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Resistance of Aedes aegypti (Diptera: Culicidae) populations to deltamethrin, permethrin and temephos in Cambodia

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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 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 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 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 Chastel showed total susceptibility of *Ae. aegypti* to DDT, fenthion, malathion and diazinon insecticides, but observed resistance to dieldrin and gamma HCH[7]. More recently, *Ae. aegypti* resistance to temephos was also investigated during two 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 WHO diagnostic dose (0.02mg/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 seven *Ae. aegypti* populations, six were found to be resistant to temephos with mortality ranging from 11.02% up to 88.62% at the WHO diagnostic concentration (To Setha, Pers. Comm.). While it seems clear that 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 two rural villages were selected as collection points within each village. Villages were selected by NDCP according to geographical representation, dengue incidence and recent use of temephos (within the previous two years) (Supplementary File 1). Twenty five households were randomly selected within each village and all

62 containers were inspected for larvae and pupae using direct pipetting for small containers and sweep
63 net method for large containers[9]. Collected larvae/pupae were pooled by location (rural/urban) in each
64 province and transported to an insectary.

65 Larvae and pupae were reared in standard conditions (temperature: 28±1°C; relative humidity: 75 ±
66 25%; photoperiod: 12 hours day/night) in 24.8 x 19.7 x 3.8 cm standard white plastic larval tray
67 containing 2 liters of purified water and fed with half a teaspoon of grounded fish food daily until adult
68 emergence. Adult *Aedes* were separated from other species by direct aspiration and each population
69 was separated by location (total of 8 populations from 4 Provinces).

70 For both larvae and adults assays, a USDA reference susceptible strain[10] was used as positive and
71 negative control with water and ethanol in plastic beakers.

72 <u>Rearing of F1 larvae for testing</u>

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

79 <u>*Ae. aegypti* larval bioassays</u>

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

82 Temephos (Sigma, Pestanal analytical grade, 250 mg) was diluted in ethanol to produce a stock solution
83 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 (e.g. the necessary concentrations needed to kill 50%/95% of mosquito larvae). Resistance ratios (RR50 and RR95) 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* mosquitoes 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 four tubes containing Permethrin (0.25%), four tubes containing deltamethrin (0.03%), and four control tubes containing silicone oil paper. Twenty-five adults at least 3 days old and non-blood fed female mosquitoes were introduced into each tube lined with untreated paper (holding tube) for 60 minutes. Mosquitoes 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 mosquitoes were transferred back to the tube without insecticide. Mortality was counted at the end of a 24 hours period and the resistance status was interpreted according to the WHO protocol.

Insecticide-synergist assay using piperonyl butoxide (PBO) was conducted to measure the effect of pre-exposure to a synergist on the expression of insecticide resistance. Adult *Aedes* were pre-exposed to this synergist for one hour before exposure to insecticide. KD and mortality were recorded the same way as standard tests.

Data management and statistical analysis

Knock down and mortality were registered at 1 hour and 24 hours post-exposure 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 (ANOVA 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 (LC_{50}=0.125±0.004 mg/L and LC_{90}= 0.221±0.008 mg/L) and Kampong Cham (Table 1). Phnom Penh and Siem Reap, the LC_{50} and LC_{90} were lowest with LC_{50} values comprised 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 two and nine-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 two provinces registered RRs twice as high as the defined resistance threshold (Kampong Cham Rural, RR=13.0; Battambang rural, RR=11.2) and one registered a RR 6 times higher than the defined threshold (Battambang urban, RR=33.6).

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). Lastly all the field populations showed higher mortality curve patterns compared to the sensitive strain over a range of concentrations (Figure 1).

<u>Adult bioassays</u>

Results showed a very high level of resistance to permethrin regardless of province or rural/urban classification (Figure 2; Supplementary File 2). The average mortality to permethrin at the WHO diagnostic dose is 2.22% ± 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 two 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 ($F=3.35$; $df=8$; $p=0.003$), 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 eight field populations had a percentage below 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 ($F=7.20$; $df=8$; $p<0.0001$).

