata resistance to endosulfan in Iran (Sharif et al. 2007). In Indonesia cyclodiene resistance had been reported in 1962 in Jogjakarta as well as in other zones of Central Java where the insecticides have been used in the rice fields to control agricultural pests. Further test showed that *Anopheles* species was resistant to both DDT and dieldrin (Soerono et al. 1965). The results indicated that agricultural use of insecticides is likely partly responsible for the development of insecticide resistance in the anopheline population. In 1991, endosulfan resistance to *Helicoverpa armigera* was reported in Sulawesi (McCaffery et al. 1991), *Plutella xylostella* L. resistance to fipronil in West Java (Udiarto & Setiawati 2007).

Since the development of resistance can affect the efficiency of available insecticides for *Anopheles* mosquito control, understanding the resistance status of *Anopheles* population would be useful. A succesful resistance management strategy involves a thorough knowledge of resistance mechanism. Resistance monitoring should be an integral part of vector or public health pest control program through bioassay, biochemical and molecular assay (Brogdon 1998). Resistance monitoring through bioassay or vulnerability test is a test to determine the level of susceptibility or immunity of insects (WHO 1992). Application of insecticides that are synergist to each other is one of the easiest and fastest procedures to obtain preliminary information about potential mechanism of resistance). The present study aims are to detect and characterized the LC_{50} to endosulfan and to perform *Anopheles* larvae *Rdl* alleles in Lampung Province.

II. MATERIALS AND METHODS

2.1 Larva Mosquitoes Sampling

Anopheles larvae were collected from breeding sites such as ground pool and fishponds in Katibung and Rajabasa sub-districts. The map of study area in Lampung Provinces showed in Appendix 6. The larvae were identified using morphological keys (Reid 1968).

2.2 Cyclodiene Insecticide

The insecticide used for this study was Endosulfan 35 % (EC 35, akodan trademark), it is derivative of cyclodiene insecticide (Bayer comp, Germany). The insecticide was diluted with double distilled water to achieve the concentrations used for susceptibility test (0.4%; 0.2%; 0.1%; 0.05%; 0.025% 0.0125%; 0.001% and 0% endosulfan for control).

2.3 Insecticide Susceptibility Test Procedure

Insecticide susceptibility tests were carried out using the WHO, CDC and publish previously study with tight modification protocol (WHO 1981; CDC 2002; Martin et al. 2006). Briefly, 20-30 of late third or early fourth instar larvae were used for bioassay in each concentration used. Using the pippetor, the appropriate volume premixed stock of endosulfan solution was added to the tray or beakers. The mosquito larvae were aspirated into each beaker glass. Mortality rate counting was done after an hour exposure. Dead larvae will either sink to the bottom of the beaker or floating to the top. This study was conducted in duplicates. The data were analyzed using a computer program to obtain the LC_{50} by probit analysis.

2.4 Molecular Techniques

Molecular DNA tests performed on *Anopheles* larva were obtained from representative different concentration of endosulfan insecticide.

2.4.1 Extraction of Mosquito Larvae DNA

Grinding buffer was added to the tube containing the larva mosquito carried homogenization. DNA extraction was performed by separating 10 µL homogenate larvae into 1.5 mL Eppendorf tube, added 200 µL saponin, then incubated for 4 hours. The tubes were centrifuge at 12 000 rpm for 10 minutes. Supernatant was discarded, 1 ml of Phosphate Buffer Saline (PBS) was added, spun at a speed of 12000 rpm for 5 minutes. Supernatant was removed, added to 100 µL and 50 µL ddH_2O 20% chelex-100 follow heated in a water bath at a temperature of 65°C for 10 minutes. The tube was at 12 000 rpm for 10 minutes. Supernatant was separated and stored as a stock of DNA on the refrigerator with -20°C.

2.4.2 Gene Amplification with PCR (Wooden et al. 1993)

The components of 10x PCR buffer reagent, 50mm $MgCl_2$, 10 mM dNTPs, *Rdl* forward primer, *Rdl* reverse primer, DNA polymerase enzyme, and ddH_2O. 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. All of PCR components are mixed in 1.5 ml Eppendorf tube, transferred into 0.2 ml microcentrifuge tube as much 22.5 µL and add 2.5 µL of DNA mosquito templates. Cycling conditions for first PCR using oligos F11 and R12 RDLF, denaturation at 94°C, annealing at 50°C, extension at extension at 72°and final polymerization at 72°C, each phase lasts for 30 seconds, 30 seconds, 1 minute and 30 seconds and 5 minutes to 30 cycles.

After the first PCR process was completed, first PCR product used as DNA template at second round PCR. A total of 1 µL DNA template and 24 µL of PCR components with oligos F10 and F12 RDLF transferred into 0.2 ml microcentrifuge tube. The second round PCR conditions used for the stages of denaturation, annealing, extension and final polymerization are 94°C, 50°C, 72°C, and 72°C, each phase lasts for 30 seconds, 30 seconds, 45 seconds and 45 seconds (40 cycles). PCR products stored at refrigerator with of 4°C temperature.

2.4.3 Electrophoresis

Amount 1g agarose powder dissolved in 50 ml of 1x TAE buffer and boiled in a microwave oven for two minutes, added 5µl EtBr. A total of 5 µL samples of the second round PCR product were in the electrophoresis buffer, and mixed with 2 µl loading dye then inserted into the gel wells. A total of 5µl marker ladder added to the wells gel as markers. Gel electrophoresis performed at a voltage 80 volts for ± 1 hour. The gel was documented using the Gel-Doc 1000.

2.4.4 Purification of DNA

Extraction and purification of DNA use a kit and performed according to procedures Wizard ® SV Gel and PCR Clean-Up System protocol (Promega).

PCR product transferred into the microcentrifuge tube then added membrane binding solution with a ratio 1:1. The tubes were incubated at 25°C for 1 minute, then transferred into a SV minicolumn tube and centrifuged at 12 000 rpm speed for 1 minute. Formed supernatant discarded and 700 µL of membrane wash solution added into the tube and centrifuged for 5 minutes. Discard supernatant and added 500 µL membrane wash solution, centrifuged the tubes for 1 minute at a speed of 12 000 rpm. SV minicolumn tube was transferred into new 1.5 ml Eppendorf tube, added to 30 µL nuclease-free water. Sample tubes were incubated at room temperature for 2 minutes and centrifuged at 12 000 rpm for 2 minutes. Supernatant containing the purified DNA was stored at 4°C or -20°C.

2.4.5 Sequencing DNA

2.4.5.1 Cycle Sequencing

DNA sequencing was carried out in accordance with the BigDye ® Terminator v3.1 Cycle Sequencing protocol (Applied Biosystem). The components of the reagent, consisting 6 µL of big-dye, 1 µL forward or reverse oligos primer F10 or F12 RDLF (4 pmol), ddH_20 and purified DNA were mixed in a 0.2 ml microcentrifuge tube. The amount of purified DNA measurements at a wavelength of 260/280 nannodrop then added ddH_20 until the total volume to 15 µL. Conditions used in thermal cycler machine was 96°C for pre-denaturation for 3 minutes and for denaturation step, annealing, polymerization were 96°C, 50°C, 60° C, each phase lasts for 10 seconds, 5 minutes and 4 minutes for 25 cycles. Cycle sequencing product was stored at 4°C.

2.4.5.2 DNA Precipitation

DNA precipitation carried out with BigDye ® Terminator v3.1 Cycle Sequencing protocol (Applied Biosystem). A total of 15 µL cycle sequencing product was transferred into 1.5 mL Eppendorf tube, added 37.5 µL 100% of ethanol, 1.5 µL of sodium acetate 3M pH 5.2 and 1.5 µL of EDTA 125 Mm. Vortex a tube briefly and incubated at room temperature for 15 minutes, centrifuged at 4°C at 12 000 rpm for 20 minutes. Supernatant discarded and the pellet formed that remains in the tube was dissolved with 250 µL 70% ethanol,

centrifuged at 12000 rpm at 4°C for 20 minutes. Discarded the supernatant and the tube was dried in a Speed-Vac ® machine at 6 000 rpm for 15 minutes. Wrapped the tube with aluminum foil paper and stored at -20°C.

2.4.5.3 Automated DNA Sequencing

The process of automated DNA sequencing performed by the laboratory staff of the Eijkman Institute sequencing services with reference to automated DNA sequencing protocol (Applied Biosystem). The results of DNA precipitation was mixed with 12 µL deionized formamide high, then vortex for 30 seconds. Inserted the tube into the plate and heated in a thermal cycler machine at a temperature of 95°C for 3 minutes, then cooled again in ice (snap cooling). Plate containing the samples mounted on a tray and placed in the machine sequencing ABI PRISM ® 3130xl Genetic Analyzer 16-capillary (Applied Biosystem). Sequencing process takes 45 minutes. Nucleotide sequence of readings recorded by a computer and converted into graphs showing by electroferogram form and arrangement of the sequence DNA.

2.5. Data Analysis

2.5.1 Susceptibility Status

Percentage mortality should be recorded on the report form. The LC_{50} was calculated using probit analysis and follow chi-square test from SPSS computer program. If the control mortality between 5-20%, the percentage mortalities should be corrected by Abbott's formula:

$$\text{Corrected} = \frac{\%\ \text{test mortality} - \%\ \text{control mortality}}{100 - \%\ \text{control mortality}} \times 100$$

(WHO 1981).

