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Genotyping F1534C mutation on dried *Aedes aegypti* preparation through direct PCR method: a proof of concept

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ABSTRACT

Aedes aegypti (*Ae. aegypti*) is the primary vector of dengue hemorrhagic fever (DHF). Various control strategies have been utilized to control its population, including the use of pyrethroid-based insecticides. However, the presence of mutations, such as the F1534C mutation, that confers resistance to pyrethroids has been increasingly reported. The increase of resistance-conferring mutation in *Ae. aegypti* population could potentially hinder DHF control measures. As such, monitoring the genotype of *Ae. aegypti* population is crucial. Mosquito rearing, DNA extraction, and PCR examination are usually employed to monitor the circulation of F1534C mutations. To simplify this process, we proposed a direct PCR workflow utilizing dried mosquito samples preserved on an in-house filter paper. To demonstrate the utility of our proposed workflow, we performed direct allele-specific PCR (AS-PCR) on 46 dried adult *Ae. aegypti.* As a comparator, conventional PCR was performed on 8 DNA extract from *Ae. aegypti*. Our results showed that direct AS-PCR successfully identified both wild-type (F allele) and mutant (C allele) genotypes from dried mosquitos with a success rate of 93.48%. These findings provide preliminary evidence supporting the use of cellulose-based in-house filter paper for genotyping insecticide-resistant mosquitoes. However, field testing must be performed before its implementation in real-world epidemiological and surveillance applications.

ABSTRAK

Aedes aegypti adalah vektor utama dari demam berdarah dengue (DBD). Berbagai strategi telah diupayakan untuk mengontrol populasinya, termasuk dengan penggunaan insektisida berbasis piretroid. Sayangnya, terjadinya mutasi yang menyebabkan resistansi pada piretroid, seperti F1534C, semakin sering dilaporkan. Terjadinya mutasi di populasi *Ae. aegypti* ini dapat menghambat upaya pengendalian DBD. Oleh karena itu, monitoring genotipe *Ae. aegypti* penting dilakukan. Proses pembudidayaan nyamuk, ekstrasi DNA, dan pemeriksaan PCR sering dilakukan untuk memonitor sirkulasi mutasi F1534C. Untuk menyederhanakan proses ini, kami mengusulkan alur *direct* PCR dengan menggunakan sampel nyamuk kering yang diawetkan di *in-house filter paper*. Untuk mendemonstrasikan utilitas alur kerja yang kami usulkan, kami melakukan *direct alelle-specific* PCR (AS-PCR) pada *Ae. aegypti* dewasa. Sebagai pembanding, PCR konvensional dilakukan pada ekstrak DNA *Ae. aegypti*. Hasil kami menunjukkan bahwa *direct* AS-PCR sukses mengidentifikasi baik genotipe *wild-type* (alel F) dan mutan (alel C) dari nyamuk kering dengan derajat sukses 93,48%. Hasil ini memberikan bukti awal yang mendukung penggunaan *in-house filter paper* berbasis selulosa untuk mengidentifikasi genotipe nyamuk resisten insektisida. Namun demikian, uji coba lapangan harus dilakukan sebelum konsep ini diimplementasikan untuk penelitian epidemiologis dan surveilan.

Keywords:
Aedes aegypti;
genomic filter paper;
F1534C;
direct PCR;
AS-PCR

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INTRODUCTION

Aedes aegypti (*Ae. aegypti*) is an anthropophilic Culicidae mosquito, which acts as a vector of several arboviral diseases, including dengue hemorrhagic fever (DHF). From January to April 2024, there were 7.6 million reported dengue cases globally, with at least 3,000 deaths attributable to this disease.[1] This mosquito species is well-adapted to urban environments, particularly in areas with human-altered landscapes that facilitate the formation of stagnant water pools, providing ideal breeding sites.[2,3] Various strategies have been implemented to control *Ae. aegypti* populations and, consequently, reduce the incidence of DHF. These approaches include the application of pyrethroid-based insecticides, the elimination of mosquito breeding sites, and the release of *Wolbachia*-infected mosquitoes. Despite these efforts, many households continue to primarily rely on pyrethroid-based insecticides for managing mosquito populations.[4]

