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Acetylcholinesterase activity and Ace-1 mutation in *Aedes aegypti* resistance to temephos

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Abstract. *Nazar YE, Hasmiwati, Rahayu R. 2025. Acetylcholinesterase activity and Ace-1 mutation in* Aedes aegypti *resistance to temephos. Biodiversitas 26: 4598-4605.* An organophosphate insecticide called temephos is frequently used to manage *Aedes aegypti*, the main Dengue Fever (DF) vector. However, prolonged and widespread application has led to resistance in many mosquito. This study aimed to assess temephos resistance in *Aedes aegypti* larvae from Tanjung Bingkuang, Solok District, West Sumatra, Indonesia, using biochemical and molecular assays, this study sought to ascertain the resistance status of *Ae. aegypti* larvae collected from Tanjung Bingkuang, Solok District, West Sumatra. The biochemical test results showed that the larvae exhibited a high level of resistance, with an Activity Value (AV) of 1.077 and Acetylcholinesterase (AChE) enzyme activity of 6.65 U/L. Further molecular analysis identified a point mutation at codon T506T in the Ace-1 gene, characterized by a nucleotide change from ACA to ACT. Molecular analysis identified a synonymous mutation (T506T; ACA → ACT) in the Ace-1 gene, potentially associated with resistance development. These results confirm the emergence of temephos resistance in local *Ae. aegypti* populations and highlight the importance of incorporating molecular and enzymatic surveillance tools into vector control programs to ensure larvicide efficacy. This study confirms that the use of the organophosphate insecticide temephos is no longer effective in controlling *Ae. aegypti* larvae in Tanjuang Bingkuang, Solok District, West Sumatra, Indonesia.

Keywords: Ace-1, acetylcholinesterase, *Aedes aegypti*, mutation, resistance

INTRODUCTION

Dengue Fever (DF), caused primarily by *Aedes aegypti* (Linnaeus, 1762) mosquitoes, remains a significant public health problem in Indonesia, with rising cases reported annually. However, limited molecular monitoring of insecticide resistance has been conducted locally, particularly in rural areas such as Solok District. Since 2009, mortality cases due to DF in West Sumatra have continued to increase (Dinkes Sumbar 2024). Various efforts have been undertaken to control the spread of dengue, one of which involves the use of insecticides (Ridha et al. 2025). Although initially effective, the continuous use of insecticides can lead to the development of resistance in mosquito populations. Resistance is a physiological adaptation that enables mosquitoes to survive exposure to insecticides that would normally be lethal. Temephos is one such insecticide that has shown a decline in efficacy due to resistance (Kandi et al. 2023). One of the primary mechanisms of resistance is the alteration of the insecticide target site, particularly the Acetylcholinesterase (AChE) enzyme encoded by the Ace-1 gene, and mutations in this gene have been associated with reduced susceptibility to organophosphate compounds, including temephos (Samal et al. 2022).

Acetylcholinesterase, an essential enzyme in the insect central nervous system, breaks down the neurotransmitter acetylcholine at neuromuscular synapses, enabling proper nerve signal transmission and muscle relaxation (Rotundo et al. 2020; Gan et al. 2021; Trang et al. 2023). Insecticides like organophosphates (e.g., temephos) inhibit this enzyme by binding to its active site, causing acetylcholine buildup, continuous nerve stimulation, and insect death (WHO 2009). This enzyme is a key target for carbamate and organophosphate resistance (Aroniadou-Anderjaska et al. 2023). Mutations can alter the enzyme's structure, reducing insecticide binding and effectiveness (Sha et al. 2025), rendering temephos ineffective (Piedra et al. 2024). Resistance among *Ae. aegypti* mosquitoes has been widely reported in Southeast Asia (Gan et al. 2021). In Colombia, resistance was linked to elevated acetylcholinesterase activity caused by an Ace-1 gene mutation at codon F443Y (Teng et al. 2022; Pineda et al. 2025).

The Ace-1 gene, which is roughly 138,970 base pairs long, encodes the enzyme acetylcholinesterase (Mori et al. 2007). It has seven introns and eight exons. In *Ae. aegypti*, the Ace-1 gene is located on chromosome 3, between markers LF106 and LF386 (Mori et al. 2007). Resistance to temephos is partly attributed to structural changes in acetylcholinesterase, which prevent the insecticide from effectively binding to the enzyme (Omuut et al. 2023). Such structural modifications often arise from mutations in the Ace-1 gene. A well-documented example is the G119S point mutation in *Ae. aegypti*, which has been linked to insecticide resistance (Binyang et al. 2022).