2.5.2 Sequences Analysis

Electroferogram of GABA gene sequences were analyzed using a Bioedit computer program and performed alignment of DNA sequences with one GABA sequence of *An. gambiae* (GenBank: XM316070). The sites of the mutated bases correspond to the amino acid residues of alanine 302, wildtype form.

Mutations at the sites will confer an amino acid change to either glycine or serine at the GABA receptor protein.

III. RESULTS AND DISCUSSION

3.1 Sampel Collection

Selection of sampling sites of *Anopheles* larvae was based on the previous malaria data, *Anopheles* population from Lampung Province and the intensity of insecticide use. Sampling sites associated with habitat and breeding site of the *Anopheles.* Katibung sub-district which has relatively few malaria cases, contains a vast agriculture area for banana and rice field. Aproximately, 400 mosquito larvae of *An. sundaicus* samples were taken from rice fields in Katibung sub-district. The other location is Rajabasa sub-district, which has a higher malaria cases and located along the coastal region of Sunda Strait. In Rajabasa sub-district, approximately 500 larvae of *Anopheles* were taken from fish ponds and shrimp ponds that are deserted. Larvae obtained from field were identified using larvae identification keys before the susceptibility test (Appendix 7).

3.2 Susceptibility Test

Susceptibility test of *Anopheles* larva to endosulfan with endosulfan, Akodan EC35 trademark was carried out using WHO, CDC and publish previously study standard procedures. Result obtained in Katibung and Rajabasa sub-districts where control mortalities were 0%, so the mortality percentage is not corrected by Abbott's formula. The purpose of the susceptibility test is to calculate the LC_{50} in *Anopheles* larvae population, that alternative control plans can be made. Larvae susceptibility test results shown the percentage of dead mosquito larva in each test concentrations were different.

In Katibung, larvae *Anopheles* were 100% mortality in 0.4% and 0.2 % concentrations, subsequently larval mortality has decreased. At 0.1%; 0.05 %; 0.025% more over 50% larvae were death and at 0.0125%; 0.001% concentration the mortality were 42.5% and 15%. The results of the susceptibility test from Katibung sub-district shown in Tables 3.2.1.

Table 3.2.1 Mortality rates of *Anopheles larva* after 1 hour exposure to endosulfan In Katibung districts

Concentration	Alive	Dead	% Mortality	% Average Mortality
0	20	0	0	0
	20	0	0	
0.001	19	1	5	15
	15	5	25	
0.0125	9	11	55	42.5
	14	6	30	
0.025	7	13	65	70
	5	15	75	
0.05	0	20	100	95
	2	18	90	
0.1	1	19	95	90
	3	17	85	
0.2	0	20	100	100
	0	20	100	
0.4	0	20	100	100
	0	20	100	

The results of the susceptibility test from Rajabasa shown in Tables 3.2.2. In Rajabasa area, at the concentrations of 0.4%; 0.2%; and 0.1% all larvae were 100% dead. At the concentrations of 0.05% or less the mortality of larvae are reduced. Generally, increase in concentration leads to increased mortality in both locations.

Table 3.2.2 Mortality rates of *Anopheles larva* after 1 hour exposure to endosulfan in Rajabasa districts.

Concentration	Alive	Dead	% Mortality	% Average mortality
0	30	0	0	0
	30	0	0	
0.001	25	5	17	12
	28	2	7	
0.0125	8	22	73	60
	16	14	47	
0.025	8	22	73	70
	10	20	67	
0.05	6	24	80	70
	12	18	60	
0.1	0	30	100	100
	0	30	100	
0.2	0	30	100	100
	0	30	100	
0.4	0	30	100	100
	0	30	100	

To find the LC_{50} with the most simple way is to graph, made in logarithmic graph paper, but the best way is using probit analysis which resumed with regression test. Endosulfan concentrations value recorded in the value of logarithms and the mortality recorded in probit (probit table shown in Appendix 8). The LC_{50} of the *Anopheles* larvae were 0.00893 (0.00332-0.01697) 0.00904 (0.00401-0.01586) from Katibung and Rajabasa sub-districts respectively (Appendixs 9 and 10). The higher value of LC_{50} shows the lower a sensitivity or toxicity. In this study LC_{50} value that obtained in both location is not significant differences, about ± 0.009 g/L, so does the LC_{90} value. The LC_{90} value from Katibung is 0.070 g/L and the LC_{90} from Rajabasa is 0.073 g/L. Although no previous data that could be used as comparison, if we compare the mortality rates percentage from Katibung and Rajabasa can also be seen that *Anopheles* larvae from Rajabasa has more susceptible to endosulfan (Figure 3.2.1).

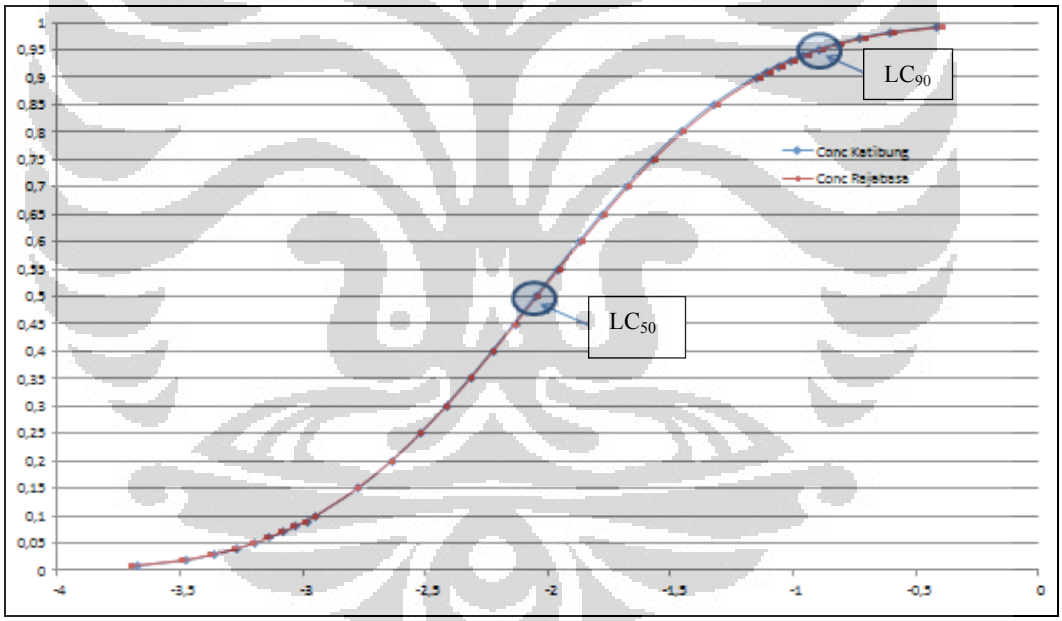

Figure 3.2.1 The LC50 and LC90 charts from Katibung and Rajabasa. The blue dot show a Katibung value and the red dot shows a Rajabasa value. X axis as a Log od endosulfan concentrartion and Y ordinat as a probit value from SPSS.

To kill 50% of *Anopheles* larva population, in Rajabasa requires lower concentration than Katibung. The findings associated to the more intensive use of endosulfan in the rice field on comparison to the fish or shrimp pond. But, if we use LC_{50} and LC_{90} as comparison to determine susceptibility, from both location there is not significant susceptibility differences.

Chi square test (X^2) showed that the withdrawl line in both locations is a good or in good fit. Can be interpreted that the result validity was based on a linear regression line is valid (Appendix 11 and 12). These data indicate that the LC_{50} value, especially on 24-hour observation can be trusted. This suggests that within 24 hours of endosulfan with ± 0.009 % concentration was able to kill 50% population of *Anopheles* larvae.

The LC_{50} value in this study warrants a regular monitoring of the mosquitoes susceptibility to the most widely used insecticide to ensure an appropriate insecticide use in the area in particular and in Indonesia in general. Detection of resistance will help public health personnel to formulate appropriate steps to counter reductions in effectiveness of control effort that may accompany with the emerging problems of insecticide resistance. Previous bioassay study in 2008-2009 in Lampung by Ministry of Health (Unpublished), *An. sundaicus* showed an *An. sundaicus* larvae susceptible to Deltamethrin 0.05 %, but tolerance to Bendiocarb 0.1%, Permethrin 0.25%, DDT 4%, Cypermethrin 0.05% and Alfacypermethrin 0.005%. Generally, most of pyrethroid insecticide, organochlorin and carbamate could still be used in agricultural area in Lampung provinces with supervision and monitoring control. However, some insecticides such as organochlorine (DDT, dieldrin, fipronil and endosulfan) have been banned for use, because the resistance has been reported elsewhere (Purworejo, Sulawesi and Bandung) (Soerono et al. 1965; McCaffery & Topper 1991; Udiarto & Setiawati 2007). Supervision, monitoring and appropriate of use this insecticides may inhibit or delay the emergence of a cross or double resistance (Mittal et al. 2004; Perera et al. 2008).

WHO (1981) was set the standard dose for dieldrin and DDT were 0.4% and 4% for adult mosquitoes, but this study cannot refer those standard dose. This study was used a larva *Anopheles* as a samples, caused there are different stage so the dosage has been different. To determine the toxicity level was measured by LC_{50}. LC_{50} values reflect both bioconcentration potential of a compound and its intrinsic toxicity (toxicological potency of the chemical once inside the organism) (McCarty 1986). LC_{50} values in this study shown that with ± 0.009 % endosulfan concentration was able to kill 50% population of *Anopheles* larvae.

