ACTA VETERINARIA INDONESIANA
P-ISSN 2337-3202, E-ISSN 2337-4373
Vol. 13, No. 3: 183-190, November 2025

Research

Specific Primer Design for *Acetylcholinesterase-1* (ace-1) as a Putative Insecticide Resistance Gene for *Aedes aegypti*

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Received: 2 November 2024, Accepted: 8 October 2025

ABSTRACT

The increase in cases of Dengue Hemorrhagic Fever (DHF) is attributed to an increase in DHF vectors, which is a consequence of a failing mosquito control program. One major factor is specific gene resistance to insecticides. Research on resistance genes is crucial for effective vector control. One of the putative insecticide-resistant genes against *Aedes aegypti* mosquitoes is ace-1 (*acetylcholinesterase-1*), currently, there is no specific primer information that can amplify the ace-1 gene. This study aimed to design a specific primer for the ace-1 gene in *Ae. aegypti*. The study is an observational descriptive study in silico using NCBI, Primer3web, and BLAST databases, followed by an in vitro confirmation stage using the Polymerase Chain Reaction (PCR). The accession number of the ace-1 gene was MK896351.1. The research results showed two potential specific primer pairs; primer 1 with an amplicon length of ±413 bp and an annealing temperature of 49.6°C, while primer 2 with an amplicon length of ±417 bp and an annealing temperature of 51.3°C. The PCR product was visualized using 2% agarose gel and EtBr dye with gel documentation. In conclusion, this research successfully acquired two potential specific primer pairs for amplifying the ace-1 gene as a putative insecticide-resistant gene in *Ae. aegypti* mosquitoes.

Keywords: gel documentation, electrophoresis, DNA isolation, PCR.

ABSTRAK

Peningkatan kasus Demam Berdarah Dengue (DBD) disebabkan oleh meningkatnya vektor DBD, yang merupakan dampak dari kegagalan upaya pengendalian nyamuk. Salah satu faktor utama penyebabnya adalah resistensi genetik spesifik terhadap insektisida. Penelitian mengenai gen resistensi sangat penting untuk pengendalian vektor yang efektif. Salah satu gen yang diduga berperan dalam resistensi insektisida pada nyamuk *Aedes aegypti* adalah ace-1 (acetylcholinesterase-1), namun saat ini belum ada informasi primer spesifik untuk memperbanyak gen tersebut. Penelitian ini bertujuan untuk merancang primer spesifik untuk gen ace-1 pada *Ae. aegypti*. Penelitian ini menggunakan pendekatan observasional deskriptif *in silico* dengan menggunakan database NCBI, Primer3web, dan BLAST, dilanjutkan dengan in vitro sebagai tahap konfirmasi dengan menggunakan *Polymerase Chain Reaction* (PCR). Nomor akses gen ace-1 yang digunakan adalah MK896351.1. Hasil penelitian menunjukkan dua pasangan primer spesifik potensial; primer 1 dengan panjang amplikon ±413 bp dan suhu annealing 49,6°C, sedangkan primer 2 dengan panjang amplikon ±417 bp dan suhu annealing 51,3°C. Produk PCR divisualisasikan menggunakan gel agarose 2% dan pewarna EtBr dengan dokumentasi gel. Sebagai kesimpulan, penelitian ini berhasil memperoleh dua pasangan primer spesifik potensial untuk memperbanyak gen ace-1 sebagai gen yang diduga bertanggung jawab atas resistensi insektisida pada nyamuk *Ae. aegypti*.

Kata kunci: dokumentasi gel, elektroforesis, isolasi DNA, PCR.

INTRODUCTION

Dengue Hemorrhagic Fever (DHF) is an endemic disease frequently found in Indonesia (Dawe *et al*., 2020). According to the Indonesian Ministry of Health, the cumulative incidence of DHF cases increased by 33% from 2018 to 2022, with the number of deaths increasing by 74% (Aida *et al*., 2023). The rise in DHF cases is caused by the proliferation of the disease's vector population, the mosquito *Aedes aegypti* (Nugraha *et al*., 2021).

