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Short Communication

Significant 24 h recovery and survival of *Culex* mosquitoes exposed to pyrethroid insecticides using the CDC bottle bioassay

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Abstract

Insecticides deployed for mosquito vector control rely on the susceptibility of the targeted vector population for continued efficacy and public health protection. The Centers for Disease Control and Prevention (CDC) bottle bioassay is a standard method used for rapid mosquito insecticide resistance assessment without measuring sublethal or delayed insecticidal effects. Ignored sublethal or delayed mortality effects may affect the accuracy of susceptibility test outcomes and appropriateness of insecticide selection decisions. This study found significant recovery and survival among *Culex* mosquito populations initially immobilized after pyrethroid insecticide exposures using the CDC bottle bioassay. Adult female *Culex* mosquitoes, reared from three different larval collection sites, were exposed to x1 and x2 intensities of deltamethrin and alphacypermethrin insecticides using the CDC bottle bioassay method. Exposed mosquitoes were held for 24 hours for comparison of 30 min exposure time and 24 h post-exposure mortalities. Subsamples of exposed mosquitoes were identified with Polymerase Chain Reaction (PCR) technique. For both x1 ($P \leq 0.009$) and x2 ($P \leq 0.048$) insecticide intensities, the percentage mosquito mortalities at 30 min exposure time (20-24%, 21-27%) reduced significantly after the 24 h post-exposure period (3-9%, 6-13%) respectively. However, after both 30 min ($P \geq 0.475$) and 24 h ($P \geq 0.090$) periods, mosquito mortalities at x2 intensity (21-27%, 6-13%) were not significantly higher than mortality at x1 (20-24%, 3-9%). All PCR-identified samples were found to be *Culex quinquefasciatus*. The significantly reduced mortality at 24 h post exposure time indicates delayed recovery and survival of mosquitoes, usually not captured when using the CDC bottle bioassay method. This result provides evidence to suggest further discussions and review of the CDC bottle bioassay procedures for improved measure of mosquito susceptibility/resistance.

Keywords: Mosquitoes, *Culex*, insecticide resistance, pyrethroids, CDC bottle bioassay

Introduction

Mosquito vectors transmit debilitating diseases such as malaria, lymphatic filariasis, dengue, encephalitis, and yellow fever. Vector control with insecticide-treated nets (ITNs) and indoor residual spraying (IRS) represents the primary means of mosquito vector control and mosquito-borne disease (MBD) prevention. However, several countries and regions across the world have reported widespread insecticide resistance in the major mosquito vectors [1-9] due to consistent insecticide deployment for vector control and crop protection. Continued use of insecticide-based strategies for vector control therefore requires insecticide resistance monitoring in the targeted mosquito populations [10]. Standard resistance bioassays used to determine phenotypic insecticide resistance (IR) occurrence and intensities in mosquito vector populations include the CDC bottle and World Health Organization (WHO) tube/bottle assays. The CDC bottle method does not involve holding of exposed mosquitoes to determine delayed mortality or recovery, it is a time mortality assay with mosquito mortality at the end of the 30 min (45 min for dichlorodiphenyltrichloroethane (DDT)) exposure time as the test end-point [11]. Justifiably, any morbid mosquito that can no longer stand is recorded as dead since such immobilized vectors, even if not dead, are likely to be eaten up by predators in field conditions

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[12]. However, this could only happen if the immobility and flight-inability of the vector remain sustained upon predator contact or disturbance. Non-evaluation of sublethal and delayed mortality effects by the CDC bottle bioassay method further amplifies the issues surrounding the lab-to-field interpretations of IR monitoring data for making operational field decisions [13]. This study was designed to determine the occurrence of delayed mortality/recovery of CDC bottle pyrethroid-exposed *Culex* mosquitoes held for 24 hours after exposure. The result is expected to give some insight into delayed mortality/recovery outcomes of such exposed mosquitoes and probably generate further discussions on this bioassay method within the vector control community.