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, pers. comm.). The RR50 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 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 (e.g. high levels of temephos use in Cambodia[14] 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 8 first weeks[16]. The use of the *Poecilia reticulate* (guppy) fish to control *Aedes* populations in water storage was tested in 2008 and after one 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 above 80% until the end of the study (34 weeks). In Kampong Cham Province in 2008 water jars were covered with LLIN Permanet 2.0 (insecticide = deltamethrin) without significant reductions in mosquitoes[17], possibly explained by the strong resistance to delamethrin that we observed in *Ae. aegypti* adults. A large-scale randomized trial comparing guppy and COMBI (Communication for Behavioural Impact) in Kampong Cham showed 92.5 % reduction in larval-positive containers and 76%-88% coverage with guppies after one year. A recently completed cluster randomized control trial showed that an integrated vector management approach using guppy fish

(*Poecilia reticulata*), a new slow release pyriproxyfen matrix (Sumilarv® 2MR), and community engagement through a clear Community for Behavioral Impact (COMBI) strategy reduced indoor adult density roughly 50% as compared to the control arm[19]. All of these methods focused on key containers, especially water cement jars that produced approximately 95% of *Ae. aegypti* larvae and pupae[9] and should be considered in Cambodia as a cost-effective replacement of temephos.

<u>Resistance to permethrin but susceptible to deltamethrin</u>

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, *Aedes aegypti* populations were either totally resistant to deltamethrin (with two populations exhibiting zero 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 USA. However there is 250-fold resistance to deltamethrin in Thailand[25].

In this study, an extremely strong resistance to permethrin was observed both with/without PBO which seems to indicate that the resistance is already fixed. Comparatively, the result with deltamethrin and deltamethrin + PBO suggest 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-resistance[7] 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 three 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, S989P[29] 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 pre-conditions 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 (LLIN) 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.

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319 Table 1. Mean Lethal Concentration (LC) 50 and LC90 (± SE) of 8 *Aedes aegypti* larval populations with
320 temephos in Cambodia. RR50 and RR90 represent the resistance ratio of the field populations compared
321 to the USDA susceptible reference strain. [a] USDA strain: LC50 = 0.0037 ± 0.00008 mg/L ; LC90= 0.0047 ±
322 0.0001mg/L

Environment	Populations [a]	LC50 (SE)	RR50	LC90 (SE)	RR90
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

323

Supplementary File 2. Percentage of mortality (± SE) of 8 *Aedes aegypti* adult populations to Deltamethrin and Permetrhin. In bold are represented the significant differences of mortality between bioassays with and without PBO.

Ae. aegypti populations	Without PBO		With PBO	
	Deltamethrin	Permethrin	Deltamethrin; p-value	Permethrin; p-value
Battambang rural	88.0 (5.1)	0.0 (0.0)	**97.8 (2.6); 0.014**	3.3 (6.5); 0.355
Battambang urban	59.6 (3.7)	0.0 (0.0)	**80.6 (9.2); 0.006**	1.2 (2.4); 0.355
Kampong Cham rural	70.0 (8.9)	1.1 (2.2)	71.8 (15.6); 0.844	**18.6 (4.4); 0.0003**
Kampong Cham urban	90.8 (2.3)	3.9 (5.4)	**98.8 (2.4); 0.003**	7.5 (3.4); 0.300
Phnom Penh rural	7.1 (2.1)	0.0 (0.0)	9.9 (7.6); 0.509	0.0 (0.0); -
Phnom Penh urban	8.3 (9.0)	0.0 (0.0)	7.3 (7.1); 0.867	0.0 (0.0); -
Siem Reap rural	6.3 (5.6)	0.0 (0.0)	**52.3 (12.6); 0.0006**	0.0 (0.0); -
Siem Reap urban	4.0 (3.1)	0.0 (0.0)	**24.6 (16.8); 0.047**	0.0 (0.0); -
USDA Sensitive Strain	100 (0.0)	100 (0.0)	100 (0.0); -	89.4 (21.2); 0.355

