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Resistance of *Aedes aegypti* (Diptera: Culicidae) Populations to Deltamethrin, Permethrin, and Temephos in Cambodia

Sébastien Boyer, PhD¹ , Sergio Lopes, Msc², Didot Prasetyo, PhD³, John Hustedt, Msc², Ay Sao Sarady, Msc², Dyna Doum, Msc², Sony Yean, Msc¹, Borin Peng, Msc¹, Sam Bunleng, Msc⁴, Rithea Leang, PhD⁴, Didier Fontenille, PhD¹, and Jeffrey Hii, PhD⁵

Abstract

Dengue fever is a major public health concern, including 185,000 annual cases in Cambodia. *Aedes aegypti* is the primary vector for dengue transmission and is targeted with insecticide treatments. This study characterized the insecticide resistance status of *Ae aegypti* from rural and urban locations. The susceptibility to temephos, permethrin, and deltamethrin of *Ae aegypti* was evaluated in accordance with World Health Organization instructions. All the field populations showed lower mortality rate to temephos compared with the sensitive strain with resistance ratio 50 (RR_{50}) varying from 3.3 to 33.78 and RR_{90} from 4.2 to 47 compared with the sensitive strain, demonstrating a generalized resistance of larvae to the temephos in Cambodia. *Ae aegypti* adult populations were highly resistant to permethrin regardless of province or rural/urban classification with an average mortality of 0.02%. Seven of the 8 field populations showed resistance to deltamethrin. These results are alarming for dengue vector control, as widespread resistance may compromise the entomological impact of larval control operations. Innovative vector control tools are needed to replace ineffective pesticides in Cambodia.

Keywords

Aedes aegypti, Cambodia, insecticide, mosquito, resistance, vector control

Introduction

Dengue fever is a major public health concern, with estimates of 400 million cases every year in urban, suburban, and rural tropical areas.¹ In Cambodia, around 185,000 cases are estimated

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annually.² The primary vector for dengue transmission is *Aedes aegypti*, which favors environments where water storage is abundant and solid waste disposal is deficient.³ As *Ae aegypti* is implicated in the transmission of arboviruses such as Zika, chikungunya, and yellow fever,⁴ vector control strategies that target *Ae aegypti* populations may have an major public health impact. Many insecticides have been used in order to control *Ae aegypti* populations, but little information exists on the susceptibility of Cambodian populations to the most commonly used insecticides.

As early as 1955, DDT (dichlorodiphenyltrichloroethane) residual spray was used in the first malaria eradication pilot in Snuol district.⁵ DDT was again used in public health programs targeting malaria and dengue in urban and rural areas and at UNHCR refugee camps along the Cambodia-Thailand border from 1981 to 1987, after which it was no longer imported.⁶ Pyrethroids, particularly permethrin and deltamethrin, were introduced to Cambodia in the late 1980s and 2000 for the control of malaria (impregnation of bednets) and dengue (thermal fogging and ULV [ultra-low volume] sprays), respectively.⁶ Since 1992, temephos has been imported with roughly 200 tons per year used mainly for larval control of dengue vectors.⁶ In 1966, Mouchet and Chastel⁷ showed total susceptibility of *Ae aegypti* to DDT, fenthion, malathion, and diazinon insecticides, but observed resistance to dieldrin and γ -HCH (γ -hexachlorocyclohexane). More recently, *Ae aegypti* resistance to temephos was also investigated during 2 field studies in Cambodia.⁸ The resistance pattern and future of temephos is increasingly important as this larvicide has been the main dengue control strategy used by National Dengue Control Program (NDCP) for more than 20 years and for biannual larvicide campaigns since 2001.^{3,6}

Using the World Health Organization (WHO) diagnostic dose (0.02 mg/L), the Phnom Penh population tested in 2001 was found to be resistant to temephos, while Kampong Cham population was still susceptible. More recently, among 7 *Ae aegypti* populations, 6 were found to be resistant to temephos with mortality ranging from 11.02% up to 88.62% at the WHO diagnostic concentration (To Setha, personal communication). While it seems clear that temephos resistance among *Ae aegypti* populations has increased over time in Cambodia, the patterns between rural and urban areas are as delineated.