Several studies shown that LC_{50} values for different species. LC_{50} for fishes species may vary widely; Tilapia has been reported to range from 10.20 µg/L (Kenneth & Seinen 2010); *Cyprinus carpio* has LC_{50} 0.001-0.0074 mg/L (Mulyaningsih 2004); *Plutella xylostella* has LC_{50} 30-237 ppm to fipronil (Udiarto & Setiawati). Standard tests employing single species provide information about the environmental risks of a chemical. However, it is not valid to rely on a narrow range of test species to set safe environmental levels for toxicants. This is further emphasized by the varying sensitivity of the widely used test species relative to other species. Different chemicals, species tested require different levels of testing to ensure comprehensive hazard evaluation. A chemical should be initially screened with a suite of short-term tests using a range of potentially vulnerable species to identify any requirement for further testing under chronic conditions (Taylor et al. 1991).

3.3 Molecular Analysis

PCR and the DNA sequencing of the *Rdl* gene fragments on 18 representative larvae treated with different concentrations of endosulfan indicated that all larvae carried the wildtype allele of the *Rdl* gene, A302 (Figure 3.3.1). Dieldrin and endosulfan is a cyclodiene insecticide group and the GABA receptor in the nervous systems of insects as a target of its insecticides. GABA receptor encodes *Rdl* (Resistance to dieldrin) gene. GABA receptor is the targets of naturally occuring compounds picrotoxinin and a wide range of synthetic insecticides (dieldrin, fipronil and endosulfan) (Buckingham et al. 2005). The relative tolerances of *Anopheles* larvae have been found to depend on both the chemical type and test period, thus highlighting the need for an increase in the range of species and exposure times used in toxicity testing. The variation in the expression of insecticide resistance in association with selection for larval time-to-hatch may induce this kind of enhanced adaptive plasticity as a consequence of pleiotropy, whereby mosquitoes are able to complete their aquatic life stages in a variable breeding environment using staggered larval time-to-hatch, giving rise to an adult population with enhanced variation in the expression of insecticide resistance (Kaiser et al. 2010).

Previous reports revealed the existence of the mutant *Rdl* alleles among the *An. vagus, An. sundaicus, An. barbirostris* and *An. kochi.* The species are well known to closely associate with agriculture area as mostly use the rice field or stream in inland area as their breeding sites. Therefore, it is highly possible that the insecticide use in the rice field select for this *Rdl* mutant alleles in those *Anopheles* species. Because there is little current data on the extent and distribution of cyclodiene resistance in field populations, or the potential for these *Anopheles* to develop cross-resistance toward endosulfan and dieldrin, the investigation of these phenomena would provide practical information on the frequency, stability of cyclodiene resistance and toxicity of endosulfan in field and the potential for long term utility of endosulfan to agriculture use control.

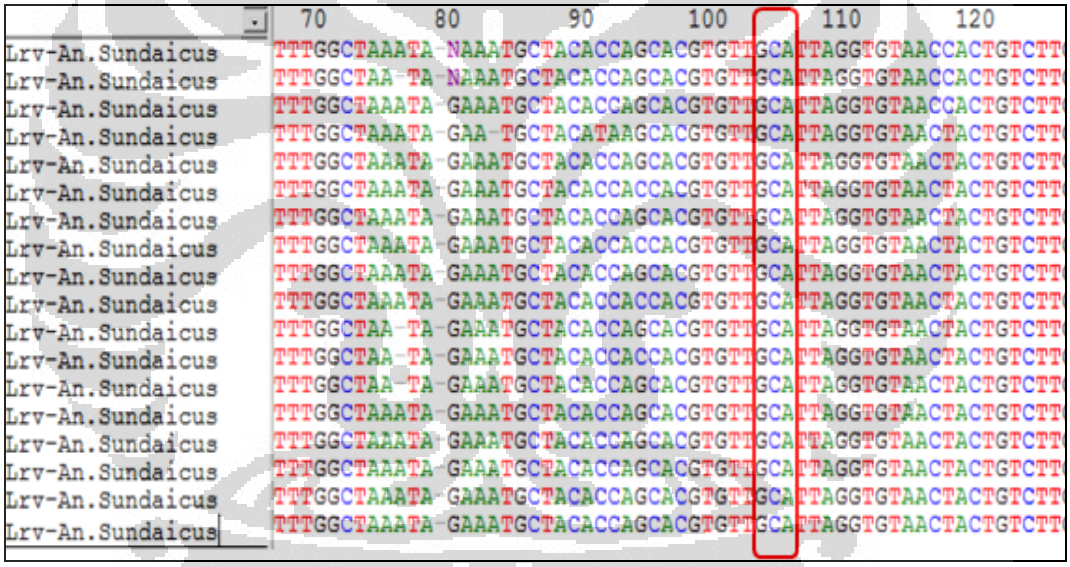

Figure 3.3.1 DNA sequence aligment of the fragment of GABA-*Rdl* gene in 18 larvae *Anopheles* from Katibung and Rajabasa District, Lampung Province. Red box shown the 302 alanine codon as point mutation of *Rdl* allele.

IV. CONCLUSIONS

Endosulfan one of cyclodiene insecticide class has been still used in Lampung Province. Commonly, endosulfan used in agriculture area and fishery. Susceptibility test using larva bioasaay in Katibung and Rajabasa districts report that LC_{50} of *Anopheles* larvae examined in both locations were 0.00893 (0.00332-

0.01697) in Katibung and 0.00904 (0.00401-0.01586) in Rajabasa. This suggests that within 24 hours of endosulfan with ± 0.009 % concentration was able to kill 50% population of *Anopheles* larvae. The LC_{50} value from both location is not significantly differences. It is indicated that the use of insecticide in both location is not differ much. From mortality percentage, we can seen that Rajabasa has lower concentration to kill 50% of *Anopheles* larvae population than concentration in Rajabasa. It is indicate that larvae from Rajabasa which were originated from fish and shrimp ponds have a significantly higher susceptibility to endosulfan than larvae from Katibung which were originated from rice field. The findings associated to the more intensive use of endosulfan in the rice field (Katibung) in comparison to the fish or shrimp pond (Rajabasa). Further study to examine more mosquito larvae from areas where the *Rdl* mutant alleles existed is warranted. The molecular analyses report, 18 representatives of *Anopheles* larvae were carried Ala302, wildtype *Rdl* allele.

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GENERAL DISCUSSION

Cyclodiene resistance is a commonly occuring phenomenon among insect species particularly in those areas where any cyclodiene-type insecticides have been used for pest control in the past. Recently, large position of the sequences of the GABA receptors in insect species have been reported including *Aedes aegypti* (Thompson et al. 1993). The initial and complete identification of one type of GABA receptor was made by ffrench-Constant et al. (1991) from a DNA clone obtained from a dieldrin resistant *Drosophila melanogaster*. The *Resistance to dieldrin* (*Rdl*) gene encode a subunit of the insect gamma aminobutyric acid (GABA) receptor and the encoded *Rdl* subunit assembles with other GABA receptor subunits to form the target site of the cyclodiene insecticides (ffrench-Constant et al. 2000). This type has been designated as *Rdl.* Subsequently, Henderson et al. (1993) reported another type of GABA receptor from a DNA cloned obtained from the same species which was named at LCCH3 belonging to β-subunit type in comparison to well-studied mammalian subunits. With regard to the site of mutation in cyclodiene-resistant insects, several reports indicating that the alanine to serine conversion of the 5th amino acid of the M2 cylinder cause resistance (Ala302 in *Rdl* terminology in *Drosophila).* Thompson et al. (1993) compare the DNA sequences of a 16 amino acid stretch of the M2 region from five species and found that in four cases the same alanine to serine conversion took place and in one case an alanine to glycine conversion was noted.

In this study, we find to detect *Anopheles species* from Indonesia that carried *Rdl* allele through molecular study. Using sequencing method to see 302 codon as a point mutation of *Rdl* allele, 19 species; 154 total sample from ten province in Indonesia has been succesfully done. Molecular study including using polymerase chain reactions (PCR) on cDNA, the DNA sequence of membrane spanning region of a GABA receptor of *Anopheles* species was identified. Using degenerate primer has been designed based on the published sequence of GABA gene form *An. gambiae* (GenBank Acc no. AF470112, AF470116), *An. stephensi* (EU883213), *Ae. aegypty* (AAU28803), *Cx. quinqifaciatus* (XM001850045) and sequence from *An. sundaicus* from Indonesia (GenBank: JN690023).