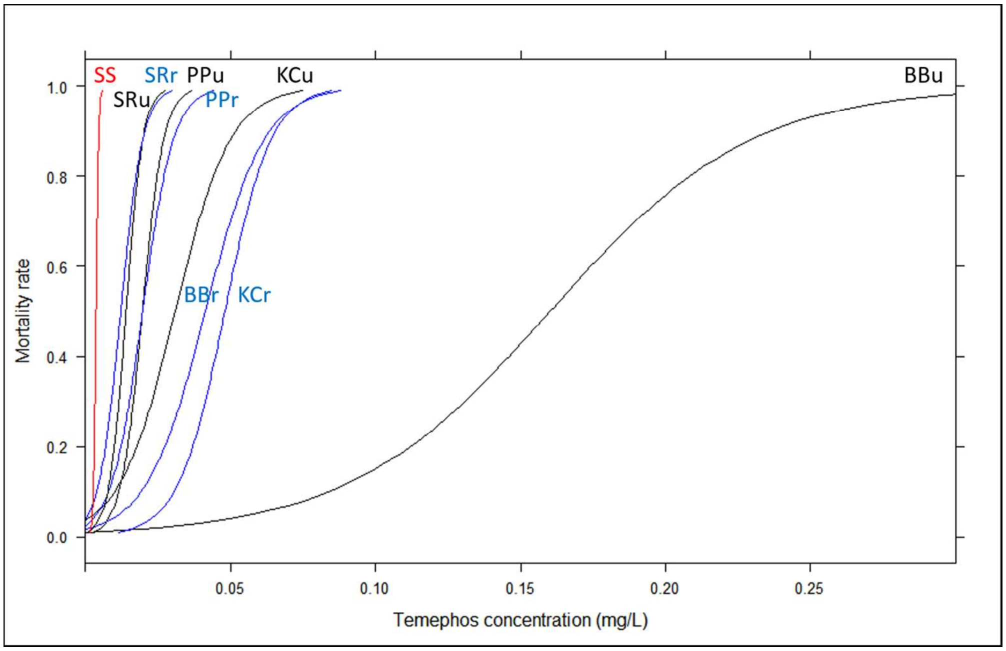

Figure 1. Mortality rate of Aedes aegypti larvae to tempehos in the 4 provinces. The 4 urban populations are represented in black, the rural populations in blue. The red line is the Sensitive strain (SS). BB Battambang, KC Kampong Chan, SR Siem Reap, PP Phnom Penh. The small letters - "r" and "u" represent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

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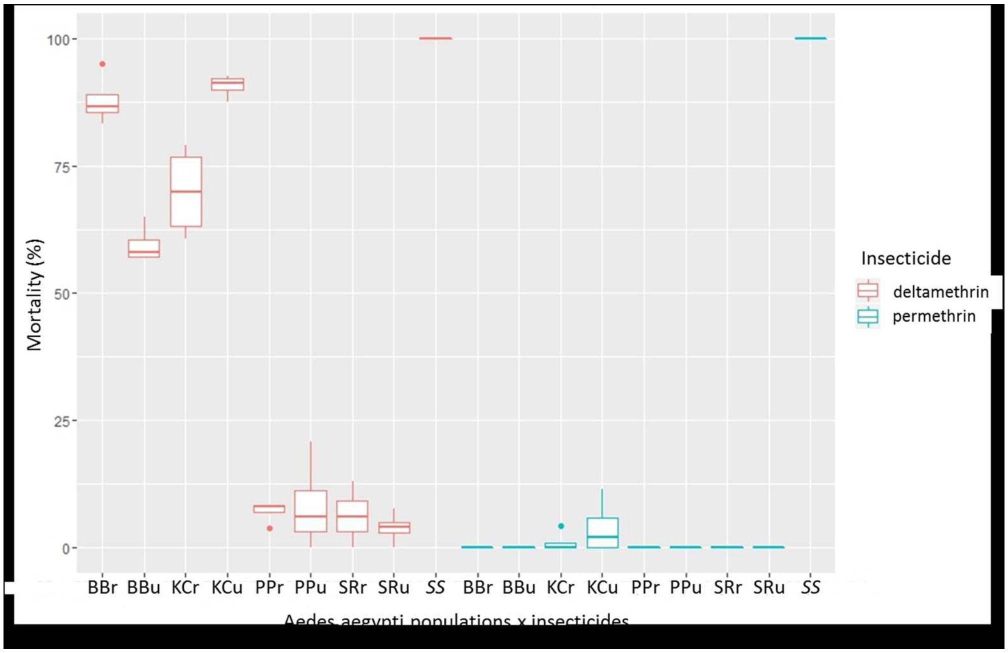

Figure 2. Mortality of Aedes aegypti populations to Deltamethrin and Permethrin. BB represents Battambang; KC Kampong Cham; PP Phnom Penh; SR Siem Reap; SS USDA Sentitive Strain. The small letters - “r” and “u” represent rural and urban areas, respectively.