The female Aedes aegypti mosquito serves as the primary vector of DHF (Podung *et al*., 2021), because the female *Ae. aegypti* mosquito is anthropophilic and prefers humans as its primary host (Putri *et al*., 2022). The female *Ae. aegypti* mosquito has a long and slender proboscis (Andrew & Bar, 2013), which makes it easier for the mosquito to suck human blood. According to Kandi (2023), the density of the *Ae. aegypti* mosquito population will increase if efforts to eradicate it fail.

Efforts to eradicate *Ae. aegypti* mosquitoes have been controlled by implementing vector control methods, including physical, biological, and chemical vector control (Armayanti & Rasjid, 2019). Physical and biological vector control are recognized to take quite a long time, while chemical control using insecticides is felt to be faster (Sugiman, 2019), so insecticides are used more often by the public.

However, the excessive and often uncontrolled use of insecticides has led to the development of resistant mosquitoes (Mu'azah *et al*., 2021). This resistance occurs because mosquitoes have developed a defense system against the frequently used insecticides (Qibtiyah *et al*., 2021). The resistance mechanism in mosquitoes involves the protein carrier gene on the cell membrane (Irawati, 2021), one of which is the ace-1 gene (*acetylcholinesterase-1*).

Mutations in the ace-1 gene have been observed in response to exposure to organophosphate and carbamate insecticides (Wijaya *et al*., 2023). Both insecticide groups are commonly used in Indonesia to control *Ae. aegypti* mosquito populations (Khairiyati *et al*., 2021). Organophosphate and carbamate insecticides inhibit the action of the acetylcholinesterase (AchE) enzyme (Marcherya, 2020), the enzyme responsible for breaking down acetylcholine into acetate and choline (Reubun *et al*., 2020), resulting in the accumulation of acetylcholine, which disrupts the central nervous system (Ananto, 2017).

Research on resistance to the ace-1 gene has been conducted in various cities in Indonesia, such as Ambon (Akollo *et al*., 2020), Padang (Hasmiwati *et al*., 2018), and Surabaya (Maftukhah *et al*., 2022). This resistance research needs to be explored to determine the presence of *Ae. aegypti* mosquitoes that have undergone mutations in the ace-1 gene in a specific region, such as Malang City, which is endemic for DHF. The exploration of the ace-1 gene is carried out using the Polymerase Chain Reaction (PCR) method, which requires a specific primer.

The specific primer is obtained by designing the primer in silico based on bioinformatics science (Fakih *et al*., 2021). Research on the design of the ace-1 gene primer in *Ae. aegypti* in Malang City has not been previously reported, making this research a primary reference for further research. This research aims to design a specific primer for the ace-1 gene in *Ae. aegypti* in Malang City, which can be used as a putative insecticide-resistant gene for organophosphate and carbamate.

MATERIALS AND METHOD

Tools and Materials

The equipment used in this research consists of two mosquito net cages, aspirator, scale, oven, beaker glass, pipette tips, 1.5 ml micro pistil, micropipette, microtubes, centrifuge, vortex, magnetic stirrer, microwave, -20℃ freezer, spin down unit, thermal cycler, comb, gel tray, flask, electrophoresis machine, PCR machine, gel documenter, and laptop.

The materials used in this research are *Ae. aegypti* mosquitoes, 10% sucrose solution, cotton, poultry pellets, parafilm paper, distilled water, 20% SDS, proteinase-K, homogenizing buffer, µL isopropanol equal volume, NaCl, ddH2O, 70% ethanol, agarose, TE buffer, ethidium bromide, gel loading buffer, loading dye, dH2O, PCR master mix, DNA template, ace-1 primers, and nuclease-free water.

Ethical Approval

This research has received ethical approval from the Health Research Ethics Commission, Faculty of Medicine, University of Muhammadiyah Malang, under approval number: E.5.a/262/KEPK-UMM/XII/2021.

