COMPARISON OF THE WHO STANDARD AND THE CDC BOTTLE BIOASSAY TESTING METHODS FOR ASSESSING THE DENGUE VECTORS' SUSCEPTIBILITY TO INSECTICIDES IN SEMARANG, INDONESIA

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Abstract. Dengue hemorrhagic fever (DHF) is a public health concern worldwide, including in Indonesia. Insecticides are the most common method to control dengue vectors. The massive use of insecticides increases the risk of resistance. The World Health Organization (WHO) impregnated paper method has been used to evaluate insecticides for decades. Since 2019, Indonesia has used the Centers for Disease Control and Prevention (CDC) bottle bioassay method in testing resistance. This study aimed to compare resistance tests conducted using the CDC and the WHO methods. Larvae of *Aedes aegypti* were collected from three villages in Semarang City. As a comparison, the *Ae. aegypti* colony captured in 1986 in Semarang City was used. Insecticides evaluated included pyrethroids cypermethrin and organophosphate malathion. Synergist piperonyl butoxide (PBO) was used to determine the resistance mechanism. The *Voltage-Gated Sodium Channels* (*VGSC*) and *ACE1* genes were sequenced as part of the molecular assays. Data from the WHO and the CDC methods showed that *Ae. aegypti* from all locations were resistant to cypermethrin and revealed different results for malathion. Using the WHO method, the test mosquitoes showed resistance but indicated susceptibility when using the CDC method. Metabolic and target site mutations were discovered as resistance mechanisms to cypermethrin. Further research and regulation are needed to implement both approaches to monitor resistance.

Keywords: *Aedes aegypti*, resistance, CDC bottle bioassay, WHO impregnated paper

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INTRODUCTION

Dengue fever is one of the world's most serious public health issues. Recent studies report 50 million dengue infections yearly, and 75 percent of the cases are in Asia (Hsan *et al*, 2019; Singhi, 2018). Dengue fever is widespread in Southeast Asia, with at least 451,000 cases reported in 2015 (Maula *et al*, 2018). In Southeast Asia, Indonesia is noted to have the highest dengue fever rate. Yearly incidence increased dramatically over the last 43 years, from 0.05 per 100,000 in 1968 to 51.48 per 100,000 in 2019. Semarang City is one of the Central Java Province's dengue-endemic areas, incidence rate 26.37 per 100,000 population in 2019 (Semarang City Health Office, 2019).

Dengue transmission is also high in Semarang. As reported by Martini *et al*, 2017a, the density of *Aedes aegypti* larvae was quite high (House index = 42.8%, container index = 21.3%, and Breteau index = 58.9%). Vector management is the best way to prevent and treat dengue fever until vaccines are available. Insecticides are the most common chemical vector control method in Indonesia, including Semarang.

However, the widespread use of insecticides has resulted in a severe problem: insecticide resistance in *Ae. aegypti*. In Southeast Asia, including Malaysia, Thailand, Singapore, and Indonesia, *Ae. aegypti* resistance to insecticide has been widely reported (Rueda, 2004; Sayono *et al*, 2016; Siti-Futri *et al*, 2020). The main insecticide resistance mechanism comes from a combination of metabolic resistance and target-site mutation (Mitchell *et al*, 2014). Organophosphates, pyrethroids, and carbamates are three insecticides currently used in Indonesia to control dengue vectors (Elyazar *et al*, 2011).

Insecticides can reduce the density of the susceptible mosquito population, allowing the resistant one to take over. The efficacy of insecticides declines and becomes no longer effective with the increasing number of resistant mosquitoes. In this situation, replacing the ineffective insecticide with another effective one is necessary (Sparks *et al*, 2020). A successful vector control needs resistance management. The key to such management is having precise information about resistance mechanisms. Understanding the processes of pesticide resistance allows us to choose the best insecticide. Resistance vector surveillance can be used as an early warning system and provide information on the development of resistance in a specific location.