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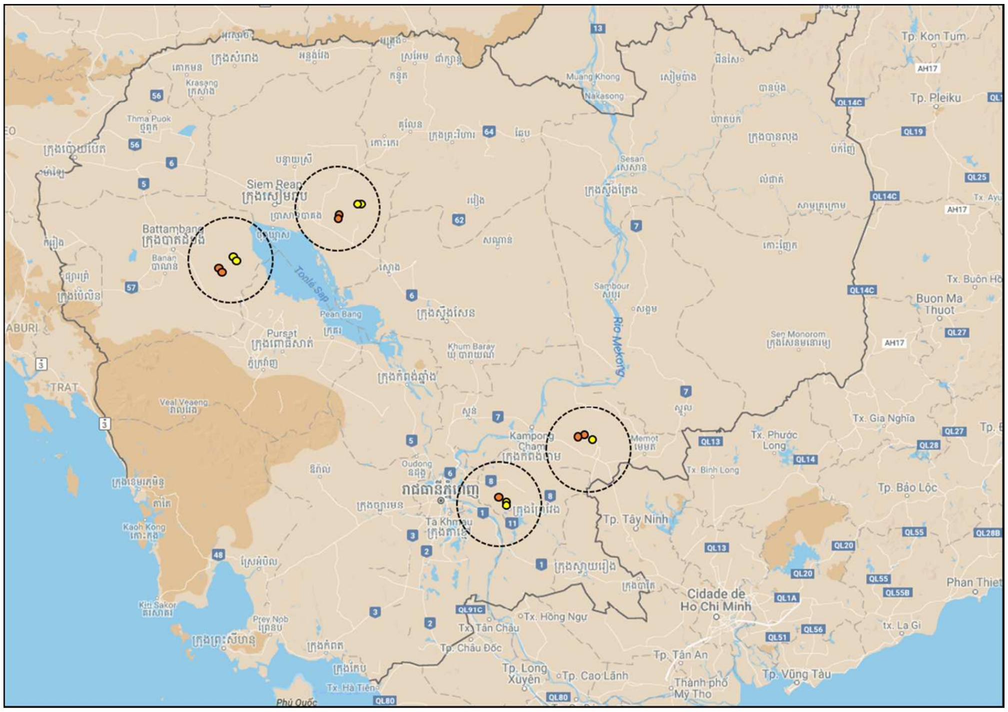

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Figure 1. Mortality rate of Aedes aegypti larvae to tempehos in the 4 provinces. The 4 urban populations are represented in black, the rural populations in blue. The red line is the Sensitive strain (SS). BB Battambang, KC Kampong Chan, SR Siem Reap,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

Figure 2. Mortality of Aedes aegypti populations to Deltamethrin (0.03%) and Permethrin (0.25%) following recommended WHO diagnostic doses. BB represents Battambang; KC Kampong Cham; PP Phnom Penh; SR Siem Reap; SS USDA Sensitive Strain. The small letters - "r" and "u" represent rural and urban areas, respectively.

Supplementary File 1. Location of collection sites in the 4 different provinces in Cambodia: Battambang, Siem Reap, Kampong Cham and Phnom Penh. The orange and yellow circles represent field collections in rural and urban areas, respectively.

Resistance of *Aedes aegypti* (Diptera: Culicidae) populations to deltamethrin, permethrin and temephos in Cambodia.