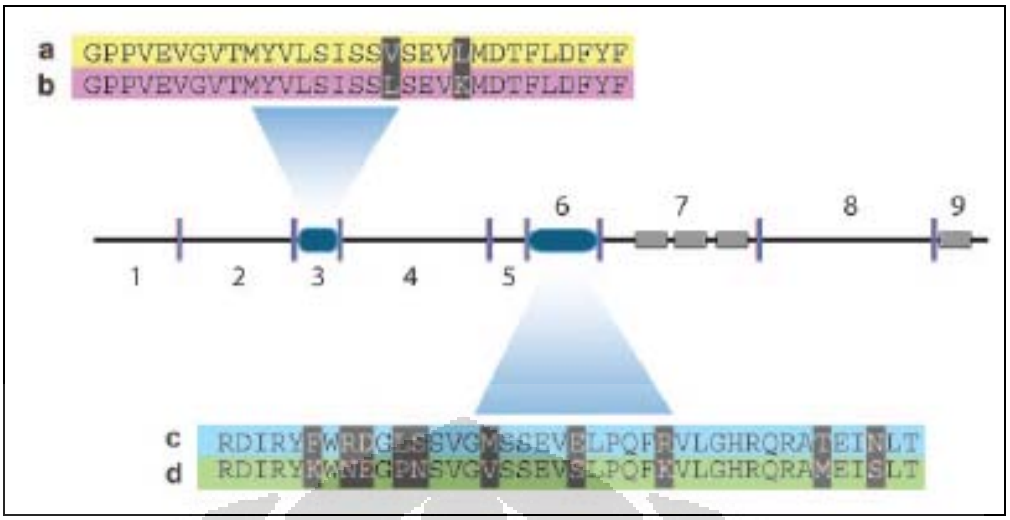

Figure 1. Modifications of RDL. A, exon structure of the *Rdl* gene; gray boxes represent transmembrane (TM) regions 1 to 3 coded by exon 7 and the TM 4 region encoded by exon 9. Alternative splicing occurs in exons 3 and 6. The two versions of exon 3 (a and b) differ by two amino acid residues. The two versions of exon 6 (c and d) differ by 10 amino acid residues (Adapted from Buckingham et al. 2005).

Oligos primer position and nucleotides of degenerate primer are shown in Figure 1. Amplicons, which is, approximately 250 bp in size, were then prepared for DNA sequencing. The sequences obtained were compared by pairwise alignment to check their homology or to confirm that the observed single point mutation site was indeed an actual change in the genomic. As expected, the DNA sequence of the GABA gene varied significantly among the *Anopheles* species analyzed but the deduced amino acid sequences indicated a high sequence conservation.

Analysis of DNA sequences of 154 amplicons representing 19 Anopheles species indicated that the majority of the *Anopheles* carried the wildtype 302A alele. Most of *Anopheles* species already identified using molecular (ITS2 fragment or *CO1* gene. There is 11% of mutated alleles *Rdl* has been detected in Indonesia, 17 samples of 154 total sample. The A302S polymorphism of the GABA gene, popularly known as *Rdl* allele was detected in 4 Provinces: North Sumatera, Central Java, Lampung and West Nusa Tenggara. The *Rdl* allele was detected *An. vagus, An. aconitus, An. barbirostris, An. sundaicus* and *An. nigerrimus*. The A302G allele was only detected in *An. farauti* from Molucca. All of the alleles was found in homozygous form. The *Rdl* 302S/G allele was not

found in any of the anopheles species examined from Aceh, North Molucca, Bangka Belitung, West Sulawesi, and East Nusa Tenggara.

The relative frequencies of these alleles varied among an *Anopheles* species from Lampung, Purworejo. In Lampung, we were obtained 3 species from 29 total mosquito samples. They are *An. vagus, An. sundaicus* and *An. barbirostris.* All species was detected have *Rdl* alelle mutation. *An. sundaicus* generally as a vector in coastal areas are in Aceh, North Sumatra, Riau, South Sumatra, Lampung, West Java, Central Java, East Java, West Nusa Tenggara, East Nusa Tenggara, Sulawesi and Kalimantan. *An. sundaicus* is exophagic (Idram et al. 1999), exophilic according to the WHO, Sundararaman study (1957) reported that in Lampung, *An. sundaicus* is endophilic and exophilic mosquito.

Lampung is also an area of farms and plantations such as banana, cofee and rice. The used of organochlorine insecticides such as dieldrin and DDT are not done anymore since its use is prohibited by the government. However, derivatives cyclodiene insecticide and organochlorines such as endosulfan is still used by citizens in the fields of agriculture, although the use of the very few and limited to certain areas. Most of farmer using cyclodiene insecticide to inhibiting pest in their farm. Bioassay test for *An. sundaicus* in Lampung was done by ministry of health using the deltamethrine 0,05% and the *Anopheles* status was susceptible. *An. sundaicus* status against bendiocarb 0,1 %, permethrin 0,25%, cypermethrine 0,05%, alfacypermethrine 0,005% and DDT 0,04% were tolerance range. Susceptibility test in this study shown that LC_{50} of *Anopheles* larva were 0.00893 (0.00332-0.01697) and 0.00904 (0.00401-0.01586) in Katibung and Rajabasa sub-districts. Followed by molecular study to detect the target site of cyclodiene insecticide, 18 larva *Anopheles* were taken from each concentrations and DNA sequenced shown that no mutation in 302Ala *Rdl*-GABA gene. Dieldrin (cyclodiene insecticide) resistance in Lampung not followed by endosulfan (cyclodiene insecticide) resistance, it caused different degrees of susceptibility to each insecticide.

The discovery of of the mutant *Rdl* allele within so many species was unexpected, since most of the strain have not undergone routine tretment and therefore selection with cyclodienes (Bass et al. 2004). Cyclodiene resistance is

able to persist in the absence of extensive insecticide selection, representing a threat for novel insecticides interacting with the cyclodiene binding site such as fipronil (ffrench-Constant et al. 2000). This leads to concern about possible cross-resistance between fipronil and cyclodiene insecticide (Ageng Li et al. 2006). The limited cross-resistance provided by the cyclodiene resistant *Rdl* allele, combined with the very low frequency of this allele in field populations (Gao et al. 2006).

Generally, this finding indicates that cyclodiene insecticides pressures along this specific target in anopheline mosquitoes are still in place in many malaria endemic areas. This insectide was only used for a short period following the growing of mosquito resistance in Central Java in 1965 (Soerono et al. 1965[2]). Therefore, the existence of *Rdl* allele in many *Anopheles* species in Indonesia might be associated with the use of this insecticide in agriculture. The fact that most of the *Anopheles* vectors that carried the *Rdl* alleles in this study were the species that commonly breed in agricultural area.

CONCLUSIONS, SUGGESTIONS AND IDEAS

Conclusions

1. *Rdl* (Resistance to dieldrin) alelles were found in Indonesia. Amount 11 % of the total 154 *Anopheles* samples examined, carried *Rdl* mutant alleles. *Rdl* allele A302S was observed in *An. vagus* (from Central Java, Lampung, and West Nusa Tenggara), *An. aconitus* (from Central Java), *An. barbirostris* (from Central Java and Lampung), *An. sundaicus* (from North Sumatra and Lampung), *An. nigerrimus* (from North Sumatra) and whereas the A302G allele was only found in *An. farauti* from Molucca.

2. The LC_{50} of *Anopheles* larvae examined were 0.00893 (0.00332-0.01697) and 0.00904 (0.00401-0.01586) in Katibung and Rajabasa sub-districts. This suggests that within 24 hours of endosulfan with ± 0.009 % concentration was able to kill 50% population of *Anopheles* larvae.

3. Insecticide pressure on the *Anopheles* population might still ongoing (though not directly associated with malaria control program, but agriculture too) or that the mutant form of the *Rdl* allele is relatively stable in the absence of insecticide.

Suggestions

Needed strict regulation, monitoring, supervision and evaluation on the use of pesticides either in agricultar or in health programme. integrated pest management is warranted in malaria endemic areas where insecticides are widely used for other purposes.

Ideas for Further Researches

1. There should be a biochemical and bioasaay study as previous study or as comparison data
2. There should be a higer amount of sample and a size of sample is homogent, this is done to determine the alelle distribution.
3. There should be more researches to several insecticides which has been still used or has banned in Indonesia.
4. It is necessary using rearing *Anopheles* species samples (larvae and adult) to check their homologous

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Appendix 1. *Rdl* GenBank Deposit

ORGANISM Anopheles species
Eukaryota; Metazoa; Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Endopterygota; Diptera; Nematocera; Culicoidea; Culicidae; Anophelinae; Anopheles.

AUTHORS Asih,P.B., Syahrani,L., Rozi,I.E., Pratama,N.R., Marantina,S., Lobo,N.F., Sukowati,S. and Syafruddin,D.

TITLE Discovery of Rdl allele among the Anopheles malaria vector in Indonesia

JOURNAL Submitted (16-SEP-2011) Malaria Laboratory, Eijkman Institute for Molecular Biology, Jl.Diponegoro No. 69, Jakarta, DKI Jakarta 10430, Indonesia

***Anopheles aconitus* isolate CentralJava-37c GABA-Rdl gene, partial cds**

GenBank: JN690008.1

LOCUS JN690008 216 bp DNA linear INV 04-FEB-2012

SOURCE Anopheles aconitus

FEATURES Location/Qualifiers

source 1..216
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/mol_type="genomic DNA"
/isolate="CentralJava-37c"
/db_xref="taxon:93947"

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CDS <1..>216
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/translation="PSGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTLMSSTNAA
LPKISYVKSIDVYLGTCFVMVFAILLE"

ORIGIN

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61 accagcacgt gttgcattag gtgtaactac tgtcttgaca atgacaacac tgatgtcgtc

121 aacgaatgct gccttaccga aaatatctta tgtaaaatcg attgacgtat atttaggcac

181 atgcttcgtc atggtatttg ccattctgct ggagga

***Anopheles balabacensis* isolate CentralJava-24D GABA-Rdl gene, partial cds**

GenBank JN690009.1

LOCUS JN690009 209 bp DNA linear INV 04-FEB-2012

DEFINITION Anopheles balabacensis isolate CentralJava-24D GABA-Rdl gene partial cds.