Research Methods

This research employed a qualitative study with a descriptive observational research design, integrating both in silico and in vitro methods, using PCR amplification techniques. The research was conducted at the Biotechnology Laboratory, University of Muhammadiyah Malang. The sample used was adult *Ae. aegypti* mosquitoes were obtained from a landing collection in Malang City.

Research procedure

In Silico Study

Ace-1 Gene Exploration

The ace-1 gene was explored through the National Center for Biotechnology Information (NCBI) database as a template for designing primers. In the search column use the keyword "ace-1 *Aedes aegypti*". The next step was to select the item with accession number MK896351.1, 473 bp from Belu, Indonesia, and then save it in FASTA format.

Primer Design

The primer candidates were designed with specific parameters (Table 3) using the Primer3web site. The specificity of these primer sequences to *Ae. aegypti* was then screened using the BLAST site from NCBI to analyze the similarity of the primers used with other organisms and to minimize off-target amplification.

Mosquito Collection, Rearing, and Identification

Mosquito eggs and larvae were collected using ovitraps and from a foot washing basin in a mosque in Malang City. The collected samples were reared and then identified morphologically using a 3.6 MP digital microscope by observing the specific characteristics of the *Ae. aegypti* mosquito.

DNA isolation

In this research, DNA was isolated from four mosquito samples using the salting-out method. The four mosquito samples were extracted using a micro pistil with the addition of 400 µL of homogenizing buffer solution. The extracted solution was added to a microtube containing 40 µL of 20% SDS and 8 µL of proteinase-K, then incubated at 65℃ for 2 hours. Subsequently, 300 µL of 6M NaCl was added, and the mixture was vortexed for 30 seconds. The resulting solution was centrifuged to separate the pellet from the supernatant. The supernatant was collected and added with an equal volume of cold isopropanol, then incubated at -20℃. The pellet containing DNA was washed with 300 µL of 70% ethanol, centrifuged again to remove the supernatant, and dried. The pellet was then added with ddH2O to proceed to the PCR step.

Polymerase Chain Reaction (PCR)

The PCR process requires forward and reverse primers for the ace-1 gene of *Ae. aegypti,* the PCR information can be seen in Table 1.

Furthermore, the PCR product was observed in a 1.5% agarose gel containing a 1x TBE solution. Electrophoresis was performed at 50 volts for 50 minutes. The electrophoresis result can be observed using gel documentation under UV light by observing the glowing DNA bands on the screen.

Table 1. PCR Component and condition

PCR Component			
Component			Volume
DNA Template			3 µL
Primer ace–1 (Forward and Reverse)			4 µL
PCR Master Mix			12.5 µL
dH2O			3 µL
PCR Condition Information			
Step	degree	times	Cycle
pre-denaturation	95℃	2 minutes	1
denaturation	95℃	30 seconds	30
Annealing Primer 1	49,6℃	20 seconds	30
Annealing Primer 2	51,3℃	20 seconds	30
elongation	72℃	20 seconds	30
Post elongation	72℃	10 minutes	1

Data analysis

Data analysis was performed descriptively by observing the presence of amplified DNA bands on the gel documentation screen. The size of the amplified ace-1 gene product was determined by comparing its migration distance with a DNA ladder of known sizes. The presence of a clear and specific band at the expected size confirmed the successful design and amplification of the ace-1 gene primer.

RESULTS

In Silico Study

The initial step of the in silico study is to determine the DNA template as a reference for primer design through the NCBI website. Selected DNA sequence data will be downloaded in FASTA format and then designed using Primer3web by setting the "General Primer Picking Condition". Based on the search results, 10 candidate primer pairs of the ace-1 gene belonging to *Ae. aegypti* mosquitoes have been obtained. The obtained primer candidate pairs will be eliminated based on the optimal range of primary design parameters. Based on the elimination results, two pairs of primers with the best criteria were obtained.