While pyrethroid and organophosphate insecticides are used in the national malaria and dengue control programs, significant use of insecticides (including larvicides, repellents, space sprays, treated materials, and coils) at home and in the private sector results in unquantifiable use of insecticides. Coupled with the lack of information on adult resistance status in Cambodia and long-term usage of space spraying by pest control companies and public health authorities, the need for characterizing the susceptibility of *Ae aegypti* to pyrethroids is urgent. This study aims to characterize the insecticide resistance status for immature and adult stages of *Ae aegypti* collected from rural and urban Cambodian environment. Eight field populations were tested using WHO test procedures against the most commonly used insecticides in Cambodia, which include temephos (for immature stages) and deltamethrin/permethrin (for adult stages).

Material and Methods

Mosquito Collection

Four different geographical areas in Cambodia were selected for field sample collections (Phnom Penh, Kampong Cham, Battambang, and Siem Reap). Two urban villages and 2 rural villages were selected as collection points within each village. Villages were selected by NDPC according to geographical representation, dengue incidence, and recent use of temephos (within the previous 2 years) (Supplementary File 1, available in the online version of the article). Twenty-five households were randomly selected within each village and all containers were inspected for larvae and pupae using direct pipetting for small containers and sweep net method for large

containers.⁹ Collected larvae/pupae were pooled by location (rural/urban) in each province and transported to an insectary.

Larvae and pupae were reared in standard conditions (temperature, $28^{\circ}\text{C} \pm 1^{\circ}\text{C}$; relative humidity, $75\% \pm 2.5\%$; photoperiod, 12 hours day/night) in $24.8 \times 19.7 \times 3.8$ cm standard white plastic larval tray containing 2 L of purified water and fed with half a teaspoon of grounded fish food daily until adult emergence. Adult *Aedes* were separated from other species by direct aspiration and each population was separated by location (total of 8 populations from 4 provinces).

For both larvae and adult assays, a USDA (United States Department of Agriculture) reference susceptible strain¹⁰ was used as positive and negative control with water and ethanol in plastic beakers.

Rearing of F1 Larvae for Testing

Adult *Aedes* mosquitos from parental generations were reared at standard conditions and fed with 10% sucrose solution. All populations were also provided with lab-reared mice for blood meal once every 3 days for 3 to 4 hours. Eggs from the F1 generation were collected on white filter paper and placed inside black plastic cups. Eggs were dried and stored in envelopes and later sent to the laboratory. F1 eggs were immersed in water according to assay needs for testing procedures and larvae were reared as previously described.

Aedes aegypti Larval Bioassays

In accordance with WHO instructions,¹¹ late third instar larvae of F1 generation were used for determining the resistance of mosquito larvae to temephos.

Temephos (Sigma, Pestanal analytical grade, 250 mg) was diluted in ethanol to produce a stock solution of 1000 mg/L. The main stock solution was diluted into several working concentrations better suited for testing. All solutions were stored in glass bottles and labeled accordingly. To obtain each of these concentrations the adequate volume of temephos was pipetted from stock solutions, adding the remaining amount of solution with ethanol into each beaker containing 99 mL of water. Four replicates were used for every concentration, and each replicate consists of 25 larvae.

Six temephos concentrations (0.2, 0.05, 0.03, 0.02, 0.01, 0.004 mg/L) were used to determine lethal concentration (LC) 50/95 (eg, the necessary concentrations needed to kill 50%/95% of mosquito larvae). Resistance ratios (RR_{50} and RR_{95}) were calculated dividing LC_{50} and LC_{95} rates from *Ae aegypti* field populations by the LC_{50} and LC_{90} rates of the USDA susceptible strain.