Cases of *Ae. aegypti* resistance have been reported globally, including in Indonesia. In 2022, *Ae. aegypti* larvae from Pesisir Selatan District, West Sumatra, were found resistant to temephos, associated with a synonymous T506T mutation (ACA → ACT) in the Ace-1 gene, though no F290V or F455W mutations were detected (Rahayu et al. 2022). Despite ongoing temephos use in Tanjung Bingkuang Village, Solok District, dengue cases have continued to rise (Solok District Health Office 2023), indicating possible resistance. This study assessed temephos efficacy, measured acetylcholinesterase activity through biochemical assays, and identified Ace-1 gene mutations in resistant *Ae. aegypti*. The findings aim to inform resistance mechanisms and enhance dengue vector control strategies. Previous studies by Hasmiwati et al. (2018) have demonstrated that mutations in the Ace-1 gene, including both non-synonymous and synonymous mutations, may contribute to resistance expression. Despite increasing reports of insecticide resistance in Southeast Asia, there is limited molecular surveillance in Indonesia, especially regarding the identification of Ace-1 gene mutations and AChE activity levels in field populations. Therefore, this study aims to evaluate biochemical and molecular markers of temephos resistance in *Ae. aegypti* larvae collected from Tanjung Bingkuang, Solok District, West Sumatra, Indonesia.

MATERIALS AND METHODS

Collecting sample

The sample was collected in Tanjung Bingkuang, Solok District, West Sumatra, Indonesia. The map below shows the sampling location in Figure 1.

Biochemical test

Biochemical tests were performed on third-instar *Aedes aegypti* as 30 larvae per treatment, obtained from residents' homes and public facilities in Tanjung Bingkuang, Solok District, West Sumatra, Indonesia, exposed to temephos at concentrations 0.012 mg/L (WHO standard), 0.018 mg/L, and 0.025 mg/L for 24 hours. After being exposed to temephos, tests were carried out according to the instructions for the acetylcholinesterase kit "QuantiChrom Acetylcholinesterase Assay Kit (DACE-100)" which refers to Magnotti et al. (1987); Ordentlich et al. (1996); Kovarik et al. (2003) with the following steps: *Ae. aegypti* instar III larvae were homogenized and 1 mL of Phosphate Buffer (0.01 M; pH 7.5) was added. Then centrifugation was carried out at a speed of 14,000 rpm for 5 minutes. After the supernatant is formed, 10 µL of supernatant is added and 190 µL of reagent is added into each microplate well. Observations are made by observing the color changes that occur in the microplate wells. If a yellow color appears, the *Ae. aegypti* larvae are declared resistant (Fernando et al. 2020). However, if there is no color change, it is declared susceptible. Color changes in the homogenate were then analyzed quantitatively. Color intensity was quantified using an Enzyme-Linked Immunosorbent Assay (ELISA) reader at 412 nm with a standard value >0.9 categories is high resistance.