SOURCE Anopheles balabacensis

FEATURES Location/Qualifiers

source 1..209

/organism="Anopheles balabacensis"

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/db_xref="taxon:59124"

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CDS <1..>209

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/db_xref="GI:374255815"

translation="SGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTLMSSTNAAL

PKISYVKSIDVYLGTCFVLVFASLL"

ORIGIN

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61 gcacgtgttg cattaggtgt aactactgtc ttgacaatga caacactgat gtcgtcaacg

121 aatgctgcct taccgaaaat atcttatgta aaatcgattg acgtatattt aggcacatgc

181 ttcgtcctgg tatttgccag tctgctgga

***Anopheles barbirostris* isolate centralJava-7a GABA-Rdl gene, partial cds**

GenBank: JN690010.1

LOCUS JN690010 203 bp DNA linear INV 04-FEB-2012

DEFINITION Anopheles barbirostris isolate centralJava-7a GABA-Rdl gene, partial cds.

SOURCE Anopheles barbirostris

FEATURES Location/Qualifiers

source 1..203
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/isolate="centralJava-7a"
/db_xref="taxon:112267"

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CDS <1..>203
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translation="SGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTLMSSTNAAL
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ORIGIN

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61 gcacgtgttg cattaggtgt aactactgtc ttgacaatga caacactgat gtcgtcaaca
121 aatgctgcct taccgaaaat atcttatgta aaatcaattg acgtatattt aggcacatgc
181 ttcgtcttgg tatttgccag ttc

***Anopheles barbumbrosus* isolate NorthMoluccas-08-2 GABA-Rdl gene, partial cds**

GenBank: JN690011.1

LOCUS JN690011 210 bp DNA linear INV 04-FEB-2012

DEFINITION Anopheles barbumbrosus isolate NorthMoluccas-08-2 GABA-Rdl gene, partial cds.

SOURCE Anopheles barbumbrosus

FEATURES Location/Qualifiers

source 1..210

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/mol_type="genomic DNA"
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mRNA <1..>210
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CDS <1..>210
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61 ccagcacgtg ttgcattagg tgtaactact gtcttgacaa tgacaacact gatgtcgtca
121 acgaatgctg ccttaccgaa aatatcttat gtaaaatcga ttgacgtata tttaggcaca
181 tgcttcgtcc tggtatttgc cattctgctg

***Anopheles farauti* isolate Moluccas-4 GABA-Rdl gene, partial cds**

GenBank: JN690012.1
LOCUS JN690012 209 bp DNA linear INV 04-FEB-2012
DEFINITION Anopheles farauti isolate Moluccas-4 GABA-Rdl gene, partial cds.
SOURCE Anopheles farauti
FEATURES Location/Qualifiers
source 1..209
/organism="Anopheles farauti"
/mol_type="genomic DNA"
/isolate="Moluccas-4"
/db_xref="taxon:69004"
mRNA <1..>209
/product="GABA-Rdl"
CDS <1..>209
/codon_start=1
/product="GABA-Rdl"

/protein_id="AEZ00790.1"
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PKISYVKSIDVYLGTCFVMVFASLL"
ORIGIN
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61 gcacgtgttg gattaggtgt aactactgtc ttgacaatga caacactgat gtcgtcaacg
121 aatgctgcct taccgaaaat atcttatgta aaatcgattg acgtatattt aggcacatgc
181 ttcgtcatgg tatttgccag tctgctgga

***Anopheles flavirostris* isolate EastNusatenggara-1017 GABA-Rdl gene, partial cds**

GenBank: JN690013.1

LOCUS JN690013 208 bp DNA linear INV 04-FEB-2012
DEFINITION Anopheles flavirostris isolate EastNusatenggara-1017 GABA-Rdl gene, partial cds.
FEATURES Location/Qualifiers
source 1..208
/organism="Anopheles flavirostris"
/mol_type="genomic DNA"
/isolate="EastNusatenggara-1017"
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mRNA <1..>208
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CDS <1..>208
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/db_xref="GI:374255823"
/translation="PSGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTLMSSTNAA
LPKISYVKSIDVYLGTCFVLVFASL"
ORIGIN
1 taccatccgg attaattgta atcatatcgt gggtatcatt ttggctaaat agaaatgcta
61 caccagcacg tgttgcatta ggtgtaacta ctgtcttgac aatgacaaca ctgatgtcgt
121 caacgaatgc tgccttaccg aaaatatctt atgtaaaatc gattgacgta tatttaggca
181 catgcttcgt cctggtattt gccagtct

***Anopheles indefinitus* isolate EastNusaTenggara-514 GABA-Rdl gene, partial cds**

GenBank: JN690014.1

LOCUS JN690014 207 bp DNA linear INV 04-FEB-2012

DEFINITION Anopheles indefinitus isolate EastNusaTenggara-514 GABA-Rdl gene, partial cds.

SOURCE Anopheles indefinitus

FEATURES Location/Qualifiers

source 1..207
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/isolate="EastNusaTenggara-514"
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CDS <1..>207
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/db_xref="GI:374255825"
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LPKISYVKSIDVYLGTCFVLVFASL"

ORIGIN

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61 ccagcacgtg ttgcattagg tgtaactact gtcttgacaa tgacaacact gatgtcgtca
121 acgaatgctg ccttaccgaa aatatcttat gtaaaatcga ttgacgtata tttaggcaca
181 tgcttcgtcc tggtatttgc cagtctg

***Anopheles kochi* isolate Lampung-24C GABA-Rdl gene, partial cds**

GenBank: JN690015.1

LOCUS JN690015 206 bp DNA linear INV 04-FEB-2012

DEFINITION Anopheles kochi isolate Lampung-24C GABA-Rdl gene, partial cds.

KEYWORDS .

SOURCE Anopheles kochi

FEATURES Location/Qualifiers

source 1..206
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/mol_type="genomic DNA"
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121 atgctgcctt accgaaaata tcttatgtaa aatcgattga cgtatattta ggcacatgct
181 tcgtcatggt atttgccatt ctgctg

***Anopheles letifer* isolate BangkaBelitung-145 GABA-Rdl gene, partial cds**

GenBank: JN690016.1
LOCUS JN690016 213 bp DNA linear INV 04-FEB-2012
DEFINITION Anopheles letifer isolate BangkaBelitung-145 GABA-Rdl gene, partial cds.
FEATURES Location/Qualifiers
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CDS <1..>213
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/db_xref="GI:374255829"
/translation="PSGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTLMSSTNAA
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ORIGIN
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61 caccagcacg tgttgcatta ggtgtaacta ctgtcttgac aatgacaaca ctgatgtcgt
121 caacgaatgc tgccttaccg aaaatatctt atgtaaaatc gattgacgta tatttaggca
181 catgcttcgt cttggtattt gccattctgc ggg

***Anopheles maculatus* isolate Aceh-9 GABA-Rdl gene, partial cds**
GenBank: JN690017.1
LOCUS JN690017 210 bp DNA linear INV 04-FEB-2012
DEFINITION Anopheles maculatus isolate Aceh-9 GABA-Rdl gene, partial cds.
SOURCE Anopheles maculatus
FEATURES Location/Qualifiers
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LPKISYVKSIDVYLGTCFVLVFASLL"
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61 ccagcacgtg ttgcattagg tgtaactact gtcttgacaa tgacaacact gatgtcgtca
121 acgaatgctg ccttaccgaa aatatcttat gtaaaatcga ttgacgtata tttaggcaca

181 tgcttcgtcc tggtatttgc cagtctgctg

***Anopheles minimus* isolate EastNusaTenggara-966 GABA-Rdl gene, partial cds**

GenBank: JN690018.1

LOCUS JN690018 209 bp DNA linear INV 04-FEB-2012

DEFINITION Anopheles minimus isolate EastNusaTenggara-966 GABA-Rdl gene, partial cds.

ACCESSION JN690018

VERSION JN690018.1 GI:374255832

FEATURES Location/Qualifiers

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CDS <1..>209
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/translation="SGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTLMSSTNAAL
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121 cgaatgctgc cttaccgaaa atatcttatg taaaatcgat tgacgtatat ttaggcacat
181 gcttcgtctt ggtatttccc attcggtgg

***Anopheles nigerrimus* isolate NorthSumatra-121A GABA-Rdl gene, partial cds**

GenBank: JN690019.1

LOCUS JN690019 209 bp DNA linear INV 04-FEB-2012

DEFINITION Anopheles nigerrimus isolate NorthSumatra-121A GABA-Rdl gene, partial cds.