Ae. aegypti Adult Bioassays

Insecticide resistance screening for adult mosquitos was conducted using the WHO tube assay.¹¹ Two synthetic pyrethroids; permethrin and deltamethrin, at diagnostic concentrations appropriate for *Aedes* mosquitos were used. WHO tube kit and impregnated permethrin (0.25%), deltamethrin (0.03%), and piperonyl butoxide for synergist assay (PBO 4%) papers were obtained from Vector Control Research Unit at the University of Science, Penang, Malaysia. Diagnostic and synergist concentrations were chosen following WHO recommendations.¹¹

For this bioassay, each tested population used 4 tubes containing permethrin (0.25%), 4 tubes containing deltamethrin (0.03%), and 4 control tubes containing silicone oil paper. Twenty-five adults at least 3 days old and non-blood-fed female mosquitos were introduced into each tube lined with untreated paper (holding tube) for 60 minutes. Mosquitos were then transferred into the exposure tube and exposed to impregnated paper for 60 minutes. Mosquito knock down (KD) was measured at the end of the exposure, after which mosquitos were transferred back to the

Table 1. Mean Lethal Concentration (LC)₅₀ and LC₉₀ (±SE) of 8 *Aedes aegypti* Larval Populations With Temephos in Cambodia.^a

| Environment | Populations ^b | LC ₅₀ (SE) | RR ₅₀ | LC ₉₀ (SE) | RR ₉₀ |
|-------------|--------------------------|-----------------------|------------------|-----------------------|------------------|
| Urban | Phnom Penh | 0.020 (0.0006) | 5.4 | 0.028 (0.0008) | 6.0 |
| | Siem Reap | 0.014 (0.0008) | 3.8 | 0.020 (0.0008) | 4.2 |
| | Kampong Cham | 0.031 (0.0012) | 8.4 | 0.052 (0.0025) | 11.1 |
| | Battambang | 0.125 (0.0044) | 33.8 | 0.221 (0.0082) | 47.0 |
| Rural | Phnom Penh | 0.014 (0.0007) | 3.8 | 0.031 (0.0011) | 6.6 |
| | Siem Reap | 0.012 (0.0006) | 3.3 | 0.021 (0.0010) | 4.4 |
| | Kampong Cham | 0.048 (0.0015) | 13.0 | 0.066 (0.0029) | 14.0 |
| | Battambang | 0.041 (0.0015) | 11.1 | 0.064 (0.0031) | 13.6 |

^aRR₅₀ and RR₉₀ represent the resistance ratio of the field populations compared with the US Department of Agriculture (USDA) susceptible reference strain.

^bUSDA strain: LC₅₀ = 0.0037 ± 0.00008 mg/L; LC₉₀ = 0.0047 ± 0.0001 mg/L.

tube without insecticide. Mortality was counted at the end of a 24-hour period and the resistance status was interpreted according to the WHO protocol.

Insecticide-synergist assay using PBO was conducted to measure the effect of preexposure to a synergist on the expression of insecticide resistance. Adult *Aedes* were preexposed to this synergist for 1 hour before exposure to insecticide. KD and mortality were recorded the same way as standard tests.

Data Management and Statistical Analysis

KD and mortality were registered at 1 and 24 hours postexposure, respectively. RRs for larvae and adult mosquitos were calculated by dividing the average mortality found in each field population by the mortality obtained with the USDA susceptible reference strain.

For larvae results, LC₅₀ and LC₉₀ were obtained by plotting the mortality using log probit analysis.

Statistical analysis (analysis of variance and mean comparison) were completed to compare the mortality of adults to permethrin and deltamethrin with or without the use of PBO. Graphs and data analysis were done with R software.¹²

Results

Larval Bioassays

The overall bioassay results for larvae are presented in Table 1. The highest LC₅₀ and LC₉₀ values were obtained with Battambang urban populations (LC₅₀ = 0.125 ± 0.004 mg/L and LC₉₀ = 0.221 ± 0.008 mg/L) and Kampong Cham (Table 1). In Phnom Penh and Siem Reap, the LC₅₀ and LC₉₀ were lowest with LC₅₀ values ranging between 0.012 mg/L (Siem Reap rural) and 0.020 mg/L (Phnom Penh rural).