Indonesia has long used the WHO impregnated paper method to test vector resistance to insecticides. The method has several advantages, *ie*, 1) insecticides are readily available on the test paper, no need to be prepared by researchers; 2) knock down resistance (KDR) and mosquito mortality are easy to observe; 3) WHO has a standardized diagnostic dose and kits are readily available and can be purchased and used. However, this method has significant drawbacks. Besides being expensive, it must run on mosquitos of the same age (homogenous), requires 24 hours observation with stable temperature and humidity, and no resistance mechanism is obvious. For these reasons, the US-CDC developed an alternative method based on the bottle bioassay (Aïzoun *et al*, 2013). The CDC bottle bioassay method has advantages, namely 1) it can detect the resistance mechanism; 2) it can use a smaller number of mosquitoes; 3) no need to move the test mosquitoes from one bottle to another; 4) simpler and faster; 5) relatively cheap and does not depend on the availability of impregnated paper; 6) can use mosquitoes in the field without having to do it in the laboratory; and 7) Better describe the status of mosquito resistance in the field. Weaknesses of the CDC' method: 1) recording mosquito deaths in Wheaton bottles is somewhat tricky; 2) need to prepare bottle coating before a test (Aïzoun *et al*, 2013).

Since 2019, Indonesia has been implementing the CDC bottle bioassay as a new insecticide resistance test method (Ministry of Health, 2018). Insecticide resistance testing is currently possible using the WHO and the CDC methods based on the guidelines of resistance monitoring published

by the Indonesian Ministry of Health. The CDC methods has only been used to test *Anopheles* mosquitoes until recently. The CDC susceptibility testing method for *Ae. Aegypti* mosquitoes has never been reported in Indonesia. Thus, the effectiveness and issues with its implementation were unknown. The researcher would use the WHO impregnated paper and the CDC bottle bioassay to determine *Ae. aegypti* susceptibility to organophosphate and pyrethroid pesticides. This study hypothesized that *Ae. aegypti* has the same resistance either using the WHO impregnated paper or the CDC bottle bioassay. To improve the analysis of the test results through these two procedures, a molecular assay was used.

MATERIALS AND METHODS

Mosquito collection

Aedes aegypti larvae and pupae were obtained from Kandri, Patemon, and Terboyo Wetan Villages, Semarang City. Such locations were chosen based on the criteria for endemicity and variances in geographical elevation and economic activity. The study ran from September 2019 to February 2020.

The larvae and pupae were collected from containers inside and outside the houses in residential areas. The larvae and pupae then reared in the insectarium of the Institute for Vector and Reservoir Control Research and Development (IVRCRD) until getting adult mosquitoes F1 and F2 for resistance testing. The species was identified using Rueda's pictorial identification key (Rueda, 2004). A susceptibility test additionally ran under the WHO and CDC protocols for the insecticides malathion (organophosphate) and cypermethrin (pyrethroid).

As a control, a resistance test was conducted using *Ae. aegypti* susceptible strain that were captured in 1986 from Semarang. The susceptible mosquito strains have been kept in the IVRCRD insectarium since then and are used for resistance testing. Malathion was chosen because it had been used since the 1980s in Indonesia to reduce *Ae. aegypti*, and cypermethrin is the cheapest type 2 pyrethroid.

The WHO impregnated paper method

The WHO bioassay was performed by exposing insects to specified impregnated papers. Malathion (0.8%) and cypermethrin (0.05%) were used in this study. The mosquitoes were put into five WHO test tubes: four with an impregnated paper containing insecticide, while one with an impregnated paper containing no insecticide. Each had 20-25 female mosquitoes aged 2-5 days from each location. After 1 hour of contact, the mosquitoes were moved to a neutral tube. Knockdown and fatality rates were determined after 60 minutes and 24 hours. The test room was 28.1°C and 60-65% humid.

The CDC bottle bioassay method

The CDC method was tested by releasing mosquitoes into an insecticide-coated Wheaton bottle. Four 250 ml Wheaton bottles were coated with pesticide dissolved in ethanol, and one was covered with ethanol only. Malathion and cypermethrin were used as insecticides.