FEATURES Location/Qualifiers
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CDS <1..>209
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121 caacgaatgc tgccttaccg aaaatatctt atgtaaaatc gattgacgta tatttaggca
181 catgcttcgt cctggtattt gccattctg

***Anopheles punctulatus* isolate NorthMoluccas4 GABA-Rdl gene, partial cds**

GenBank: JN690020.1
LOCUS JN690020 212 bp DNA linear INV 04-FEB-2012
DEFINITION Anopheles punctulatus isolate NorthMoluccas4 GABA-Rdl gene, partial cds.
FEATURES Location/Qualifiers
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121 caacgaatgc tgccttaccg aaaatatctt atgtaaaatc gattgacgta tatttaggca
181 catgcttcgt cctggtattt gccattctgc tg

***Anopheles subpictus* isolate WestNusaTenggara-69 GABA-Rdl gene, partial cds**

GenBank: JN690021.1
LOCUS JN690021 215 bp DNA linear INV 04-FEB-2012
DEFINITION Anopheles subpictus isolate WestNusaTenggara-69 GABA-Rdl gene, partial cds.
FEATURES Location/Qualifiers
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CDS <1..>215
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/db_xref="GI:374255839"
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ORIGIN
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121 cgaatgctgc cttaccgaaa atatcttatg taaaatcgat tgacgtatat ttaggcacat

181 gcttcgtcct ggtatttgcc agtctgctgg aaaaa

***Anopheles sulawesi* isolate WestSulawesi-646 GABA-Rdl gene, partial cds**

GenBank: JN690022.1

LOCUS JN690022 212 bp DNA linear INV 04-FEB-2012

DEFINITION Anopheles sulawesi isolate WestSulawesi-646 GABA-Rdl gene, partial cds.

FEATURES Location/Qualifiers

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/mol_type="genomic DNA"

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CDS <1..>212

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ORIGIN

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61 ccagcacgtg ttgcattagg tgtaactact gtcttgacaa tgacaacact gatgtcgtca

121 acgaatgctg ccttaccgaa aatatcttat gtaaaatcga ttgacgtata tttaggcaca

181 tgcttcgtcc tggtatttgc cagtctgctg ga

***Anopheles sundaicus* isolate Lampung-6C GABA-Rdl gene, partial cds**

GenBank: JN690023.1

LOCUS JN690023 207 bp DNA linear INV 04-FEB-2012

DEFINITION Anopheles sundaicus isolate Lampung-6C GABA-Rdl gene, partial cds.

FEATURES Location/Qualifiers

source 1..207

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/db_xref="taxon:34692"
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CDS <1..>207
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ORIGIN
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121 aacgaatgct gccttaccga aaatatctta tgtaaaatcg attgacgtat atttaggcac
181 atgcttcgtc ttggtatttg ccattct

***Anopheles tessellatus* isolate NorthMoluccas-31c GABA-Rdl gene, partial cds**
GenBank: JN690024.1
LOCUS JN690024 208 bp DNA linear INV 04-FEB-2012
DEFINITION Anopheles tessellatus isolate NorthMoluccas-31c GABA-Rdl gene,
partial cds.
FEATURES Location/Qualifiers
source 1..208
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/protein_id="AEZ00802.1"
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PKISYVKSIDVYLGTCFVLVFASLL"
ORIGIN
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61 gcacgtgttg cattaggtgt aactactgtc ttgacaatga caacactgat gtcgtcaacg
121 aatgctgcct taccgaaaat atcttatgta aaatcgattg acgtatattt aggcacatgc
181 ttcgtcctgg tatttgccag tctgctgg

***Anopheles vagus* isolate Lampung-25C GABA-Rdl gene, partial cds**

GenBank: JN690025.1

LOCUS JN690025 209 bp DNA linear INV 04-FEB-2012
DEFINITION Anopheles vagus isolate Lampung-25C GABA-Rdl gene, partial cds.
FEATURES Location/Qualifiers
source 1..209
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/isolate="Lampung-25C"
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mRNA <1..>209
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CDS <1..>209
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61 accagcacgt gttgcattag gtgtaactac tgtcttgaca atgacaacac tgatgtcgtc
121 aacgaatgct gccttaccaa aaatatctta tgtaaaatcg attgacgtat atttaggcac
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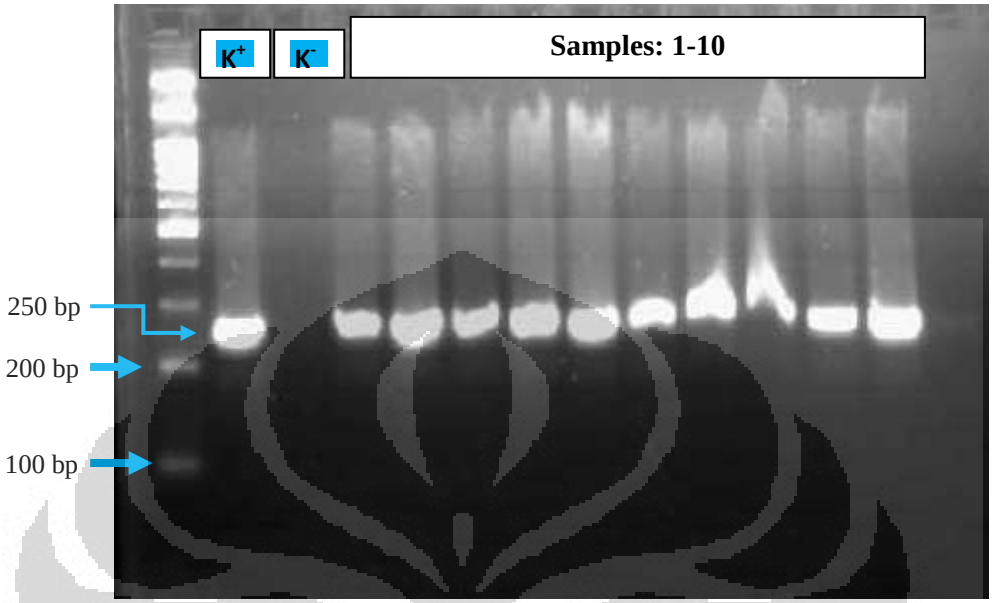
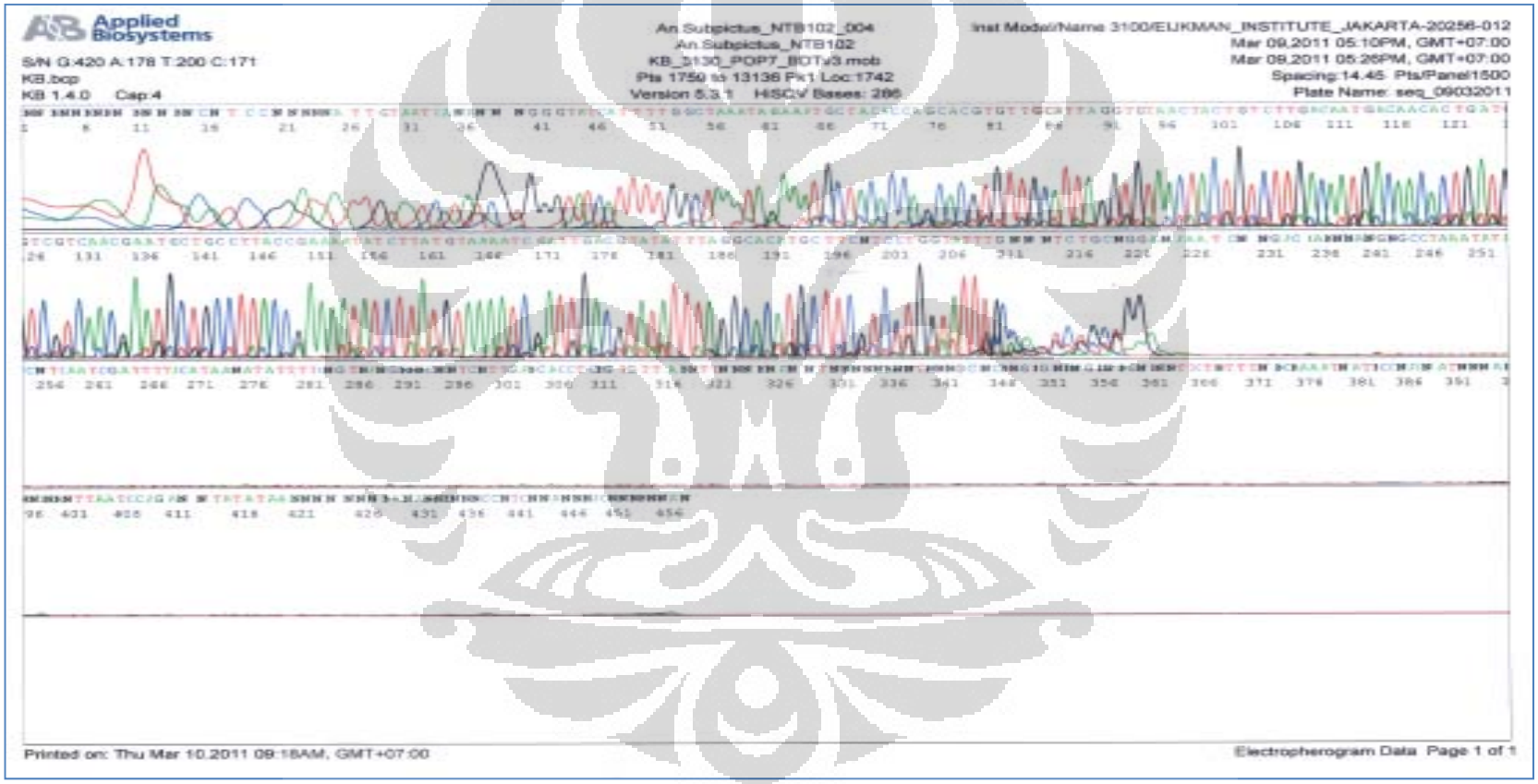
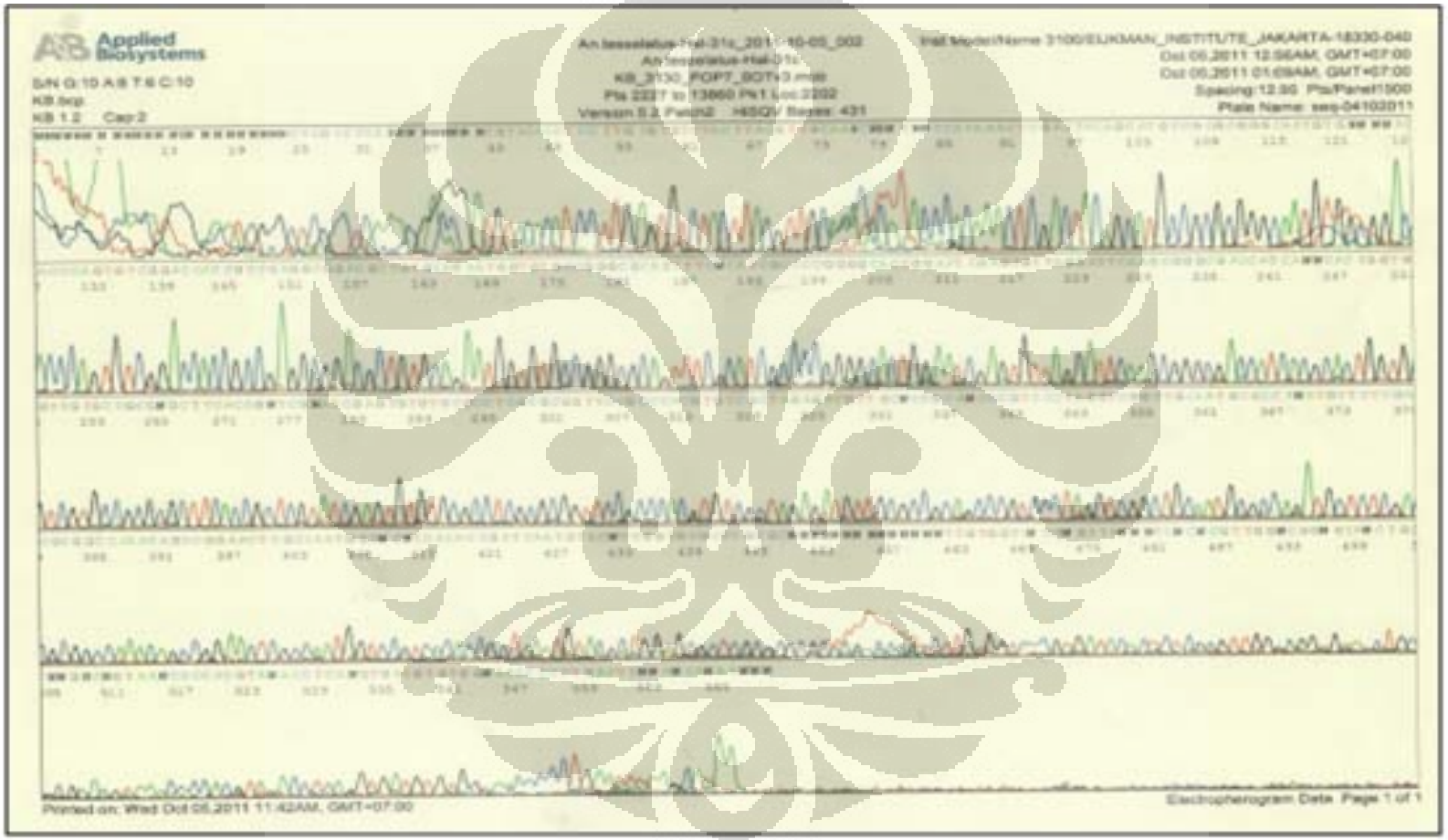
Appendix 2. Electropherograph showing the amplification of GABA gene from *Anopheles* species. Banding pattern of expected *Rdl* (250 bp).