The RR for urban and rural populations of Siem Reap and Phnom Penh provinces were mostly above the threshold, which is defined as a resistant population with RR ≥ 5. RR values of Kampong Cham and Battambang urban and rural populations were 2- and 9-fold higher than the threshold, respectively. While these results may be linked to the continued distribution of temephos and consequent exposure of populations to this chemical, it is of great concern that 2 out of 4 populations in these 2 provinces registered RRs twice as high as the defined resistance threshold (Kampong Cham Rural, RR = 13.0; Battambang rural, RR = 11.2) and 1 province registered an RR 6 times higher than the defined threshold (Battambang urban, RR = 33.6).

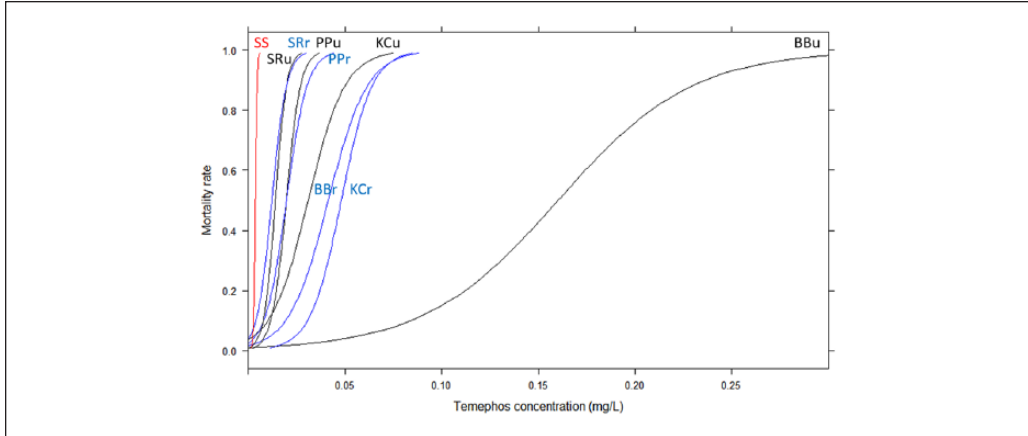


Figure 1. Mortality rate of *Aedes aegypti* larvae to temephos in the 4 provinces. The 4 urban populations are represented in black, the rural populations in blue. The red line is the sensitive strain (SS). BB, Battambang; KC, Kampong Chan; SR, Siem Reap; PP, Phnom Penh. The lowercase letters “r” and “u” represent rural and urban areas, respectively.

Higher lethal doses (LC_{50} or LC_{90}) are needed to kill *Ae. aegypti* larvae from Battambang and Kampong Cham populations as depicted on the four mortality curves on the right side of the graph compared to Siem Reap and Phnom Penh populations (Figure 1). Finally, all the field populations showed higher mortality curve patterns compared with the sensitive strain over a range of concentrations (Figure 1).

Adult Bioassays

Results showed a very high level of resistance to permethrin regardless of province or rural/urban classification (Figure 2; Supplementary File 2, available in the online version of the article). The average mortality to permethrin at the WHO diagnostic dose is $2.22\% \pm 0.02\%$ for all the populations. While all populations showed resistance to permethrin, six of the eight populations showed no mortality to permethrin at all. The additional 2 Kampong Cham populations had 1.1% and 3.9% of mortality. Adult bioassays showed a significant difference in mortality to permethrin depending on the population and the presence of PBO ($F = 3.35$; $df = 8$; $P = .003$), particularly a significant increase in mortality from 1.1% to 18.6% in rural population from Kampong Cham province (Supplementary File 2).

Seven of the 8 field populations had a percentage less than 90% of mortality due to deltamethrin, meaning that these populations are resistant. The average mortality of *Ae aegypti* populations from Phnom Penh and Siem Reap provinces ranged between 4.0% and 8.3% only. A significant difference in mortality to deltamethrin among the five highest mortality populations (>52%) tested were observed in the presence of PBO ($F = 7.20$; $df = 8$; $P < .0001$).