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 WHO instructions. All the field populations showed lower mortality rate to temephos compared to the sensitive ~~strains~~strain with Resistance Ratio 50 (RR50) varying from 3.3 to 33.78 and RR90 from 4.2 to 47 compared to 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 eight 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 annually.[2] The primary vector for dengue transmission is *Aedes aegypti* ~~that~~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 residual spray was used in the first malaria eradication pilot in Snuol district[5] ~~followed by~~. 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 ~~during~~from 1981 to 1987, after which ~~DDT~~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 sprays), respectively[6]. Since 1992, Temephos ~~was~~has been imported ~~from 1992 to present~~ with roughly 200 tons per year used mainly for larval control of dengue vectors[6]. In 1966, Mouchet and Chastel showed total susceptibility of *Ae. aegypti* to DDT, fenthion, malathion and diazinon insecticides, but observed resistance to dieldrin and gamma HCH[7]. More recently, *Ae. aegypti* resistance to temephos was also investigated during two 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 WHO diagnostic dose (0.02mg/L), the Phnom Penh population tested in 2001 was found to be resistant to temephos ~~(LC50=0.02mg/l and LC95=0.03mg/L),~~, while Kampong Cham population was still susceptible ~~(LC50=0.009mg/l and LC95=0.015mg/L).~~. More recently, among seven *Ae. aegypti* populations, six were found to be resistant to temephos with mortality ranging from 11.02% up to 88.62% at the WHO diagnostic concentration (To Setha, Pers. Comm.). While it seems clear that that temephos resistance among *Ae. aegypti* populations has increased over ~~last years~~time in Cambodia, the patterns between rural and urban areas are ~~not clear~~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 ~~of~~on adult resistance status in Cambodia and long-term usage of space spraying by pest control companies and public health ~~outbreak response, it is timely to characterize~~authorities, the need for characterizing the susceptibility of *Ae. aegypti* to ~~pyrethroïds~~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. ~~Using WHO test procedures, 8~~Eight field populations were tested ~~with~~using WHO test procedures against the most commonly used insecticides in Cambodia~~:~~ which include temephos (for immature stages~~),~~) and deltamethrin ~~and~~ /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~~. Within each Province, two~~). Two urban villages ~~in an urban setting~~ and two rural villages were selected as collection ~~point.~~points within each village. Villages were selected by ~~Cambodian National Center for Entomology, Parasitology, and Malaria Control (CNM)~~NDCP according to geographical representation, dengue incidence and ~~history of~~ recent use of temephos (within the previous two years) (Supplementary File 1).

~~In each village, 25~~ 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±1°C; relative humidity: 75 ± 25%; photoperiod: 12 hours day/night) in 24.8 x 19.7 x 3.8 cm standard white plastic larval tray containing 2 liters 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 adults assays, a USDA reference susceptible strain[10] was used as positive and negative control with water and ethanol in plastic beakers. ~~LC50 and LC90 results obtained were used to calculate Resistance Ratios (RR).~~

Rearing of F1 larvae for testing~~.~~

Adult *Aedes* ~~mosquito~~mosquitos from parental ~~generation~~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 ~~in~~ every three days for 3-4 hours. Eggs ~~of~~from the F1 generation were collected on white filter paper and placed inside black plastic cups. Eggs were dried and stored in envelops and later sent to the laboratory. F1 eggs were ~~later~~ immersed in water according to assay needs for testing procedures and larvae were reared as previously described.

Ae. 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. ~~Larvae showing any abnormalities were discarded before the experiment.~~

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 ~~other solutions~~ several working concentrations better suited for testing~~, denominated as stock solutions.~~. All solutions were stored in glass bottles and labeled accordingly. ~~To determine Lethal Concentrations 50 and 90 (LC 50, LC 90), six temephos concentrations were used: 0.2, 0.05, 0.03, 0.02, 0.01, 0.004 mg/L.~~ To obtain each of ~~this concentration, we pipetted~~ these concentrations the adequate volume of temephos was pipetted from stock solutions, ~~completing~~adding the remaining ~~value~~amount of solution with ethanol into each beaker containing 99 ml of water. Four replicates were used for every concentration, and each replicate ~~consist~~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 (e.g. the necessary concentrations needed to kill 50%/95% of mosquito larvae). Resistance ratios (RR50 and RR95) 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. ~~*Ae. aegypti* adult bioassays.~~

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aegypti adult bioassays

Insecticide resistance screening for adult ~~mosquito~~mosquitos was conducted using the WHO tube assay[11]. Two synthetic pyrethroids; permethrin and deltamethrin, at diagnostic concentrations appropriate for *Aedes* mosquitoes 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 ~~(VCRU) in~~at the University of Science~~ (USM),~~, Penang, Malaysia. Diagnostic and synergist concentrations were chosen following WHO recommendations[11].