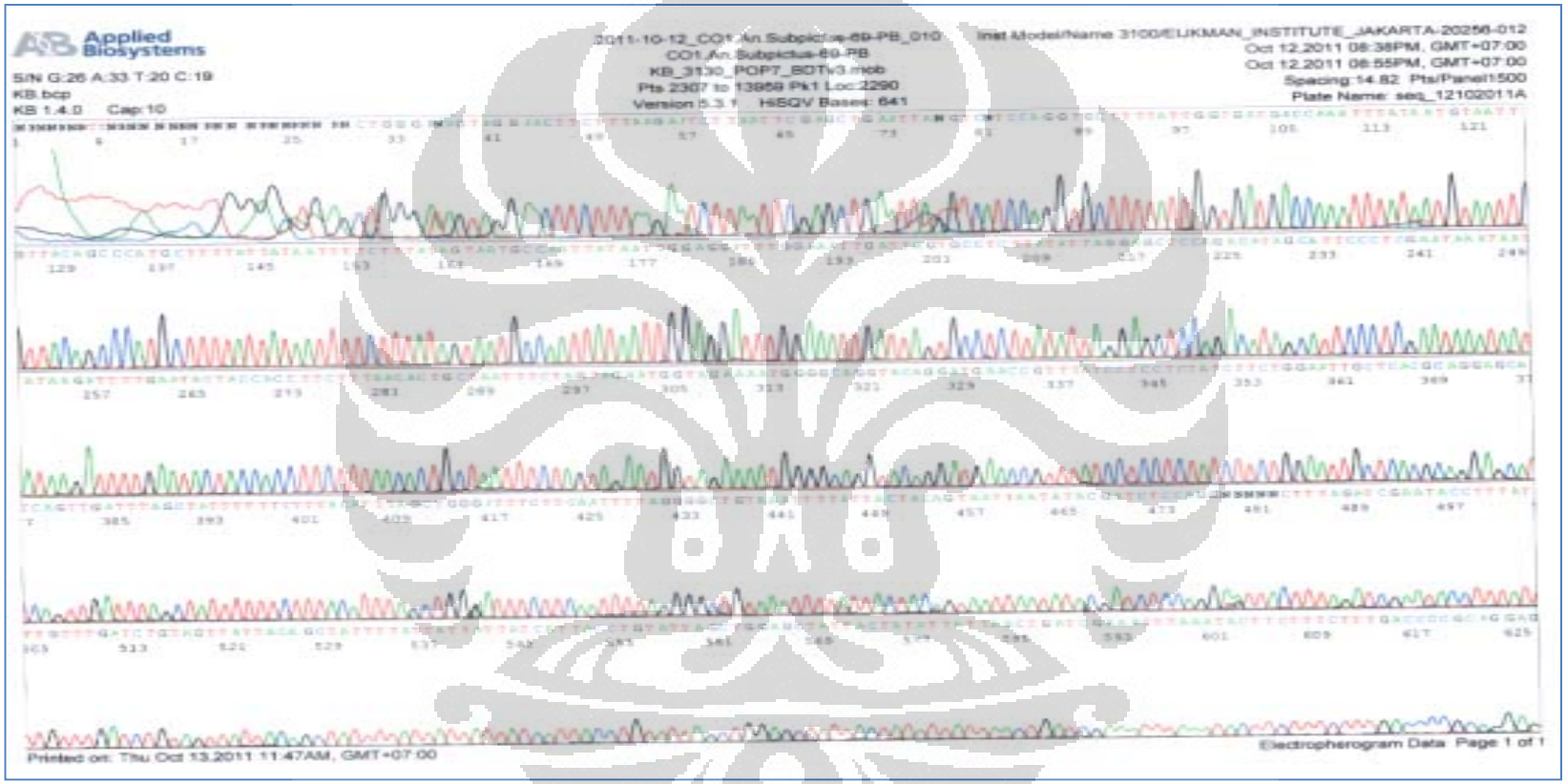
Appendix 3. *Rdl* allele sequences of *Anopheles subpictus* from West Nusa Tenggara



Appendix 4. *CO1* gene of *Anopheles subpictus* from West Nusa Tenggara



Appendix 5. *ITS2* gene of *Anopheles tessellatus* from North Mollucas



Appendix 6. Map of Study Area in Lampung Provinces: Rajabasa and KatibungDistricts

Appendix 7. Key Identification of *Anopheles* Larvae (Reid 1968)

KEY TO LARVAE

I.- Bases of clypeal hairs (*ic)* much loser to one another than to the outer clypeals (oc), antennal shaft hair branched, often large (in a few forms either the inner clypeals are rather wide apart but antennal hair is branched, or the antennal hair is simple but the inner clypeals are close together and the frontal hairs are reduced)

(subgenus Anopheles)

Bases of inner clypeal hairs wide apart and closer to the bases of the outer clypeals than to one another, antennal shaft hair small or minute and nearly always simple

(subgenus Cellia)

(Reid 1968)