Discussion

Resistance to Temephos: Implications for Public Health

Observed *Ae aegypti* resistance to temephos is consistent with a recent study where 6 of 7 populations showed similar resistance in Cambodia (To Setha, personal communication). The RR_{50} range of the 8 populations to temephos between 3.8 and 33.6 reflects the intensity of insecticide control. In Thailand, despite mosquito resistance to deltamethrin and permethrin, temephos is

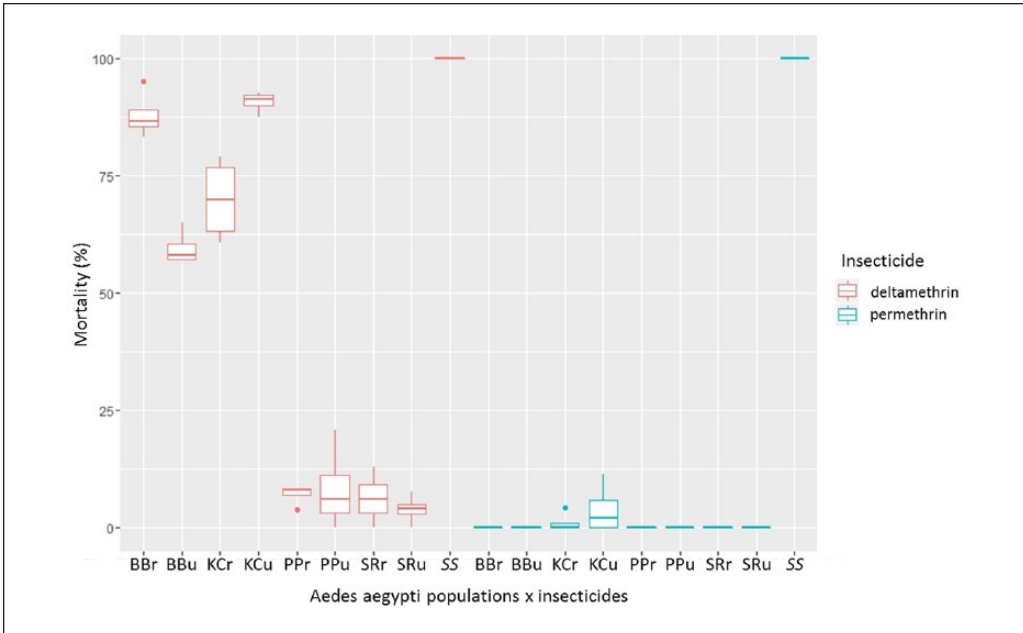


Figure 2. Mortality of *Aedes aegypti* populations to deltamethrin (0.03%) and permethrin (0.25%) following recommended WHO diagnostic doses. BB, Battambang; KC, Kampong Cham; PP, Phnom Penh; SR, Siem Reap; SS USDA sensitive strain. The lowercase letters “r” and “u” represent rural and urban areas, respectively.

still an effective insecticide to control *Ae aegypti* larvae.¹³ On the basis of data showing temephos resistance in Phnom Penh over 17 years,⁸ a review of prevention and control strategies should be conducted and highlight the effects of reliance on a single method of control (eg, high levels of temephos use in Cambodia¹⁴ may compromise the entomological impact of larval control operations).

Bacillus thuringiensis var *israelensis* (Bti) was tested with success in 2005 around Phnom Penh.¹⁵ A new Bti strain AM65-52 was tested in 2016 against *Ae aegypti* field population from Kandal province that was resistant to temephos. Results showed a reduction in the number of pupae over 13 weeks, with an average 70% reduction during the first 8 weeks.¹⁶ The use of the *Poecilia reticulata* (guppy) fish to control *Aedes* populations in water storage was tested in 2008 and after 1 year, a 79% reduction in *Aedes* larvae in community was observed with a presence of guppies in only 57% of the containers.¹⁷ In 2008, a new formulation of pyriproxifen was tested in water containers against *Ae aegypti* in Phum Thmei near Phnom Penh.¹⁸ The study identified an inhibition of adult emergence in treated jars reaching 90% for 20 weeks, and remaining >80% until the end of the study (34 weeks). In Kampong Cham Province in 2008 water jars were covered with long-lasting insecticide net Permanet 2.0 (insecticide = deltamethrin) without significant reductions in mosquitoes,¹⁷ possibly explained by the strong resistance to deltamethrin that we observed in *Ae aegypti* adults. A large-scale randomized trial comparing guppy and COMBI (Communication for Behavioral Impact) in Kampong Cham showed 92.5% reduction in larval-positive containers and 76% to 88% coverage with guppies after 1 year. A recently completed cluster randomized control trial showed that an integrated vector management approach using guppy fish (*Poecilia reticulata*), a new slow release pyriproxifen matrix (Sumilarv 2MR), and community engagement through a clear COMBI strategy reduced indoor adult density roughly 50% as compared with the control arm.¹⁹ All these methods focused on key containers, especially