For this bioassay, each tested population ~~use~~used four tubes ~~contained~~containing Permethrin (0.25%), four tubes ~~contained~~containing deltamethrin (0.03%), and four control tubes ~~contained~~containing silicone oil paper. Twenty-five ~~adult with~~adults at least 3 days old and non-blood fed female mosquitoes were introduced into each tube lined with untreated paper (holding tube) for 60 ~~min~~minutes. Mosquitoes were then transferred into the exposure tube and exposed to impregnated paper for 60 ~~min.~~minutes. Mosquito Knock Down (KD) ~~mosquito were counted~~was measured at the end of the exposure, after which mosquitoes were transferred back to the tube without insecticide. Mortality was counted at the end of a 24 hours period and the resistance status was interpreted according to the WHO protocol.

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Insecticide-synergist assay using piperonyl butoxide (PBO) was conducted to measure the effect of pre-exposure to a synergist on the expression of insecticide resistance. Adult *Aedes* were pre~~-~~-exposed to this synergist for one hour before exposure to insecticide. KD and mortality were recorded the same way as standard tests.

Data ~~Management~~management and statistical analysis~~.~~

Knock down and mortality were registered at ~~1h~~1 hour and ~~24h~~24 hours post~~-~~-exposure respectively. RRs for larvae and ~~adults mosquito~~adult mosquitos were calculated by dividing the average mortality found in each field ~~populations~~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. ~~LC50 and LC90 results obtained from field populations were then divided by results obtained in USDA strain to obtain RRs for each field population.~~

Statistical analysis (ANOVA ~~then~~and mean comparison) were ~~realized~~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 ~~with~~ (LC_{50}=0.125±0.004 mg/L and LC_{90}= 0.221±0.008 mg/L~~,~~ ~~followed by~~) and Kampong Cham (Table 1). ~~These two outlying provinces are distant from big urban centers and have experienced large outbreaks and significant outbreak responses. Hence, in~~ Phnom Penh and Siem Reap, the LC_{50} and LC_{90} were lowest with LC_{50} values comprised 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 ~~mainly~~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 two and nine-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 two provinces registered RRs twice as high as the defined resistance threshold (Kampong Cham Rural, RR=13.0; Battambang rural, RR=11.2) and one registered a RR 6 times higher than the defined threshold (Battambang urban, RR=33.6).

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). Lastly all the field populations showed higher mortality curve patterns compared to 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). The average mortality to permethrin at the WHO diagnostic dose is 2.22% ± 0.02~~% ± 0.0002~~ for all the populations. ~~All the~~While all populations showed resistance to permethrin~~;~~, six of the eight populations showed no mortality to permethrin ~~while the 2~~at all. The additional two 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*=0.003), particularly a significant increase in mortality from 1.1% to 18.6% in rural population from Kampong Cham province (Supplementary File 2).

~~With deltamethrin, seven~~Seven of the eight field populations ~~have~~had a ~~mortality~~ percentage below 90% of mortality due to deltamethrin, meaning that these populations are resistant ~~to deltamethrin~~. 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*<0.0001).

Discussion

Resistance to temephos: ~~implication~~implications for ~~Public Health~~public health

Observed *Ae. aegypti* resistance to temephos is ~~fully~~ consistent with a recent ~~analysis among~~study where 6 of 7 populations ~~showing~~showed similar resistance in Cambodia (To Setha, pers. comm~~.): the RR (at LC50)~~.). The RR50 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 still an effective insecticide to control *Ae. aegypti* larvae[13]~~-~~. On the basis of data showing ~~-~~temephos resistance in Phnom Penh ~~since~~over 17 years[8], ~~we suggest~~ a review of prevention and control strategies~~,~~ should be conducted and highlight the effects of reliance ~~of~~on a single method of control~~; i.~~ (e.~~-~~g. high levels of temephos ~~which is the most widely used larvicide to control~~ use in Cambodia[14]~~*Ae. aegypti*~~[14]~~,~~ may compromise the entomological impact of larval control operations~~.~~).