Appendix 8. Probit Table

%	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
—	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Appendix 9. Analysis Probit of Endosulfan Susceptibility Test in Katibung

a. LC_{50} Probit Analysis in Katibung

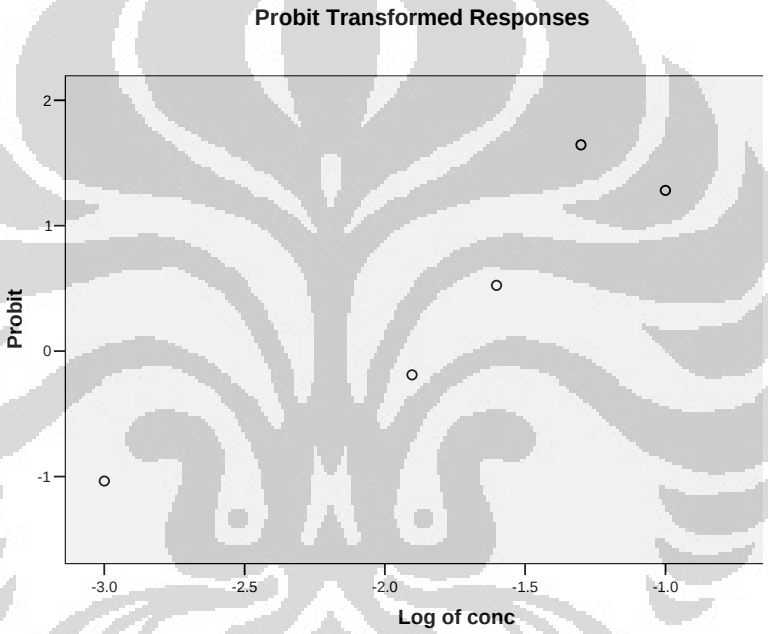
Conc. (%)	log 10 conc	% mortality	probit	LC_{50}
0,4	-0,39794	100		
0,2	-0,69897	100		
0,1	-1	90	6,28	
0,05	-1,30103	95	6,64	0.00893
0,025	-1,60206	70	5,52	
0,0125	-1,90309	42,5	4,81	
0,001	-3	15	3,96	
0		0		

SPSS output

		95% Confidence Limits	
Prob	conc	Lower	Upper
,01	,00021	,00001	,00089
,02	,00033	,00002	,00123
,03	,00043	,00002	,00151
,04	,00053	,00003	,00177
,05	,00063	,00005	,00200
,06	,00073	,00006	,00223
,07	,00083	,00007	,00246
,08	,00093	,00009	,00267
,09	,00103	,00010	,00289
,10	,00113	,00012	,00311
,15	,00168	,00023	,00420
,20	,00230	,00039	,00536
,25	,00301	,00061	,00664
,30	,00383	,00090	,00808
,35	,00480	,00128	,00974
,40	,00594	,00179	,01170
,45	,00730	,00245	,01406
,50	,00893	,00332	,01697
,55	,01094	,00445	,02069
,60	,01345	,00592	,02560
,65	,01664	,00785	,03236
,70	,02082	,01037	,04213
,75	,02652	,01373	,05718
,80	,03473	,01834	,08225
,85	,04756	,02501	,12907
,90	,07062	,03582	,23473

,91	,07770	,03892	,27228
,92	,08619	,04252	,32037
,93	,09661	,04680	,38368
,94	,10974	,05200	,47005
,95	,12690	,05854	,59363
,96	,15053	,06713	,78263
,97	,18568	,07923	1,10228
,98	,24544	,09839	1,74424
,99	,38100	,13753	3,61857

b. Probit Transformed Responses



Appendix 10. Analysis Probit of Endosulfan Susceptibility Test in Rajabasa

a. LC_{50} Probit Analysis

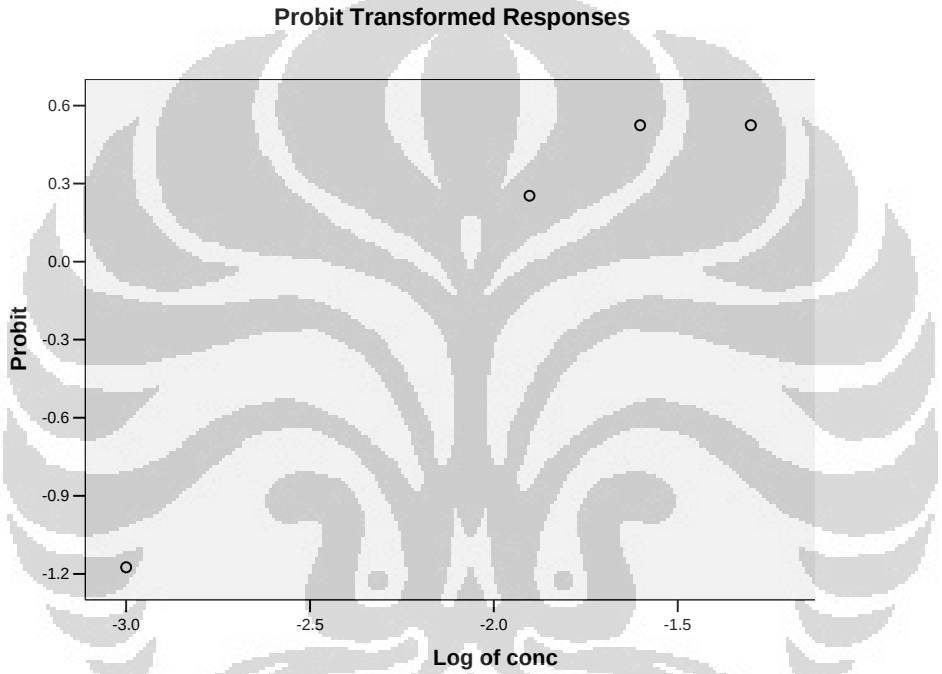
Conc. (%_	log conc	% mortality	probit table	LC_{50}
0		0		0.0094
0,001	-3	12	3,82	
0,0125	-1,903089987	60	5,25	
0,025	-1,602059991	70	5,52	
0,05	-1,301029996	70	5,52	
0,1	-1	100		
0,2	-0,698970004	100		
0,4	-0,397940009	100		

SPSS output

		95% Confidence Limits	
Prob	conc	Lower	Upper
,01	,00020	,00000	,00096
,02	,00032	,00001	,00132
,03	,00042	,00001	,00162
,04	,00052	,00002	,00189
,05	,00062	,00003	,00214
,06	,00071	,00004	,00238
,07	,00081	,00005	,00262
,08	,00091	,00006	,00285
,09	,00101	,00007	,00307
,10	,00112	,00008	,00330
,15	,00166	,00017	,00445
,20	,00229	,00029	,00566
,25	,00301	,00047	,00700
,30	,00384	,00071	,00851
,35	,00482	,00105	,01026
,40	,00598	,00150	,01232
,45	,00736	,00211	,01481
,50	,00904	,00292	,01791
,55	,01109	,00401	,02189
,60	,01366	,00546	,02721
,65	,01695	,00737	,03465
,70	,02127	,00992	,04562
,75	,02717	,01333	,06291
,80	,03569	,01801	,09259
,85	,04905	,02477	,15002
,90	,07319	,03565	,28560
,91	,08061	,03875	,33518

,92	,08954	,04236	,39948
,93	,10049	,04664	,48534
,94	,11432	,05182	,60434
,95	,13243	,05833	,77767
,96	,15740	,06686	1,04835
,97	,19464	,07885	1,51794
,98	,25813	,09778	2,49286
,99	,40278	,13629	5,48647

b. **Probit Transformed Responses**



Appendix 11. The validity test of linear regression line with chi-squared (x^2) in Katibung

The mortality percentage of *Anopheles* larvae from Katibung in eight concentrations of endosulfan (log concentration) for 24 hour in probit.

Aim: to test the validity of the linear regression line of the influence endosulfan concentration against mortality of *Anopheles* larvae.

Hypothesis :

H_0 : linear regression line on Katibung is valid

H_A : linear regression line on Katibung is not valid

Statistical testing

X^2 obtained from calculations with computer program

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	48,000(a)	42	,243
Likelihood Ratio	30,498	42	,906
Linear-by-Linear Association	2,999	1	,083
N of Valid Cases	8		

a 56 cells (100,0%) have expected count less than 5. The minimum expected count is ,13.

Testing criteria

If the count of $X^2 < X^2$ of table, H_o accepted and H_A rejected

The results of calculations

Based on the calculation results obtained table X^2 (df = 42, α = 0.05) = 55.8

X^2 count = 48, because the count $X^2 < X^2$ table, H_0 is accepted

Conclusion

The line on the graph 1 (Katibung) is valid, so the LC_{50}-24 hour obtained acceptable.

Appendix 12. The validity test of linear regression line with chi-squared (x^2) in Rajabasa

The mortality percentage of *Anopheles* larvae from Rajabasa in eight concentrations of endosulfan (log concentration) for 24 hour in probit.

Aim: to test the validity of the linear regression line of the influence endosulfan concentration against mortality of *Anopheles* larvae.

Hypothesis:

H_0 : linear regression line on Rajabasa is valid

H_A : linear regression line on Rajabasa is not valid

Statistical testing

X^2 obtained from calculations with computer program*

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	32,000(a)	28	,275
Likelihood Ratio	23,907	28	,686
Linear-by-Linear Association	3,128	1	,077
N of Valid Cases	8		

a 40 cells (100,0%) have expected count less than 5. The minimum expected count is ,13.

Testing criteria

If the count of $X^2 < X^2$ of table, H_o accepted and H_A rejected

The results of calculations

Based on the calculation results obtained table X^2 (df = 24, $\alpha = 0.05$) = 36.4

X^2 count = 32, because the count $X^2 < X^2$ table, H_0 is accepted

Conclusion

The line on the graph 2 (Rajabasa) is valid, so the LC_{50}-24 hour obtained acceptable.