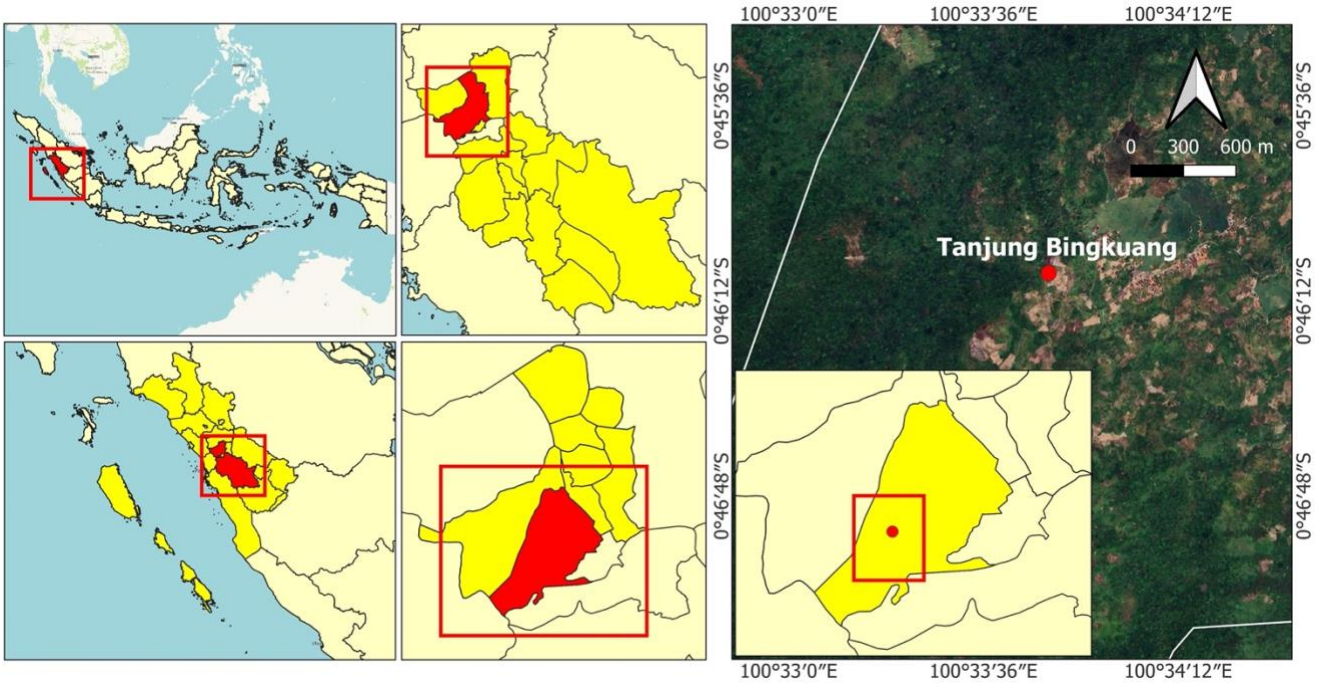

Figure 1. Map of Solok District, West Sumatra, Indonesia with an inset marking Tanjung Bingkuang as the sampling location for *Aedes aegypti* larval collection

Molecular test

The procedures of Hasmiwati et al. (2018) and Rahayu et al. (2022) were followed for doing molecular testing. Following the manufacturer's instructions, DNA was extracted from *Ae. aegypti* larvae DNA was isolated using the kit DNA PureLink Genomic DNA Minikit (Invitrogen, USA) with the provider's protocol. DNA extraction was made until the supernatant was formed and inserted into the GD column in the collection tube, centrifuged, and then incubated. Following the extraction procedure, a supernatant was created, transferred to a GD column in a collection tube, centrifuged, and allowed to incubate. Polymerase Chain Reaction (PCR) was used to amplify the target gene fragment using the primer pair forward F4 (5'-GTTTGGTGAAAGTGCAGGTG-3') and reverse R4 (5'-CATAGGTTGTGTTGAGCCCA-3'). Electrophoresis of the PCR products was performed on a 1.5% agarose gel that was made with 1X TAE buffer and 5 µL of GelRed per 50 µL of agarose. Purification and sequencing of the PCR products were carried out by Macrogen Korea. The resulting sequence data were analyzed using the Genius software program. Mutation analysis focused on the gene region spanning nucleotides 125,107 to 126,169. At this point there are two codons known to cause changes in the structure of acetylcholinesterase in mosquitoes, namely F290V and F455W (Mori et al. 2007). In another study, Temeyer et al. (2025) described the biochemical characterization of recombinant AChE with mutations F290V and F331W (homologous F455W in mosquitoes) (Mori et al. 2007). Additionally, Temeyer et al. (2025) described the biochemical characterization of recombinant acetylcholinesterase with mutations F290V and F331W (the latter homologous to F455W in mosquitoes).

Data analysis

By utilizing an ELISA reader to measure the Absorbance Value (AV) at a wavelength of 412 nm, the findings of biochemical tests were quantitatively examined. The following is how the resistance status was ascertained using the AV criteria:

AV<0.700 : Indicates susceptibility (SS)

AV 0.700-0.900 : Indicates a moderate level of resistance (RS)

AV>0.900 : High Resistance (RR)

Lejbman et al. (2025) stated that the range of Absorbance Values (AV) is critical for categorizing resistance levels, with AV values greater than 0.900 indicating a high resistance category. To calculate the acetylcholinesterase enzyme (AChE) activity, the following formula is used:

$$\text{AChE Activity} = \frac{OD_{10} - OD_{2}}{OD_{CAL} - OD_{H20}} \times 200 \ \text{(U/L)}$$

Where :

OD_{10} : Optical density measured at 10 minutes

OD_{2} : Optical density measured at 2 minutes

OD(cal) : Optical density of the calibrator solution

ODH_2O) : Optical density of the distilled water (blank control)

All values were measured using the QuantiChrom™ Acetylcholinesterase Assay Kit (DACE-100).