The selected primers were obtained based on the settings in table 2. The selected primer candidates were rechecked using BLAST, and the results showed that primer 1 and primer 2 are specific to *Ae. aegypti* mosquitoes, as shown in the gene map in Figure 1.

Based on the results of the in-silico primer design through NCBI, Primer3web, and BLAST, two pairs of primers were obtained and ordered through Integrated DNA Technologies (IDT) (Table 4).

Morphological Identification

Mosquito larvae and eggs were collected from a landing site in a dengue-endemic area in the Malang region (Figure 2). Morphological identification of *Ae. aegypti* mosquitoes from the landing collection and rearing was conducted using a 3.6 MP digital microscope. The identification results are shown in Figure 3.

Table 2. Primer setting using Primer3web

Parameter	Primer 1	Primer 2
Primer size	opt: 21	opt: 21
Tm	opt: 56.0	opt: 58.0
Primer GC %	opt: 55.0	opt:59.0
Product size	400-420	400-420

Table 3. Results of BLAST primer 1 and primer 2

No	Primer 1	Primer 2
1	ace-1 of *Aedes aegypti* (XM_021851339.1)	ace-1 of *Aedes aegypti* (XM_021851339.1)
2	ace-1 of *Aedes aegypti* (XM_001656927.3)	ace-1 of *Aedes aegypti* (XM_001656927.3)
3	ace-1 of *Aedes aegypti* (XM_021851338.1)	ace-1 of *Aedes aegypti* (XM_021851338.1)
4	ace-1 of *Aedes aegypti* (XM_021851337.1)	ace-1 of *Aedes aegypti* (XM_021851337.1)
5	ace-1 of *Aedes aegypti* (XM_021851336.1)	ace-1 of *Aedes aegypti* (XM_021851336.1)
6	ace-1 of *Aedes aegypti* (XM_021851334.1)	ace-1 of *Aedes aegypti* (XM_021851334.1)
7	ace-1 of *Aedes aegypti* (XM_021851333.1)	ace-1 of *Aedes aegypti* (XM_021851333.1)
8	ace-1 of *Aedes aegypti* (XM_021851332.1)	ace-1 of *Aedes aegypti* (XM_021851332.1)
9	ace-1 of *Aedes aegypti* (XM_021851331.1)	ace-1 of *Aedes aegypti* (XM_021851331.1)

Table 4. Primer design results for ace-1 *Aedes aegypti* through IDT

No		Primary Pair	Length	Tm	GC%	Product Size
Primer 1	Forward	AACGTTCGCTAGCCAGTG	18	55.3 °C	55.6	413 bp
	Reverse	GTCACCATGCATCACACC	18	53.9 °C	55.6	
Primer 2	Forward	CAACGTTCGCTAGCCAGTG	19	56.4 °C	57.9	417 bp
	Reverse	CTCGTCACCATGCATCACAC	20	56.2 °C	55.0	

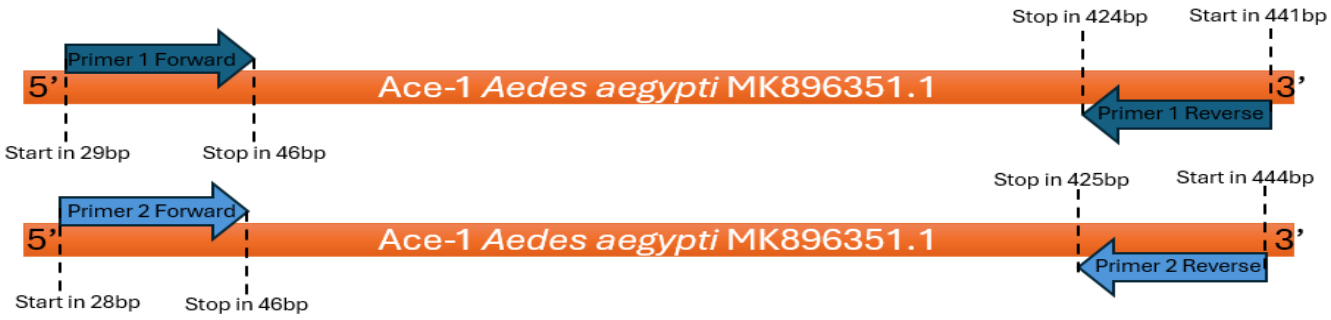

Figure 1. Map of *annealing primer Ace-1 Ae. aegypti*

Figure 2. Map of *Aedes aegypti* landing collection. can access by coordinate: latitude: -7.931457, longitude: 112.657534.