Material and Methods

Culex mosquito larvae and pupae were collected from natural breeding sites in Akalambi (N08°50.645' E004° .55128'), Baruba (N08°50.41' E004° 54.75') and shao garage (N08°50.67' E004°50.52') areas hereafter referred to sites 1-3, in urban Ilorin, the Capital city of Kwara State, Nigeria. The preadult mosquitoes were transported to and reared (27-29°C, 78-82%) to adults in the insectary at the Department of Zoology, Kwara State University, Malete, Nigeria. Adult female *Culex* mosquitoes emerging from the larvae were maintained with 10% sugar solution in mosquito cages until exposure. All male mosquitoes were removed from the cages. Only 2-3 days old non-blood fed adult female mosquitoes were used for the experiments. The CDC-supplied technical grade x1 and x2 insecticide was diluted separately, each in 50 mL of acetone as stock solution. Each of the Wheaton 250 mL CDC bottle was coated along with its cap with 1 mL of stock solution (12.5 µg/bottle). The control bottle for each test was coated with 1 mL of acetone. Coating and drying of the CDC bottles with deltamethrin (12.5 µg/bottle) and alphacypermethrin (12.5 µg/bottle) insecticides at x1 and x2 intensities and subsequent introduction of 10-25 *Culex* mosquitoes into coated bottles for 30 min were done following the standard CDC bottle bioassay protocol [11]. Each insecticide exposure had at least seven replicates with a control. For each intensity, female mosquito mortalities were recorded at 5 min intervals up to 30 min diagnostic time period [11]. After recording mortality results at 30 min, all exposed female mosquitoes (dead or alive) were immediately transferred from each CDC bottle into a holding cage containing 10% sugar solution in order to determine delayed recovery or mortality after 24 hours. Exposed *Culex* mosquito samples were identified morphologically [14]. Sub-samples (at least 10%) of the morphologically identified mosquitoes were identified with PCR using standard primers; ACEquin (5'CCTTCTTGAATGGCTGTGGCA-3'), ACEpip (5'-GGAAACAACGACGTATGTACT-3') and B1246s (5'TGGAGCCTCCTCTTCACGG-3') and protocols [15]. Genomic DNA used for the PCR identification was extracted from each mosquito sample following the procedure of Collins et al. [16]. The PCR products were amplified on 1.5% agarose gel and visualized in a gel documentation machine. Percentage mosquito mortality <90% was taken as resistant population. Percentage mortalities at 30 min and 24 h post-exposure periods were compared using Pearson's chi-squared test (P<0.05).

Results

Exposed mosquito mortalities at x2 insecticide intensity after 30 min (21-27%) and 24 h (6-13%) were higher than those of x1 (20-24, 3-9%) respectively. However, after both 30 min ($P \geq 0.475$) and 24 h ($P \geq 0.090$) periods, mosquito mortalities at x2 intensity (21-27%, 6-13%) were not significantly different from mortality at x1 (20-24%, 3-9%). For both x1 ($P \leq 0.009$) and x2 ($P \leq 0.048$) insecticide intensities, the percentage mosquito mortalities at 30 min exposure period (20-24%, 21-27%) were significantly higher than after the 24 h post-exposure period (3-9%, 6-13%) respectively (**Table 1**). All PCR-identified samples were found to be *Culex quinquefasciatus*.

Sites			Deltamethrin (30 min %MT)	Deltamethrin (24 h %MT)	*P*	Alphacypermethrin (30 min %MT)	Alphacypermethrin (24 h %MT)	*P*
Site 1	x1	N	484	484	0.003	455	455	0.002
		% MT	23	7		21	5	
	x2	N	449	449	0.005	416	416	0.048
		%MT	24	8		21	10	
Site 2	x1	N	313	313	0.009	296	296	0.002
		% MT	24	9		21	5	
	x2	N	314	314	0.037	367	367	0.033
		%MT	26	13		25	12	
Site 3	x1	N	353	353	0.005	256	256	<0.0001
		%MT	22	7		20	3	
	x2	N	256	256	0.003	173	173	0.004
		%MT	27	9		21	6	