RESULTS AND DISCUSSION

Detection of *Aedes aegypti* resistance using biochemical tests

Aedes aegypti samples for this study were collected in Tanjung Bingkuang, Solok District, West Sumatra, Indonesia. The samples taken were instar III larvae of the *Ae. aegypti* species. The larvae were then treated (exposed to temephos) with a concentrate 0.012 mg/L (WHO standard), 0.018 mg/L, and 0.025 mg/L for 24 hours. After being exposed to temephos for 24 hours, the results showed that *Ae. aegypti* larvae were resistant to temephos. To confirm this statement, the samples were further tested biochemically to determine the activity of the acetylcholinesterase enzyme in the larvae.

Biochemical observations were conducted both qualitatively and quantitatively. The results of the qualitative test indicated that *Ae. aegypti* larvae collected from Tanjung Bingkuang, Solok District, West Sumatra, were resistant to temephos, as evidenced by a color change in the homogenate within the microplate wells, which shifted toward yellow.

Quantitative analysis was then performed to determine the Absorbance Value (AV) and the activity of the Acetylcholinesterase (AChE) enzyme using an ELISA reader. The AV values obtained from each larval sample are presented in Figure 2 below.

The AV values obtained from Figure 2 range from 0.8 to 1.4. The graph shows that under temephos exposure at a concentration of 0.012 mg/L, 90% of *Ae. aegypti* larvae exhibited AV values >0.9. At concentrations of 0.018 mg/L and 0.025 mg/L, 100% of the larvae exhibited AV values >0.9. These findings indicate that the larvae fall into the high resistance category. This suggests that the use of temephos at the concentration recommended by the WHO is no longer effective (Boyer et al. 2022). Muthusamy et al. (2014) also reported that an AV value >0.9 classifies insects as highly resistant. This is further supported by the acetylcholinesterase activity values presented in Table 1.

Identification of Ace-1 gene mutations

Molecular detection of resistance can be performed by identifying mutations in genes responsible for increased enzyme levels that detoxify insecticides (Wang et al. 2024). Molecular detection of resistance was conducted through a series of steps, including DNA isolation, PCR amplification, electrophoresis, and sequencing. The concentration of DNA obtained during the isolation process ranged from 20 to 40 ng/µL, with absorbance ratio values between 1.8 and 2.0, indicating good purity. Once high-quality DNA was obtained, amplification was carried out using the PCR method. A pair of forward and reverse primers was used. The forward primer was adopted from Mori et al. (2007), while the reverse primer was custom-designed. The primers were designed using the “Geneious version 11.1.2” software. The design process began by downloading the required DNA sequences from National Center for Biotechnology Information (NCBI), followed by identifying the target mutation sites based on Mori et al. (2007). The following section presents the primer binding positions for amplification. The amplified PCR product is 1082 bp in length. To

confirm the presence of mutations in the Ace-1 gene, nucleotide sequences obtained from field samples were aligned with a reference sequence from GenBank. The alignment revealed a synonymous point mutation at codon 506 (T506T), where the codon ACA was substituted with ACT. This mutation, although it does not alter the amino acid sequence (threonine), may influence gene expression or mRNA stability. The aligned sequences are presented in Figure 3.