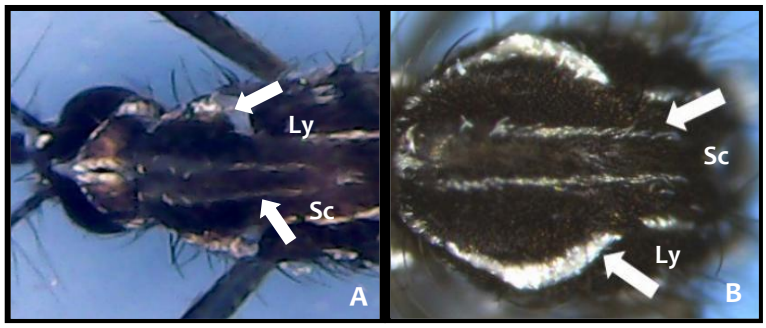

Figure 3. Dorsal Morphology of Aedes aegypti, showing scutum (Sc) and white marks in the shape of a lyre (Ly). A: Personal documentation, B: Buxton et al., 2019

PCR Results

Amplified PCR products are analyzed by horizontal electrophoresis and visualized using gel documentation to display DNA bands (Figure 4). The PCR amplicon in this study was successfully amplified at a size of 413 bp (primer 1) and 417 bp (primer 2). The sizes of these bands were determined by comparing them to a DNA ladder. The successful amplification of the target gene confirmed the effectiveness of the designed primers.

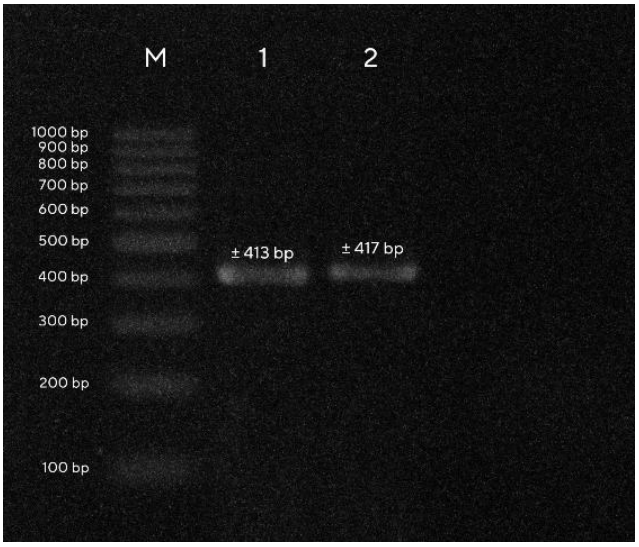

Figure 4. Results of PCR visualization using electrophoresis show a 1Kb marker (M), primer 1 band in 413bp ; primer 2 band in 417bp.

DISCUSSION

The in-silico primer design successfully yielded two pairs of primers that align with the optimal primer design parameters (Table 2). The optimal parameters for the primer design used in this study include a primer length of 18-24 bp, a GC content of 50%-60%, and a melting temperature (Tm) of 52°C-58°C (Hafsah *et al*., 2023). According to Anika *et al.* (2019), a primer that is too short will reduce the primer's specificity because it may bind to any location, while a primer that is too long will make the PCR reaction ineffective.