Each bottle contained 10-25 *Ae. aegypti* female adults. Resistance was assessed by examining percent mortality at 30 minutes (diagnostic time). If test results showed mosquito resistance, then the test was repeated with a synergist. As per the CDC test protocol, all mosquitoes were placed in a synergist-coated bottle for one hour before being transferred to the test bottle. Analyzing mortality rates with and without synergist revealed metabolic enzyme activity. Piperonyl Butoxide (PBO) was the synergist, and SSS-tribulyphosphorotrithioate (DEF) bound the esterase enzyme.

Molecular detection: *Acetylcholinesterase* (*AChE*) and *kdr* mutations

The *kdr* mutation was identified using the polymerase chain reaction (PCR) and sequencing methods. PCR was performed using the SimpliAmpTM Applied Biosystems thermal cycler (Perkin Elmer, Branchburg, NJ). Primers specific that were used for the *kdr* gene were Forward (5'-GGTGGAACTTCACCGACTTC-3') and Reverse

(5'-GGACGCAATCTGGCTTGTTA-3') targeting domain II of the *VGSC*. The PCR reaction was initiated with a 10-minute denaturation step at 94°C, followed by 40 cycles of amplification at 94°C for 1 minute, 63°C for 45 seconds, and 72°C for 1 minute, followed by a final 7-minute elongation step at 72°C. Following SYBR™ Safe DNA Gel Stain in 0.5X TBE (Thermo Fischer Scientific, Waltham, MA) Invitrogen labeling, all PCR products were put onto a 2% agarose gel electrophoresis and run for 60 minutes at 90 Volts in ready-for-use TAE buffer UltraPure™, 10X (Thermo Fischer Scientific, Waltham, MA) to ensure the quality of the PCR results.

To identify mutations on *ACE1* gene, AceF (5'-CGATAACG-AATGGGGAACG-3') and AceR (5'-TCAGAGGCTCACCGAACACA-3') primers were used. The following conditions were used: a 3-minute denaturation phase at 94°C, followed by 35 cycles of amplification at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes, followed by a 10-minute elongation step at 72°C. Purified PCR products were sequenced directly in both directions using the same primer for PCR amplification, G119S. Following that, the sequencing analysis was performed using an Applied Biosystems 3500 series genetic analyzer (Applied Biosystems® Sanger Sequencing 3500 Series Genetic Analyzers; Thermo Fischer Scientific, Waltham, MA).

Data analysis and interpretation

The CDC and the WHO techniques for susceptibility testing were compared to each other and were studied descriptively in terms of susceptibility to different types of insecticides and resistance to those with and without synergists. According to the WHO guidelines, the following criteria were used to establish resistance status: 1) Resistant when mortality is ≤90%; 2) Tolerant when mortality ranges from 90-97%; and 3) Susceptible when mortality is ≥98%. The susceptibility testing findings obtained through both the WHO and the CDC methods were then analyzed together with molecular assays.

This study is part of a research on the relationship between genetic population of *Aedes aegypti* and *Aedes aegypti* resistance which involved

human subjects. It was approved by the Ethics Health Research Commission, Faculty of Public Health, Universitas Diponegoro under the reference number 169/EA/KEPK-FKM/2019.

RESULTS

Aedes aegypti catching location

The dengue hemorrhagic fever (DHF) circumstances varied significantly in the three study locations. According to the research sites, the incidence rates in Patemon from 2016 to 2019 were 38.5, 59.6, 0, and 18.43/100,000 populations, respectively (Table 1). Terboyo Wetan and Kandri did not have endemic rates of any kind, except Kandri, which had an incidence rate of 0.8 per 100,000 people in 2019. Kandri and Patemon each have an elevation of over 250 meters above sea level (masl), while Terboyo wetan is a seaside area with an elevation of 10 masl (Fig 1).