Table 1. After being exposed to temephos for 24 hours, *Aedes aegypti* larvae from Tanjung Bingkuang, Solok District, West Sumatra, Indonesia showed activity of the acetylcholinesterase enzyme

Temephos concentration (mg/L)	Number of samples	Mean value of acetylcholinesterase enzyme activity (U/L)±standard deviation
0.012	10	6.39±0.93
0.018	10	6.61±0.95
0.025	10	6.83±0.82

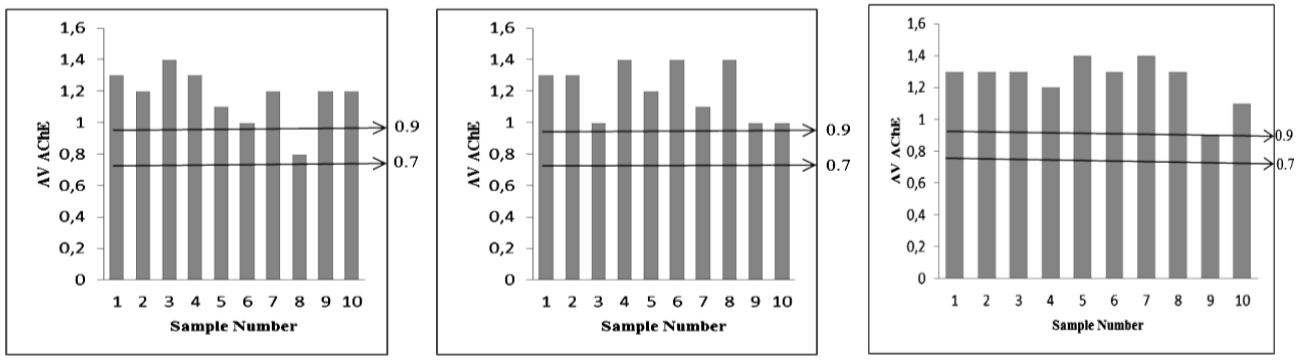

Figure 2. Average Acetylcholinesterase (AChE) activity levels in *Aedes aegypti* larvae collected from Tanjung Bingkuang Village, Solok District, West Sumatra, after 24-hour exposure to temephos at the following concentrations: A. 0.012 mg/L, B. 0.018 mg/L, and C. 0.025 mg/L

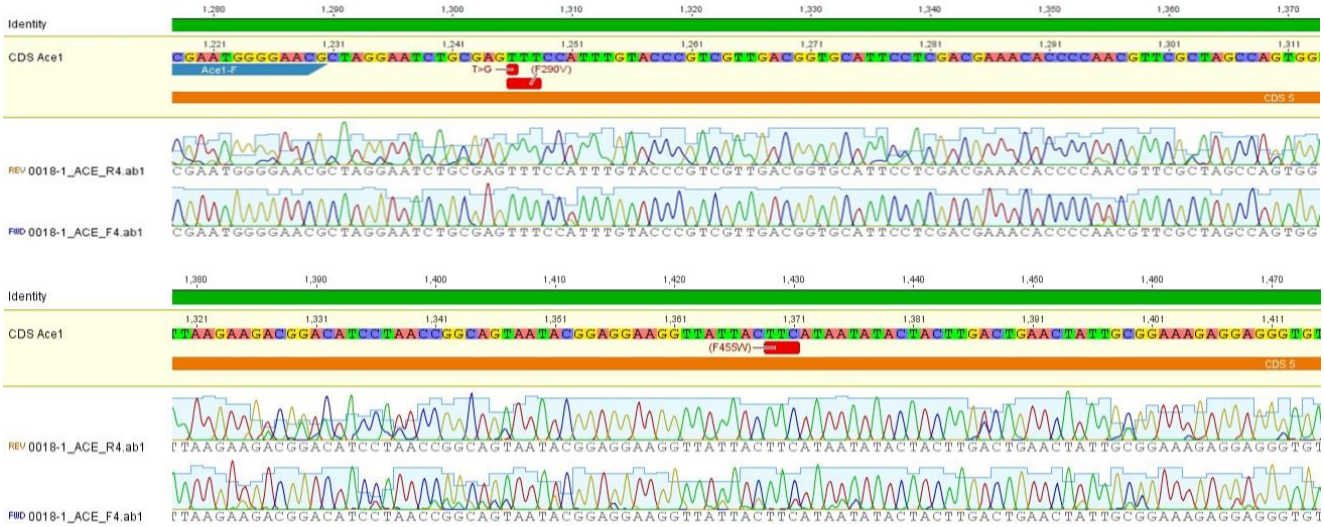

Figure 3. Nucleotide sequence alignment of the Ace-1 gene between field-collected *Aedes aegypti* samples from Tanjung Bingkuang and the GenBank reference sequence. A synonymous mutation at position T506T (ACA → ACT) was identified and is highlighted in the alignment

Based on the alignment results, no mutations were detected at positions F290V and F455W, as shown in Figures 4 and 5, respectively.