The massive application of pyrethroid-based insecticides to control *Ae. aegypti* population potentially increases the evolutionary pressure that selects for pyrethroid-resistant population. Pyrethroids act by interfering with the mosquito's voltage-gated sodium channel (VGSC), leading to paralysis and even death.[5] This class of insecticides is favored due to its ability to selectively bind to insect VGSC, but not to mammalian VGSCs, resulting in low toxicity to human.[6] Several mechanisms are known to cause pyrethroid resistance. These include enhanced activity of detoxification enzymes like cytochrome P450 monooxygenases, glutathione transferases, and esterases; reduced permeability of mosquito cuticle, and decreased sensitivity of the VGSC because of mutations in the gene, commonly referred to as knockdown resistance (*kdr*) mutations.[7] The presence of *kdr* mutations has been well-documented in Indonesia. Among these, the V1016G, S989P, and F1534C mutations are the most frequently reported across various regions of the country.[8-12]

The F1534C mutation is among the most prevalent kdr mutations across the world.[13,14] This mutation has been demonstrated to confer at least a seven-fold increase in resistance to multiple pyrethroid-based insecticides in *Ae. aegypti* mosquitoes.[15] As a result, the widespread application of pyrethroid-based insecticides in communities may become ineffective. Furthermore, given the endemic nature of DHF in Indonesia, the presence of this resistant allele within local *Ae. aegypti* populations could significantly undermine DHF control efforts in the country. Recently, *Ae. aegypti* mosquitoes with the homozygous resistant genotype (F1534C) were identified in Yogyakarta, Indonesia.[16] This report emphasizes the importance of genotypic surveillance to monitor the development of *kdr* mutations in the country.

To date, mosquito genetic surveillance involves sample collection, mosquito rearing, DNA isolation, and PCR. This procedure requires dedicated facilities and logistics for the mosquito rearing process.[17] DNA isolation techniques require high-cost kits, which might hinder mosquito surveillance efforts. In addition to the expensive DNA isolation step, a DNA sequencing step is necessary to detect mutations from the DNA or PCR product, thus further increasing the cost of surveillance.[18]

Therefore, we proposed a direct allele-specific PCR (AS-PCR) protocol to

lower the surveillance cost. Direct AS-PCR could detect the specific mutated alleles, using a specific set of primers, thus bypassing DNA sequencing step and further lowering the mosquito genetic surveillance cost.[19] In direct AS-PCR, DNA templates were generated using genomic filter paper to simplify PCR steps. Utilization of genomic filter paper for PCR has been used to detect pathogens and genetic diseases in human population.[20,21] By utilizing this approach in mosquito genetic surveillance, field sample collectors can directly prepare mosquito samples on-site and send them to a laboratory to be examined. This not only simplifies the workflow, but also potentially reduces the overall cost of mosquito genetic surveillance.

In this study, we proposed a mosquito genetic surveillance workflow using an in-house genomic filter paper. Our filter paper has previously been tested as a sample collection tool for genetic screening from blood samples.[21–23] However, its utility as a sample collection tool for genetic screening from solid tissue samples has not been tested. Therefore, the current study primarily aims to evaluate the reliability of our genetic filter paper in screening *kdr* mutation from dried mosquito samples using AS-PCR. We utilized the adult stage of *Ae. aegypti* reared in the Entomology Laboratory of the Department of Parasitology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta.

MATERIAL AND METHODS

All examinations were performed in the Entomology Laboratory of the Department of Parasitology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia. This study was approved by the Medical and Health Research Ethic Committee, Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada – Dr. Sardjito General Hospital (Ref. No.: KE/FK/0360/EC/2024).

Mosquito Samples and Dried Mosquito Preparation

Adult *Ae. aegypti* mosquitoes used in this study were obtained from a mosquito colony that has been perpetually reared in the Entomology Laboratory of the Department of Parasitology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta. The colony was originally collected from Gondokusuman SubDistrict, Yogyakarta City. Before the procedure, the mosquitoes were euthanized by putting them in a -4°C freezer for 5 min.