The WHO and CDC methods' insecticide test results

The results of the WHO test of the susceptibility against the insecticide cypermethrin in Table 2 indicated that the death rates were less than 90%

Table 1
Incidence rate of larva sampling locations for resistance test in Semarang City in 2016-2019

Sub-district	Incidence rate by year (/100,000 populations)			
	2016	2017	2018	2019
Patemon	38.5	59.65	0	18.43
Terboyo Wetan	0	0	0	0
Kandri	0	0	0	0.80

Fig 1 - Locations of catching the resistance of larvae and pupae in Semarang City in 2019

in the three locations. According to the WHO guidelines, a death rate of less than 90% shows mosquitoes are resistant to cypermethrin. The test using the CDC method revealed that mosquitoes in all study locations were resistant. Following the addition of the PBO synergist, the death rate increased, and the mosquitoes became tolerant in Patemon and Kandri but remained resistant in Terboyo Wetan.

In Patemon, Terboyo Wetan, and Kandri, the WHO test revealed death rates of 91.7, 86.7, and 81.7 percent, respectively. When the CDC method was used, the death rates were 98.3, 96.7, and 93.3 percent, respectively. Because the CDC method's numbers show the susceptible status, the test was not repeated using the synergist DEF.

Table 2
Comparison of the CDC bottle bioassay and the WHO impregnated paper resistance tests

Location	% mosquito death (Malathion)		% mosquito death (Cypermethrin)		
	WHO standard (after 24 hours)	CDC bottle bioassay (after 30 minutes)	WHO standard (after 24 hours)	CDC bottle bioassay (after 30 minutes)	+PBO
Patemon	91.7^{++}	98.3^{+++}	62.4^{+}	90^{++}	96^{++}
Terboyo Wetan	86.7^{+}	96.7^{++}	30^{+}	55.0^{+}	71^{+}
Kandri	81.7^{+}	98.3^{+++}	75.3^{+}	84.7^{+}	93.3^{++}
Semarang 1986*	100^{+}	99.2^{+}	100+	100^{+}	---

Note: WHO test refers to WHO Impregnated Paper Method; CDC test refers to CDC Bottle Bioassay Method with insecticide only; +PBO: test refers CDC Method with insecticide and synergist PBO.

+Resistant; ++Tolerant; +++Susceptible; ---PBO assay was not done, because the mortality rate was 100% (susceptible)

*Test using *Aedes aegypti* mosquitoes that were caught from Semarang in 1986. *Aedes aegypti* strain susceptible which was captured in 1986 in Semarang City is still being rearing in the IVRCRD insectarium, Indonesia.

PBO: piperonyl butoxide

Resistance mechanisms based on the CDC bottle bioassay test using a synergist

Synergists bind pesticide-detoxifying enzymes. Synergists can reveal pesticide-resistant enzymes. When resistant mosquitoes are exposed to the synergist, their death rate or susceptibility changes, indicating that enzyme activity plays a role in resistance. Esterase detoxifies pyrethroid insecticides, and PBO binds them. Monooxygenase enzymes are involved in the detoxification of organophosphate pesticides, and DEF is the binding synergist. The test mosquitoes were resistant to cypermethrin but susceptible to malathion, as shown in Table 2. Based on these data, a test was conducted with the insecticide cypermethrin and the synergist PBO. Since the test mosquitoes continued to be susceptible to malathion, the DEF synergist was not used (Fig 2).

Resistance mechanisms of *Ae. aegypti* are depicted in Fig 2. In Patemon, the cypermethrin and PBO test charts are identical, indicating that the mutation of the target site mutation caused resistance. Kandri's major resistance was a mutation at the target location, while the metabolic resistance was modest. Terboyo Wetan possessed two mechanisms: mutation at the target site and metabolic resistance.

VGSC and *ACE1* gene mutations

Pyrethroid pesticides specifically target the voltage-gated sodium channel (*VGSC*) of insect nerve cells. Mutations in the *VGSC* gene, particularly in codons S989P and V1016G reduce pesticide sensitivity and result in resistance in *Ae. aegypti* mosquitoes. Organophosphate and carbamate pesticides target acetylcholinesterase, especially the *ACE1* gene, in the mosquito's neural synaptic gap. The mutation associated with resistance is codon G119S.