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Bacillus thuringiensis var. *israelensis* (Bti) was tested with success in 2005 around Phnom Penh[15]. ~~In 2016, a~~A new *Bti* strain AM65-52 was tested in 2016 against ~~Kandal~~ *Ae. aegypti* field population from Kandal province that was resistant to temephos~~, with~~ . Results showed a reduction in the number of pupae over 13 weeks, with an average 70% reduction during ~~13~~the 8 first weeks[16]. The use of the *Poecilia reticulate* (guppy) fish to control *Aedes* populations in water storage was tested in 2008 and after one year~~,~~ a 79% reduction ~~of~~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]. ~~Their main result was~~The study identified an inhibition of adult emergence in treated jars reaching 90% for 20 weeks, and remaining above 80% until the end of the study (34 weeks). In ~~2008, in~~ Kampong Cham ~~province~~Province in ~~Cambodia,~~2008 water jars were covered with LLIN Permanet 2.0 (insecticide = deltamethrin) without significant ~~reduction~~[17]reductions in mosquitoes[17], possibly explained by the strong resistance to delamethrin that we observed in *Ae. aegypti* adults. A large-scale randomized trial comparing guppy and COMBI (Communication for Behavioural Impact) in Kampong Cham showed 92.5 % reduction in larval-positive containers and 76%-88% coverage with guppies after one year. A recently completed cluster randomized control trial showed that an integrated vector management approach using guppy fish (*Poecilia reticulata*), a new slow release pyriproxyfen matrix (Sumilarv® 2MR), and community engagement through a clear Community for Behavioral Impact (COMBI) strategy reduced indoor adult density by 83% (pre- versus post-intervention) or 44% (intervention *versus* control arms)[19] . roughly 50% as compared to the control arm[19]. All of these methods focused on key containers, especially water cement jars that produced approximately 95% of *Ae. aegypti* larvae and pupae[9] and should be considered in Cambodia as a cost-effective replacement of temephos.

Resistance to permethrin but susceptible to deltamethrin

Resistance of *Ae. aegypti* to deltamethrin has public health implications. *Aedes aegypti* compare favorably deltamethrin-resistant populations have been described in different countries in Asia[20], Latin America[21], Africa[22], Oceania[23], Caraibes[24]. Surprisingly, we did not observe the same pattern of resistance with permethrin. and the Caribbean[24]. In our study, *Aedes aegypti* populations were either totally resistant to deltamethrin (with two populations exhibiting zero mortality,) or withhad tolerance patterns. Recently, the same pattern was observed in Thailand: where *Ae. aegypti* F1 females were susceptible to deltamethrin, but resistant to permethrin[13]. In a recent review (Smith et al. 2016), there isA substantial geographic variation exist to pyrethroid resistance, with lower adult resistance levels in Asia, Africa and the USA (based on both RRs and % mortality values), although. However there is 250-fold resistance to deltamethrin in Thailand[25].