A total of 46 dried mosquito samples were prepared in this proof-of-concept study. Each dried mosquito sample was prepared by pressing one euthanized adult *Ae. aegypti* mosquito on an in-house genetic filter paper. Two drops of absolute methanol were subsequently applied to the preparation, which was then allowed to air dry (FIGURE 1A). Dried mosquitos were stored in sealed zip-lock bags containing desiccant and kept in a dark room at ambient temperature until further analysis. Up to five individual mosquitoes were prepared on one in-house filter paper. Each mosquito was treated as a distinct sample.

Mosquito DNA isolation

A total of 8 different mosquito DNA samples were isolated in this study and were used as a reference template in the PCR to be compared with direct AS-PCR from dried mosquitoes. The mosquito DNA isolation was performed using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit, in accordance with the manufacturer's instruction from one adult *Ae. aegypti*. The concentration and purity of the isolated DNA were then evaluated using a nanodrop spectrophotometer.

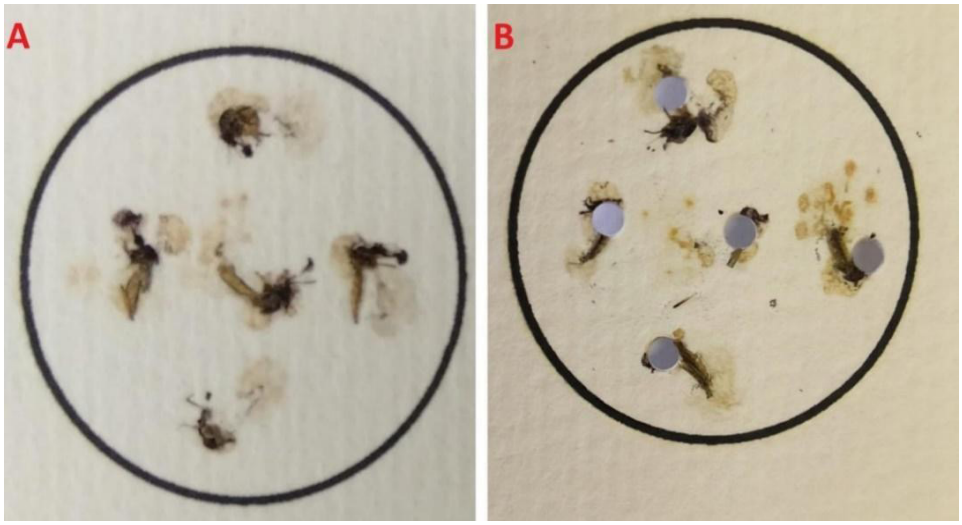

FIGURE 1. Dried mosquito preparations on in-house genetic filter paper (A). A 2.0 mm hole was punched through each mosquito to be utilized as a sample for direct AS-PCR (B). Each mosquito represents one sample.

Genetic analysis

We examined the F1534C mutation in *Ae. aegypti* using AS-PCR approach.[19] Briefly, 10ng of template DNA or 2.0 mm punch of dried mosquito preparation (FIGURE 1B) were added into a PCR mix containing 1×PCR buffer, 0.4 µM common reverse primer, 0.4 µM of each allele-specific forward primer, 400 µM dNTP mixture, and 0.5 units KOD FX NEO (Toyobo Inc. Osaka, Japan). The mix underwent initial denaturation step at 98°C for 2 min, followed by 40 cycles of denaturation at 98°C for 30 seconds, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. Then, the process was closed with a final extension step at 72°C for 5 min. The primer design can be seen in TABLE 1. The product of AS-PCR was visualized using 4% TBE agarose gel using electrophoresis. The AS-PCR produced a 93 bp band for the wild-type (F allele), a 113 bp band for the resistant mutant (C allele), and both bands for the heterozygous. To evaluate the successful rate of direct PCR from dried mosquitoes, we calculated the proportion of successful PCR which produced clear amplicon over total number of PCR being performed and reported the results as percentage.