The alignment results revealed a substitution-type mutation at a different site, identified as a polymorphism. Although this substitution does not alter the amino acid sequence, a change from ACA to ACT was detected at position 506 bp, as shown in Figure 6.

After identifying the appropriate primer binding sites, forward and reverse primers were designed to flank the target mutation region. Once the primers were constructed, they were validated using NCBI Primer-BLAST to ensure their specificity for the Ace-1 gene of *Ae. aegypti*. The following figure shows the primer design used in the amplification process in Figure 7.

The primers used were 20 bases in length and amplification was performed in 30 cycles at an annealing temperature of 63°C. The primers annealed at base positions 125,107 and 126,169 bp, producing an amplicon of 1,082 bp in length. Following amplification, electrophoresis was carried out to visualize the PCR products. The results of the electrophoresis are presented in Figure 8 below.

Figure 8 shows the presence of the target band at nucleotide positions 125,107 to 126,169 bp. After confirming the target band, sequencing was performed at Macrogen, Korea. The identification results show a genotype change from ACA (threonine) to ACT (threonine) at position T506T. The T506T substitution represents a synonymous mutation at the 506th nucleotide, which does not alter the encoded amino acid, threonine.

Discussion

Acetylcholinesterase activity in the test sample was declared high. Acetylcholinesterase is an enzyme involved in transmitting nerve impulses to the central nervous system. It has a highly complex and flexible active site (Reynoso-García et al. 2025). This enzyme is responsible for hydrolyzing acetylcholine into choline and acetic acid. Acetylcholine is a neurotransmitter that triggers the opening of Na^+ and K^+ channels, facilitating synaptic signaling through the action of acetylcholinesterase (Foley 2005).

Temephos is expected to paralyze and kill larvae by inhibiting acetylcholinesterase, which regulates the hydrolysis of acetylcholine in the larval body. However, in this study, temephos was found to be ineffective, as the homogenate exhibited a yellow coloration. Fernando et al. (2020) reported that a yellow color in the homogenate is an indicator of resistance, with moderate to strong yellow intensity correlating with increased acetylcholinesterase enzyme activity. A strong yellow coloration thus reflects elevated enzyme activity. Acetylcholinesterase activity was detected in *Ae. aegypti* larvae, indicating that temephos is unable to effectively inhibit this enzyme. Acetylcholine functions as a neurotransmitter in the synaptic cleft (Wei et al. 2022). After impulse transmission, acetylcholine is hydrolyzed by acetylcholinesterase into choline and acetic acid. The choline is removed, and acetic acid recombines to form acetylcholine, continuing the cycle (Ming et al. 2024).

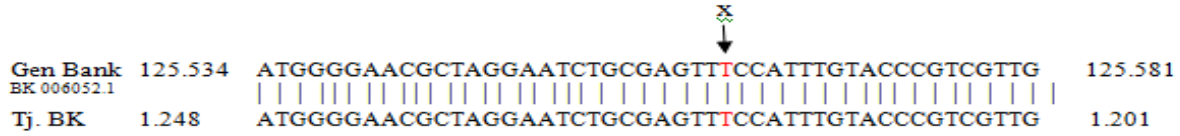

Figure 4. Nucleotide sequence alignment of the Ace-1 gene between samples from Tanjung Bingkuang and *Aedes aegypti* (GenBank). At location F290V (nucleotides 125 and 167), no mutation was found (X: Mutation site F290V, Tj BK: Tanjung Bingkuang)

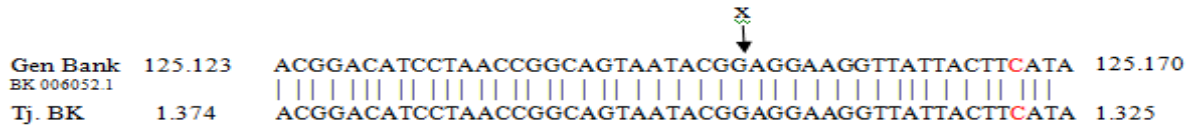

Figure 5. Alignment of the Ace-1 gene nucleotide sequences from *Aedes aegypti* (GenBank) and samples from Tanjung Bingkuang. No mutation was detected at position F455W (nucleotides 125 and 562) (X: Mutation point F455W, Tj BK: Tanjung Bingkuang)