High GC content can complicate the separation of double-stranded DNA chains in primers and DNA templates (Purwakasih & Achyar, 2021), while low GC content can affect the efficiency of PCR by preventing primers from binding to the target (Praja & Rosalina, 2021). This is because the bond between guanine (G) and cytosine (C) bases is stronger than the bond between adenine (A) and thymine (T) bases (Sihotang *et al*., 2021). The GC/AT content also affects the melting temperature (Tm), the higher GC/AT content, the higher Tm (Winder *et al*., 2010). According to Sitepu *et al.* (2022), the melting temperature difference between the forward and reverse primers should not exceed 5°C to facilitate denaturation time determination.

Two primer pairs that have met the criteria will be subjected to BLAST analysis to assess the similarity of the primers used with other genomes (Muhsinin *et al*., 2018). The BLAST results (Table 3) indicate that primer 1 and primer 2 are specific to the *Aedes aegypti* mosquito and do not bind to genes in other organisms.

The results of the identification in Figure 2 show specific characteristics of *Ae. aegypti* mosquitoes. The thorax has a scutum with a pair of longitudinal white lines and a lyre-shaped white pattern (Buxton *et al*., 2019).

In the PCR step, the tested temperature is the annealing temperature (Ta). The Ta setting used is based on a range of (-5°C) to (+5°C) from the factory Tm (Nuryady *et al*., 2020), resulting in Ta of primer 1

= 49.6°C and primer 2 = 51.3°C. Annealing temperature plays an important role in the process of primer annealing with the DNA template. A temperature that is too low causes non-specific binding, while a temperature that is too high causes poor primer annealing (Annisa *et al*., 2024). The denaturation to elongation stage is a repeated (cycling) stage, where DNA duplication occurs in each cycle (Abdurrahman & Purnamasari, 2023). In this study, 30 cycles were used.

The PCR product was analyzed using electrophoresis with a 2% agarose gel. In the process of electrophoresis, a marker is also used to determine the size of the amplified DNA (Sundari & Priadi, 2019). The marker used is a 1 Kb bp DNA ladder. The visibility of the DNA bands in the electrophoresis results is determined by the presence of the DNA stain (Anggraeni *et al*., 2023). The staining is done by immersing the agarose gel in water containing EtBr (Ibrahim, 2023; Nataprawira *et al.*, 2022). The electrophoresis results show that the designed primer can be used as a specific primer candidate for further research.

The research successfully designed and validated this ace–1 primer as a crucial first step for developing a molecular diagnostic tool to detect insecticide resistance in *Aedes aegypti* in endemic areas. The application of this result is directly relevant to managing resistance against organophosphate (OP) and carbamate insecticides (Maftukhah *et al*., 2022). Specifically, the ace–1 gene encodes the AChE enzyme, and a mutation (like G119S) in this region is the genetic mechanism for resistance to OPs/carbamates (Wijaya *et al*., 2023). This primer enables rapid surveillance of the ace–1 allele frequency, providing essential data to inform public health decisions regarding insecticide rotation and replacement. Crucially, the ace–1 primer differs from others, such as those targeting KDR or VGSC, because it addresses a distinct resistance mechanism (OP/Carbamate resistance via AChE modification) compared to the KDR/VGSC gene, which confers resistance to pyrethroids and DDT via a modification of the sodium channel (Akollo *et al*., 2020; Hasmiwati *et al.*, 2018). Both ace–1 and KDR primers are necessary for a comprehensive, parallel resistance monitoring program.

The study successfully designed and validated two primer pairs through rigorous in-silico design and subsequent electrophoresis, confirming their specificity and functionality. This verified primer is a crucial molecular tool for the rapid surveillance of organophosphate and carbamate resistance, enabling effective, evidence-based management of vector control programs.

ACKNOWLEDGEMENT

Author expresses gratitude to the Directorate of Research and Community Service University of Muhammadiyah Malang with the Fundamental research scheme for funding the research, Dr. rer.nat Hans-Peter Fuehrer from Institute of Parasitology, veterinary Medicine University of Vienna for his advice and guidance, as well as to all parties who have participated directly or indirectly in this research.

"*The authors declare that there is no conflict of interest with any parties involved in this research*."

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