RESULTS

Direct AS-PCR successfully genotype dried mosquitoes

To comprehensively evaluate the feasibility of direct AS-PCR from dried mosquito specimens, we conducted a comparative analysis between PCR results obtained directly from dried mosquitoes—bypassing the conventional DNA extraction step—and those derived from purified genomic DNA. The direct AS-PCR method employed mosquito samples blotted onto and stored on our in-house filter paper. Notably, this streamlined approach successfully amplified distinct allele-specific products, with a 93 bp fragment corresponding to the wild-type allele and a 113 bp fragment corresponding to the mutant allele (FIGURE 2). The clarity and specificity of these bands were comparable to those produced by conventional AS-PCR using extracted DNA, thereby demonstrating that direct AS-PCR is a viable alternative for genotyping dried mosquito samples. This approach not only reduces processing time and reagent costs but also minimizes potential sample loss during extraction, making it particularly advantageous for high-throughput or field-based applications.

TABLE 1. Primer design for detecting F1534C mutation on *A. aegypti.*[19]

Primer	Sequence
F1534C-Falel-For	5'-GCGGGCTCTACTTTGTGTTCTTCATCATATT-3'
F1534C-Calel-For	5'-GCGGGCAGGGCGGCGGGGGCGGGGCCTCTAC TTTGT-GTTCTTCATCATGTG-3'
F1534C-Rev	5'-TCTGCTCGTTGAAGTTGTCGAT-3'

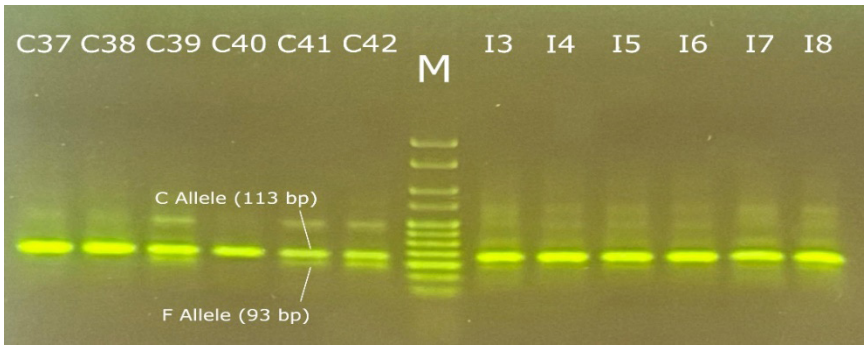

FIGURE 2. The amplicon band produced from dried mosquito group (C37 – C42) and DNA isolate group (I3 – I8) were compared qualitatively. Distinct non-specific amplicons were observed in dried mosquito group. The allele-specific amplification PCR produced a 113 bp fragment for the C allele, a 93 bp fragment for the F allele, and both bands for the heterozygote. M = 50 bp DNA Ladder Marker.

A total of 46 dried mosquito specimens were analyzed using the direct AS-PCR approach. Of these, 43 samples yielded successful amplification and were accurately genotyped, while three samples failed to produce a detectable PCR product. In parallel, all 8 samples that underwent conventional DNA extraction were successfully amplified and genotyped using AS-PCR, achieving a 100% success rate. The success rate of 93.48% for direct AS-PCR underscores the reliability and robustness of the method, though it also highlights the importance of sample quality.

We further evaluated the allele frequency of the dried mosquito from mosquitoes bred in our facility. The mosquito bred in our facility was initially collected from Gondokusuman SubDistrict, Yogyakarta City. Allele frequency analysis based on the direct AS-PCR results (TABLE 2) revealed that the wild-type F allele was present at a frequency of 19.77%, whereas the mutant C allele was dominant, at a frequency of 80.23%. These findings provide insight into the genetic makeup of the mosquito population maintained in our facility and highlight the potential application of this simplified genotyping method in population genetic studies, insecticide resistance monitoring, and vector control programs.