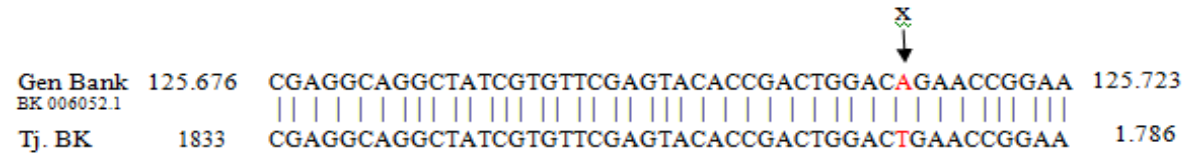

Figure 6. Alignment of the Ace-1 gene nucleotide sequences from *Aedes aegypti* (GenBank) and samples from Tanjung Bingkuang. A synonymous substitution was detected at position T506T (nucleotide 125,714), where ACA was changed to ACT (X: Mutation point T506T, Tj BK: Tanjung Bingkuang)

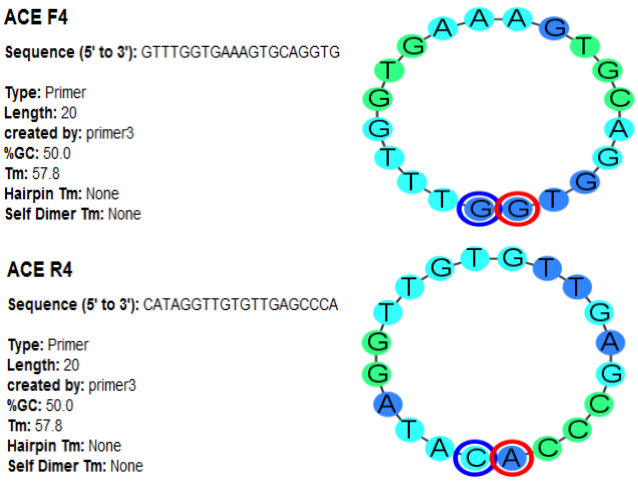

Figure 7. Forward and reverse primers designed to flank the mutation region of the Ace-1 gene in *Aedes aegypti*

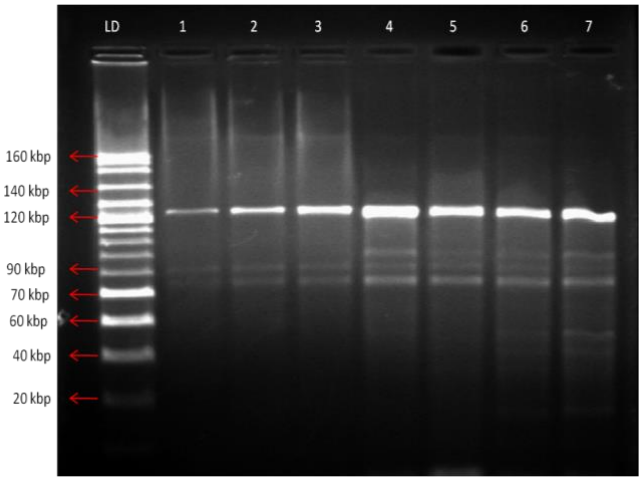

Figure 8. PCR product of the Ace-1 gene amplification at nucleotide positions 125,169-126,169 bp in *Aedes aegypti* from Tanjung Bingkuang, Solok District, West Sumatra, Indonesia

Upon exposure to temephos, acetylcholinesterase binds to the insecticide, producing thiocholine. This thiocholine leads to acetylcholine accumulation, disrupting impulse transmission, which results in impaired muscle coordination, convulsions, and ultimately death in insects (WHO 2009). However, the results of this study do not support this. Temephos was unable to paralyze the larvae, allowing them to survive, indicating resistance (Davila-Barboza et al. 2024). Larval resistance to temephos is evidenced by elevated AV values and increased acetylcholinesterase enzyme activity (Pineda et al. 2025). The heightened enzyme activity is thought to result from structural changes in acetylcholinesterase (Scott 1995).