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In this study, an extremely strong resistance to permethrin was observed both with/without PBO experiment thatwhich seems to indicate that the resistance is already fixed. Comparatively, the resultsresult with deltamethrin and deltamethrin + PBO suggest the involvement of detoxifying enzymes. ButHowever, generally, multiple resistance between pyrethroids are possible, and we could expect the intervention of the 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-resistance[7]~~,~~ 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]. ~~According to the point mutations the resistant phenotype could not be the same according to the pyrethroids.~~ Heterozygous V1016G, and F1534F and F1534C mutants were ~~find~~found in Thailand[28], and the same mutation ~~were~~was also described ~~in South of~~southern China with ~~also~~ V1016G mutants[29]. ~~Whilst there~~There is substantial variation in kdr in the Southeast Asian region~~,~~ that has effects on resistance (arising from different combinations of three mutations~~:~~ - S989P, V1016G and F1534C - in *Ae. aegypti*~~,~~). Although there are other ~~few~~ mutations detected in *Ae. aegypti* ~~but~~ they do not appear to have effect on resistance based on current evidence. For example~~-~~, combinations of F1534 ~~and/or~~, C1534C ~~and / or~~, V1016G ~~and / or~~, S989P[29] are present in Cambodia~~,~~ and ~~acting~~may act together with metabolic resistance. The resistance patterns to deltamethrin and permethrin in the Cambodian villages ~~would~~ 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 ~~Peng~~Penh and Siem Reap~~,~~ (perhaps in combination with 1534~~, though they tend to occur on alternate chromosomes so co-occurrence is uncommon, although this has certainly been observed. These will be key mutations to screen, perhaps with a little sequencing to check whether others are found – the *Ae. aegypti* sodium channel is full of non-synonymous mutations – which is very strongly resistance-associated, and resistance links are yet to be established.~~).

Our results question the resistance mechanisms~~, and even if classically expressed, the multiple resistance is considered as evident when talking about pyrethroids, it shouldn't or requires a different explanation.~~. Indeed~~, for instance~~, the absence of correlation between permethrin and deltamethrin

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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 pre-conditions 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 (LLIN) 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 as well as the results of the neighboring countries are alarming. From a regional point of view, it seems essential to rapidly change control methods based on the use of a larvicide and to replace the temephos with another larvicide that remains to be determined. Finally, and perhaps more worrying, it seems that in the event of an epidemic, the adulticides used in the South East Asia region are no longer effective. We must quickly find an alternative.

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Table 1. Mean Lethal Concentration (LC) 50 ~~(LC50)~~ and LC90 (± SE) of 8 *Aedes aegypti* larval populations with temephos in Cambodia. RR50 and RR90 represent the resistance ratio of the field populations compared to the USDA susceptible reference strain. [a] USDA strain: LC50 = 0.0037 ± 0.00008 mg/L ; LC90= 0.0047 ± 0.0001mg/L

Environment	Populations [a]	LC50 (SE)	RR50	LC90 (SE)	RR90
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

Supplementary File 2. Percentage of mortality (± SE) of 8 *Aedes aegypti* adult populations to Deltamethrin and Permetrhin. In bold are represented the significant differences of mortality between bioassays ~~realized~~ with and without PBO.

Ae. aegypti populations	Without PBO		With PBO		
	Deltamethrin	Permethrin	Deltamethrin; p-value		Permethrin; p-value
Battambang rural	88.0 (5.1)	0.0 (0.0)	**97.8 (2.6~~)~~); 0.014**		3.3 (6.5~~)~~); 0.355
Battambang urban	59.6 (3.7)	0.0 (0.0)	**80.6 (9.2~~)~~); 0.006**		1.2 (2.4~~)~~); 0.355
Kampong Cham rural	70.0 (8.9)	1.1 (2.2)	71.8 (15.6~~)~~); 0.844		**18.6 (4.4~~)~~); 0.0003**
Kampong Cham urban	90.8 (2.3)	3.9 (5.4)	**98.8 (2.4~~)~~); 0.003**		7.5 (3.4~~)~~); 0.300
Phnom Penh rural	7.1 (2.1)	0.0 (0.0)	9.9 (7.6~~)~~); 0.509	~~0.0 (0.0)~~	0.0 (0.0); -
Phnom Penh urban	8.3 (9.0)	0.0 (0.0)	7.3 (7.1~~)~~); 0.867	~~0.0 (0.0)~~	0.0 (0.0); -
Siem Reap rural	6.3 (5.6)	0.0 (0.0)	**52.3 (12.6~~)~~); 0.0006**	~~0.0 (0.0)~~	0.0 (0.0); -
Siem Reap urban	4.0 (3.1)	0.0 (0.0)	**24.6 (16.8~~)~~); 0.047**	~~0.0 (0.0)~~	0.0 (0.0); -
USDA Sensitive Strain	100 (0.0)	100 (0.0)	100 (0.0~~)~~); -		89.4 (21.2~~)~~); 0.355

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