As shown in Table 3, mutations in the *VGSC* domain II gene have occurred, most notably in the S989P V1016G variant. In the S989P allele, mutations were detected in 57.9 percent of cases and 42.1 percent of controls. In allele 1016, the mutation was detected in 78.9% of cases, heterozygous in 15.8%, and wild type in only 5.3% of cases.

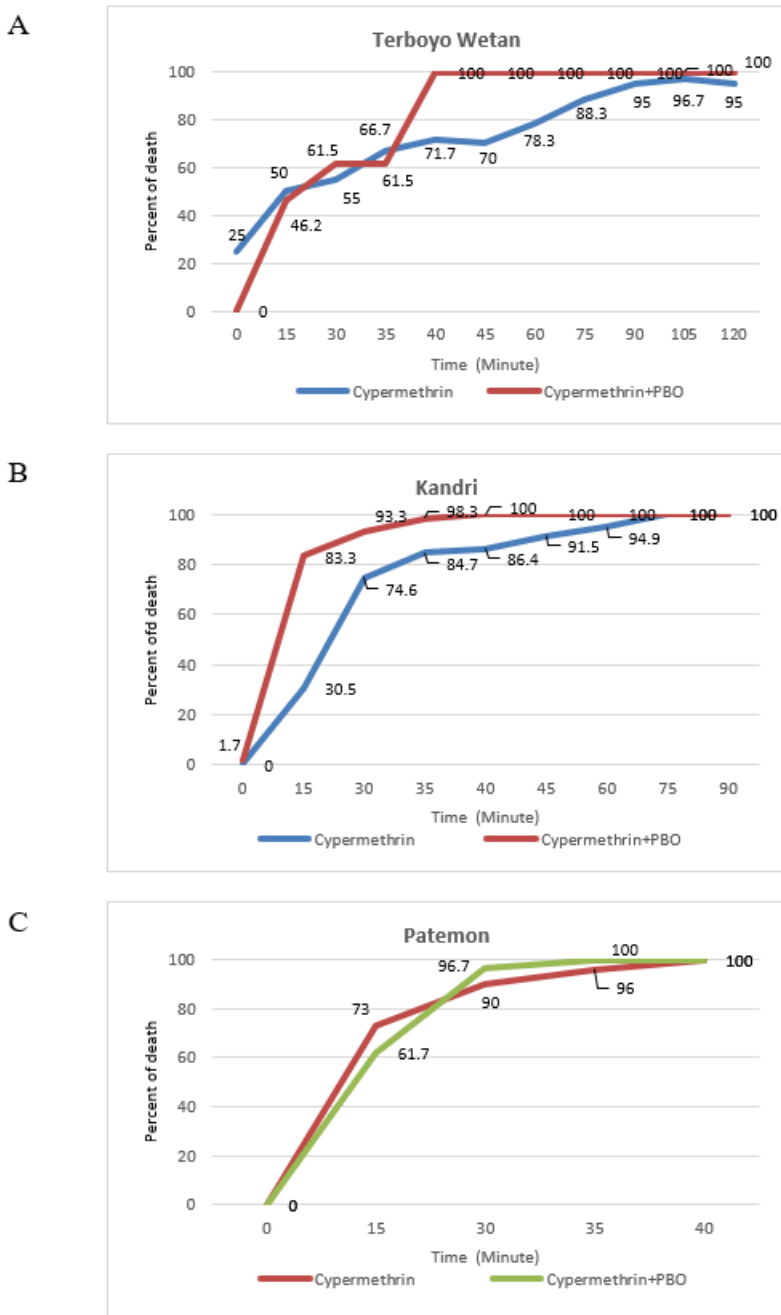

Fig 2 - Results of test using cypermethrin and PBO as the synergist

A: Test result from Terboyo Wetan Village; B: Test result from Kandri Village; C: Test cesult from Patemon Village

PBO: piperonyl butoxide

Table 3
Mutation of the *VGSC* gene

Location	Allele	Number	% Alleles occurred
VGSC			
Alel S989P	S	8	42.1
	SP	0	0
	P	11	57.9
Alel V1016G	V	1	5.3
	VG	3	15.8
	G	15	78.9
ACE 1			
Alel G119S	G	16	100
	GS	0	0
	S	0	0