water cement jars that produced approximately 95% of *Ae aegypti* larvae and pupae⁹ and should be considered in Cambodia as a cost-effective replacement of temephos.

Resistant to Permethrin but Susceptible to Deltamethrin

Ae aegypti deltamethrin-resistant populations have been described in different countries in Asia,²⁰ Latin America,²¹ Africa,²² Oceania,²³ and the Caribbean.²⁴ In our study, *Ae aegypti* populations were either totally resistant to deltamethrin (with 2 populations exhibiting 0 mortality) or had tolerance patterns. Recently, the same pattern was observed in Thailand where *Ae aegypti* F1 females were susceptible to deltamethrin, but resistant to permethrin.¹³ A substantial geographic variation exist to pyrethroid resistance, with lower adult resistance levels in Asia, Africa, and the United States. However, there is 250-fold resistance to deltamethrin in Thailand.²⁵

In this study, an extremely strong resistance to permethrin was observed both with and without PBO, which seems to indicate that the resistance is already fixed. Comparatively, the result with deltamethrin and deltamethrin + PBO suggests the involvement of detoxifying enzymes. However, generally multiple resistance between pyrethroids are possible and it can be expected that there is a *kdr* mutation for resistance in both insecticides. As the mechanisms of resistance between permethrin and DDT are expected to be the same, via a *kdr* mutation,²⁶ the already existing DDT resistance⁷ may explain the current fixed resistance observed with permethrin. There are several *kdr* mutations common in *Aedes* species that synergize with each other when they are associated.²⁷ Heterozygous V1016G, and F1534F and F1534C mutants were found in Thailand,²⁸ and the same mutation was also described southern China with V1016G mutants.²⁹ There is substantial variation in *kdr* in the Southeast Asian region that has effects on resistance (arising from different combinations of 3 mutations—S989P, V1016G, and F1534C—in *Ae aegypti*). Although there are other mutations detected in *Ae aegypti*, they do not appear to have effect on resistance based on current evidence. For example, combinations of F1534, C1534C, V1016G, and S989P²⁹ are present in Cambodia and may act together with metabolic resistance. The resistance patterns to deltamethrin and permethrin in the Cambodian villages fit with the variation in frequencies of the three mutations and especially in low 989/1016 but high 1534 in permethrin (but not deltamethrin) resistant locations, but higher 989/1016 in Phnom Penh and Siem Reap (perhaps in combination with 1534).

Our results question the resistance mechanisms. Indeed, the absence of correlation between permethrin and deltamethrin may involve different effects induced by type I pyrethroid (permethrin) and a pseudo pyrethroid (nonester pyrethroid; deltamethrin), and so different resistance mechanisms.³⁰

Limitations and Conclusion

We acknowledge the lack of baseline data on temephos distribution in the villages sampled. While temephos distribution has been acknowledged as the main outbreak response tool in Cambodia,³ the timing and concentrations used in the villages sampled in this study were not discriminated. Hence, we cannot fully characterize the existing preconditions of each village in terms of previous larviciding activities, but temephos distribution is organized annually at a national and province scales. Likewise, pyrethroid based interventions like thermal fogging, long-lasting insecticide nets usage and pyrethroid-based aerosol spray use was not characterized during field collection, limiting the possibility to ascertain potential drivers for the resistance patterns registered.

Nevertheless, our results and those of neighboring countries are alarming. From a regional point of view, it seems essential to rapidly change control methods and replace temephos with

another larvicide that remains to be determined. Finally, and perhaps most worrying, it seems that in the event of an epidemic the adulticides used in the Southeast Asia region are no longer effective. We must quickly find an alternative.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

The supplementary material for this article is available online.

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