TABLE 2. The frequency of F1534C mutation

Group	Genotype frequency (%)			Allele frequency (%)	
	F/F	F/C	C/C	C (resistant) allele	F (sensitive) allele
Dried mosquito	0/43 (0%)	17/43 (39.53)	26/43 (60.47)	80.23	19.77

DISCUSSION

Our results demonstrated that 43 out of 46 dried mosquito samples (93.48%) were successfully amplified and genotyped. This successful amplification highlighted the feasibility and reliability of the direct AS-PCR method applied to crude samples like dried mosquitoes, bypassing the conventional, labor-intense DNA extraction step. The direct AS-PCR approach also simplifies storage and handling requirements, which is particularly beneficial for field sample collection in remote or resource-limited settings.[24–26] Furthermore, the straightforward process minimizes DNA loss during handling and transfer, thereby improving the overall efficiency and reliability of the genotyping process.[25–27] Our results also indicate the applicability of our in-house filter paper as a carrier for dried mosquito samples, in addition to its previously demonstrated utility for dried blood spots in genetic analysis such as AS-PCR and PCR-RFLP. This cellulose-based card of about 180 gsm was enough to store solid tissue of crude samples like dried mosquitos.[23] To our knowledge, this was the first comparative evaluation between dried mosquitoes on a cellulose-based, in-house filter paper with pure gDNA for PCR-based genetic analysis.

Although direct PCR offers practical benefits, such as reduced processing time and minimal sample handling, it also presents several limitations that can impact the reliability and accuracy of the results.[28] A primary concern is the omission of a DNA purification step, which can significantly impact the quality and consistency of PCR results. In conventional PCR workflows, DNA purification is essential for removing cellular components that may inhibit DNA polymerase activity.[29,30] However, in direct PCR, the crude sample is used as-is, without undergoing this purification process. As a result, various cellular components present in the mosquitos (like proteins, polysaccharides, and secondary metabolites) can act as inhibitors of the DNA polymerase enzyme, compromising the PCR efficiency. This interference may lead to partial or complete failure of target amplification, increasing the risk of false-negative results.[25,26] In our study, we observed a failure to amplify DNA in a dried mosquito sample, presumably due to such inhibitory effects interfering with the PCR process.

Additionally, the use of unpurified DNA in direct PCR reactions often leads to other technical issues, including the appearance of smeared bands or non-specific amplification on agarose gel electrophoresis.[31,32] These phenomena not only complicate the interpretation of results but also reduce the specificity and reliability of the assay if not evaluated carefully. Consequently, while direct PCR offers faster turnaround time and a simplified workflow, its limitations must be carefully considered and mitigated.

To address these issues, we implemented several strategies. The simplest approach was to employ a high fidelity, inhibitor-resistant DNA polymerase like KOD FX NEO.[33] This polymerase was derived from

the hyperthermophilic archaeon *Thermococcus kodakarensis*.[33] This enzyme exhibits enhanced resistance to common PCR inhibitors, thereby improving amplification success from crude samples like dried mosquitos used in this study.[33,34] Our findings confirm that the use of KOD FX NEO significantly improves the amplification success rate.

Additionally, we optimized the amount of input template to balance between providing sufficient DNA for amplification and minimizing inhibitor presence. For each reaction, a 2 mm punch of the dried mosquito sample was used, which provided an adequate DNA quantity while limiting the concentration of inhibitors that might interfere with the PCR. These simple yet effective strategies enabled us to achieve a high success rate in direct genotyping from dried mosquitoes.

Considering the success rate of our method, we observed a predominance of the C allele within our dried mosquito population. Previous studies, such as one conducted in Yunnan, China, have shown a similarly high prevalence of the C allele in *Ae. aegypti* populations resistant to 3.20% permethrin.[35] The elevated mutant allele frequency in our samples was likely due to the use of a laboratory-reared mosquito strain originally derived from field populations with a high incidence of pesticide resistance. Therefore, the proportion of mosquitoes carrying the C allele was higher than that of the wild-type allele. Nevertheless, our findings suggest that direct AS-PCR is a viable method for detecting resistance-associated alleles in mosquito populations, with potential applications in field-based genetic surveillance programs.

CONCLUSION

Our proposed workflow offers a practical and reliable solution for epidemiological surveillance of *kdr* mutation in *Ae. aegypti*, especially in resource-limited settings. Our in-house filter paper method and its accompanying workflow present a promising, cost-effective alternative; however, field validation is essential before it can be scaled up. Additionally, we plan to investigate whether this workflow can be adapted to detect other targets, such as dengue virus in mosquitoes, which could enhance its applicability for outbreak investigations and broader public health monitoring.

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