P: Probability value; MT: Mortality; N: Number of mosquitoes exposed

Discussion

This study compared standard time and delayed mortalities of *Culex quinquefasciatus* mosquitoes exposed to deltamethrin and alphacypermethrin insecticides in CDC bottle bioassays. The low mosquito mortalities (20-27%) observed, after the 30 min standard time exposure to x1 and x2 intensities of the insecticides, signifies confirmed resistance of the *Culex quinquefasciatus* mosquito populations from the various sites to the two pyrethroid insecticides tested. The confirmed pyrethroid resistance observed in these urban *Culex* mosquito populations may threaten the effectiveness of the widely used pyrethroid ITNs for mosquito vector control and MBD prevention in the location, leading to increased *Culex* mosquito nuisance biting [17], perceived ITN inefficacy and nightly ITN non-compliance. Such loss of ITN protection against pyrethroid-resistant *Culex* mosquito bites have been recorded in earlier studies [18]. *Culex* mosquito resistance to pyrethroid insecticides have also been reported in other studies conducted in kwara state [19] and other parts of Nigeria [20-22]. The allowance of 24 h post-exposure period by other mosquito bioassay method [23] is to capture delayed recovery or mortality of the exposed mosquitoes. Rather than increased delayed mortality [24], the results of this study showed significantly reduced mortalities at 24 h, implying significant recovery of the earlier completely immobilized mosquitoes. This implies that such mosquitoes usually recorded as dead according to the CDC bottle bioassay protocol [11] may have recovered if given adequate time such as the 24 h recovery time allowed in this study. Careful observations during annual routine mosquito IR monitoring over the years have revealed the sudden flights of CDC bottle-exposed mosquitoes shortly after the mortality result recording (Obembe A, Unpublished observations). Such mosquitoes appearing lifeless and already recorded as dead have often been seen flying off at the slightest disturbance or attempt to pick and transfer them into Eppendorf tubes a few minutes after results recording. The number of these reviving and flying mosquito samples tends to increase as the delay in time taken to transfer them in Eppendorf tubes increased. Interestingly, some samples have been found flying off upon opening of the Eppendorf tubes into which they have been transferred few minutes earlier. Similar low percentage mortalities (10-30%) like the one in this study (7-13%) have also been recorded in another 24 h CDC bottle alphacypermethrin bioassay involving *Culex quinquefasciatus* mosquitoes in Nigeria [22]. While the latter allowed 24 h recovery time in order to compare the CDC bottle bioassay results with those of WHO tube, the study did not include the results of *Culex* mosquito mortality after the 30 min standard exposure time. This present study represents the first report of 30 min and 24 h post CDC bottle bioassay mosquito exposure mortality comparison in order to determine the occurrence of delayed mortality or recovery. Since death/survival ultimately determines mosquito susceptibility/resistance, accurate measure of mortality, including delayed mosquito recovery/deaths is required to measure the susceptibility or resistance status of any specific mosquito population. Already, it is a known fact that caution must be applied in interpreting IR bioassay data directly for making operational field mosquito control decisions [23]. The results of IR bioassays generated for cautious vector control decision-making must therefore be as accurate as possible. The mosquitoes alive after insecticide exposure survival therefore represent a major part of insecticide susceptibility bioassay results that must be captured for accurate measure of mosquito population susceptibility or resistance. Richards *et al*. [23] earlier noted that non-evaluation of sublethal and delayed mortality effects by the CDC bottle bioassay method could amplify the issues surrounding the lab-to-field interpretations of IR monitoring data for making operational field decisions. The results of significant *Culex* mosquito recovery after allowance of 24 h post-exposure period in this study represents a preliminary report that could generate further research and discussions on the CDC bottle bioassay procedure within the mosquito vector control community.

Conclusion

This study reports significant 24 h recovery and survival of *Culex* mosquitoes that died after 30 min exposure to the CDC bottle pyrethroid bioassay. The significant mosquito mortality reversal found between 30 min exposure and 24 h post-exposure times indicates delayed recovery and survival of mosquitoes, usually not captured when using the CDC bottle bioassay method. This result calls for further discussions and review of the CDC bottle bioassay procedures for improved measure of mosquito susceptibility/resistance.

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