DISCUSSION

The use of vector control and household pesticides in Indonesia and its challenges

Insecticides are a critical component of public health to eradicate mosquitoes. In Indonesia, the chemical control campaign for dengue vector is directed at adult mosquitoes and larvae (Ministry of Health, 2012). Indonesia uses organophosphate, carbamate, and pyrethroid insecticides. Since chemical control began in Indonesia, malathion has been used. Indonesia banned the use of the organochlorine insecticide dichlorodiphenyltrichloroethane (DDT) in 1989. For decades, malathion has been chemically used to control dengue vectors in Semarang City. Since 1999, pyrethroid synthetic have been used interchangeably with those with organophosphate active ingredients (Sayono *et al*, 2016).

Patemon's last fogging conducted four times in 2016, but Terboyo Wetan and Kandri have had no fogging in the last four years. Most exposure may come from household insecticides, which has pyrethroid as an active ingredient. Prior study reported that household insecticide was widely used. Reported, households using household insecticides in 2016 were 56.5% (Sayono *et al*, 2016). Synthetic pyrethroid active components are used in household pesticides.

Resistance testing method for *Aedes aegypti* to pesticides used in Indonesia

For decades, Indonesia's employed WHO-standard method for *Ae. aegypti* mosquito pesticide resistance test. This WHO method measures population mosquito resistance. The method can't provide information on resistance mechanisms, and that the type of insecticide tested is restricted to those available on impregnated paper (Aïzoun *et al*, 2013). District-level health institutions lack equipment and capacity to conducted the WHO testing method. The CDC has created a test method that addresses the WHO approach's several limitations.

Since 2019, Indonesia has adopted the CDC bottle bioassay method to test mosquito resistance (Brogdon and Chan, 2018). In Indonesia, the CDC bottle bioassay is used to determine *Anopheles* mosquito resistance for malaria elimination. This is the first publication in Indonesia comparing CDC bottle bioassay with WHO impregnated paper. his CDC method can provide information on the resistance mechanism without molecular or enzyme-linked immunosorbent assay (ELISA) tests by using a synergist. This resistance mechanism is crucial for selecting insecticides, especially in areas where mosquitoes are resistant to all classes of insecticides. This information is crucial for resistance surveillance.

Numerous studies have documented *Ae. aegypti* resistance in Semarang using the WHO method. According to Martini *et al* (2019), Sayono *et al* (2016), Widiastuti *et al* (2015), *Ae. aegypti* is resistant to pyrethroid and

organophosphate insecticides in Semarang City. Other Southeast Asian countries such as Malaysia, Thailand, and Singapore have also confirmed *Ae. aegypti* resistance to pesticides using the WHO technique (Rueda, 2004; Sathantriphop *et al*, 2020; Siti-Futri *et al*, 2020). *Ae. aegypti* resistance is a crucial problem because of the very wide spread of *Ae. aegypti*, not only in the lowlands but also in the highlands (Martini *et al*, 2017b). As reported by Wijanarko *et al* (2017), resistance of *Ae. aegypti* to insecticides in the highlands has also found. s

Comparison of *Aedes aegypti* resistance test using the WHO, CDC, and molecular methods