Acetylcholinesterase is an enzyme encoded by the Ace-1 gene. Molecular analysis revealed a substitution mutation at the T506T site, identified as a polymorphism. Although this mutation does not alter the amino acid sequence, it involves a base substitution from ACA to ACT at position 506 bp. The T506T substitution is hypothesized to influence the susceptibility of *Ae. aegypti* by increasing acetylcholinesterase enzyme activity. This increase may occur because the genetic code produces a target enzyme variant with a modified structure. In other words, there is a structural change in the acetylcholinesterase enzyme, particularly at the enzyme's active site (Scott 1995). This finding aligns with studies by Hasmiwati et al. (2018) and Rahayu et al. (2022), which reported a substitution at T506T changing ACA to ACT. This mutation is believed to contribute to increased acetylcholinesterase activity through structural modifications, serving as a mechanism for enhanced enzyme function.

Ace-1 directly encodes the enzyme acetylcholinesterase. Therefore, enzyme activity is influenced not only by changes in amino acids but also by genotypic variations within the Ace-1 gene itself. Polymorphisms in Ace-1 can lead to structural alterations in the encoded enzyme (Scott 1995). Maestre-Serrano et al. (2014) reported that mutations in the Ace-1 gene can modify acetylcholinesterase activity, rendering the enzyme insensitive to temephos. This phenomenon represents a phenotypic expression observable at the population level, whereby exposure to toxicants results in resistance, making the substance ineffective against the strain (Scott 1995).

The identification results reveal a genotype change from ACA to ACT at position T506T. This substitution at the 506th nucleotide is synonymous and does not alter the encoded amino acid, threonine. Scott (1995) stated that genotypic changes in Ace-1 can affect the regulation and structure of the gene product. The gene's ability to modify the product's structure increases the likelihood that resistance genes will continue to evolve, ultimately causing substrate insensitivity of the enzyme (Maestre-Serrano et al. 2014). Acetylcholinesterase, an enzyme belonging to the esterase group, undergoes structural changes as part of a target site resistance mechanism, resulting in insensitivity at the enzyme's active site. In target site resistance, the enzyme's quantity remains unaffected, but its quality is altered. Variants of acetylcholinesterase produced through such mutations enable *Ae. aegypti* larvae to survive temephos exposure (Scott 1995). The link between enzymatic and genetic resistance mechanisms observed in this study suggests a potential interaction between elevated AChE activity and the presence of Ace-1 mutations. Although the T506T mutation is synonymous and does not result in a change in the amino acid sequence, it may influence gene expression, mRNA stability, or translation efficiency, which could contribute to the increased enzymatic activity observed. This relationship highlights the complexity of resistance mechanisms, where even silent mutations may play a functional role in phenotypic resistance. Understanding this connection is critical for the development of molecular diagnostic tools and for refining larvicide application strategies based on resistance profiles in vector populations.

The resistance of *Ae. aegypti* to temephos observed in this study is supported by both biochemical and molecular findings, where high AChE activity values (AV>0.9) indicate strong enzymatic resistance, and the detection of

an Ace-1 gene mutation (T506T) suggests a potential genetic contribution to the reduced susceptibility.

The main limitation of this study lies in the resistance detection, which focused solely on acetylcholinesterase enzyme activity and Ace-1 gene mutations. In fact, various other enzymes are also involved in the resistance mechanism of *Ae. aegypti*, such as Glutathione S-Transferase (GST), non-specific esterases, and cytochrome P450s (Scott 1995). However, this study only assessed the activity of AChE. Detection of Ace-1 gene mutations was also not fully comprehensive due to limitations in primer design, which only amplified a fragment spanning base positions 125,107 to 126,169. It is possible that mutations in other regions of the Ace-1 gene also influence AChE activity. Moreover, in the molecular context, resistance is not solely associated with the Ace-1 gene, but may also involve other genes such as the Voltage-Gated Sodium Channel (VGSC) gene and additional genetic factors.

In conclusion, the results of the identification of the causes of resistance stated that *Ae. aegypti* larvae obtained in Tanjung Bingkuang Village, Solok District, West Sumatra in 2017 with a temephos concentration of 0.012 mg/L had an Absorbance Value (AV) of 1.21 which indicated high resistance in the larvae and an acetylcholinesterase enzyme activity value (AChE) of 6.39 u/L and a substitution (polymorphism) was found in t506t which changed aca to act in the Ace-1 gene. The appropriate dosage of temephos should be reconsidered or optimized for effective larval control in Nagari Tanjung Bingkuang, Solok District, West Sumatra, Indonesia.

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