According to WHO and CDC methods, *Ae. aegypti* in Semarang were resistant to cypermethrin. The WHO methods revealed *Ae. aegypti* resistant to cypermethrin in three locations, while CDC method found it resistant in Terboyo Wetan and Kandri, but tolerant in Patemon. After adding the synergist PBO, the death rate rose but didn't become susceptible. The percentage of deaths (Fig 2) demonstrates that resistance occurred due to the target site's mutation in Patemon, whereas in Terboyo Wetan and Kandri resistance occurred through two mechanisms: target site mutation and metabolic resistance. It was observed that the rise in mortality did not affect the state of susceptibility at 30 minutes. This finding was compatible with that of the *VGSC* gene molecular test. Codon S989P and V1016G mutations were observed in the *VGSC* domain II gene sequences. Susceptibility testing to malathion revealed multiple resistance status. *Ae. aegypti* was tolerant (91.7% mortality) in the Patemon but resistant (86.7% mortality) in Terboyo Wetan and kandri. The CDC method yielded very different findings. Mosquitoes in Terboyo Wetan were resistant to malathion (96.7% mortality), while those in Patemon and Kandri villages were susceptible (98.3% mortality). The test using the DEF synergist was not carried out because the mosquitoes were not resistant to malation. Susceptibility testing in three regions revealed the same pattern. The three research locations had a range of environmental circumstances. Kandri and Patemon are high-altitude locations at 360 masl, while Terboyo Wetan is a low-altitude coastal area at

10 masl. Patemon was an endemic settlement, although Kandri and terboyo Wetan were not.

Comparing WHO and CDC methods in other countries using different mosquito species has produced inconsistent results. Owusu *et al* (2015) reported that using the WHO and CDC methods, *Aedes aegypti* from the ROCK strain was resistant to Malathion, permetrhin and DDT. *Ae. aegypti* was susceptible to lambda-cyhalothrin when tested by WHO but resistant by CDC. Prior research used laboratory *Aedes aegypti* colonies, while this study used field-caught mosquitoes.

Vatandoost *et al* (2019) in Iran observed that, while the resistance status of *An. stephensii* mosquitoes to DDT, bendiocarb, and deltamethrin was equivalent using the WHO and the CDC methods, the median lethal time (time to death: LT50) values differed (Vatandoost *et al*, 2019). The WHO and the CDC methods determined the susceptibility of *An. gambiae* mosquitoes to be 98.33 and 97.95 percent, respectively (Owusu *et al*, 2015). Fonseca-González *et al* (2009) found that using the WHO approach, *An. nuneztovary* mosquitoes were still vulnerable to phenytotrion, while the mortality rate was only 20% when using the CDC method.

Potential use of the CDC method in monitoring *Aedes aegypti* resistance

This is the first report in Indonesia using the CDC method to assess dengue vector pesticide resistance. Differences in susceptibility test status could complicate resistance surveillance and insecticide selection after Indonesia adopted the CDC method. The CDC's methods have potential for susceptibility and resistance testing. Along with providing information on resistance mechanisms based on the use of synergists, this method has several advantages over the WHO method (Brogdon and Chan, 2018). With these several advantages, health workers in rural places can do this procedure without a complex insectarium.

This study had limitations: the diagnostic dose and diagnostic time were based on CDC standards, not the local strain of *Ae. aegypti* mosquitoes in Indonesia. In resistance testing using the CDC bottle bioassay method, it is

suggested that the diagnostic dose (DD) and diagnostic time (DT) standards for each area be determined.

This study's results could inform dengue vector resistance surveillance. Due to its benefits, the CDC bottle bioassay should complement the WHO method. The WHO method measures vector insecticide resistance, while the CDC method is for ascertaining the state and resistance mechanism. Both strategies should produce comparable outcomes when these objectives are accomplished. This study demonstrated that the concurrent use of the CDC and WHO resistance testing methods requires additional regulation. Variations in *Ae. aegypti* resistance to malathion in this study could lead to incorrect data and errors in pesticide rotation management.

In summary, the use of the WHO, CDC and genetic methods to assess the *Ae. aegypti* susceptibility to pesticides revealed no variations in cypermethrin. However, there were differences in malathion. The CDC bottle bioassay has several advantages for resistance testing, notably its portability and synergist use. With this disparity in resistance status, additional research is necessary to compare the CDC to the WHO approaches to surveillance of *Ae. aegypti* resistance in Indonesia. The possibility of employing the CDC method will improve the sensitivity and efficacy of testing, resulting in more accurate recommendations than the WHO method does. Molecular data analysis is required to confirm the possibility of lasting resistance to target gene alterations.

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CONFLICT OF INTEREST DISCLOSURE

The authors state that they have no conflicts of interest.

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