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Note

Comparison of a novel high-throughput screening system with the Bottle assay for evaluating insecticide toxicity

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Insecticide toxicity is commonly evaluated for disease vectors by either the WHO test or Bottle assay. More recently, a high-throughput screening (HTS) system was developed for testing insecticide effects on mosquito behavior and mortality. We compared HTS with the Bottle assay to evaluate the toxicity of insecticides in a population of *Aedes aegypti* from Thailand. Both the HTS and Bottle assay system were determined to be equivalent. The two systems mainly differed (1) in reaction time, with mosquitoes reacting faster in the Bottle assay than HTS, (2) in knock-down and mortality at low doses. This information will guide the testing protocol for evaluating chemical effects on behavioral responses in various vector populations. ©Pesticide Science Society of Japan

Keywords: Bottle assay, HTS, insecticide toxicity, mosquitoes resistance.

Introduction

For decades, insecticides for use in vector-borne disease control have been evaluated based on their toxic effects; however, the rapid development of insecticide resistance in targeted populations has limited their efficacy. Therefore, evaluating the efficacy of insecticides against resistant vector populations has become the main focus for the development of novel compounds to be used in vector control strategies. The World Health Organization developed a bioassay system to detect resistance levels in adult mosquito populations.¹⁾ This system exposes insects to chemical impregnated filter paper placed inside a plastic cylinder. Stan-

dardization of diagnostic doses and the establishment of baseline data for susceptible populations has facilitated the monitoring of resistance and has guided the decision making process for the use of pesticides.¹⁾ Brogdon and MacAllister modified the WHO resistance test kit using insecticide-coated glass bottles with solutions of standard grade insecticides and synergists.²⁾ The bottle assay answers the question: will an insecticide at a concentration that gives 100% mortality for a susceptible population kill test mosquitoes during the same time interval? This system has also been used to evaluate the diagnostic dose/time within different populations of mosquitoes^{3–5)} and to determine the standardized diagnostic dose for new insecticides.⁶⁾ Recently, Grieco *et al.* developed a high-throughput screening system (HTS) which exposes mosquitoes to insecticides *via* a treated nylon net placed inside a metal cylinder.⁷⁾ This assay was designed to test the contact irritant and spatial repellent activity as well as toxic effects of chemicals to insects. Better knowledge of these effects at a specific level could have a role in improving vector control strategies by disrupting contact more efficiently between humans and vectors. This system has previously been used to evaluate behavioral responses and the mortality of *Aedes aegypti* in response to topical repellents and other standard compounds used for vector control.^{7–8)} The results were reproducible but no further comparisons were made to evaluate HTS performance against more standard assays.

The current approach aimed to assess HTS as a potential new system for evaluating the effects of chemicals on insects. Therefore, this study compared HTS to the bottle assay system to evaluate the toxicity of alpha-cypermethrin (pyrethroid), malathion (organophosphate), bendiocarb and propoxur (carbamates) against a Thai population of *Ae. aegypti*, the primary vector of dengue. Therefore, the ultimate goal was to use HTS as a unique assay to compare resistant and susceptible populations of disease vectors by evaluating their level of resistance and their behavioral responses to contact irritant and spatial repellent chemicals.

Materials and Methods

1. Mosquitoes

Aedes aegypti were colonized at Kasetsart University, Bangkok, Thailand, from a population collected in Pu Teuy Village, Sai Yok District, Kachanaburi Province, Thailand (14°20'11"N, 98°59'45"E). F₁ or F₂ eggs from this colony were shipped to the Uniformed Services University of the Health Sciences (USUHS) (Bethesda, Maryland) to establish a colony. The colony was maintained at 28°C and 80%RH under a photoperiod of 12 : 12 (L : D) h. Baseline testing against DDT 4%, deltamethrin 0.05%, malathion 0.8%, propoxur 0.1% were performed with the WHO filter paper test and the population was determined to only be resistant to DDT (Chareonviriyaphap, unpublished data).

Females (4–7 days old) used in testing were from the F₂–F₄ generations, non-bloodfed, unmated and starved from 10% sucrose solution 24-h prior to conducting an assay.

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Chemicals and doses: Five insecticides comprising four different chemical classes commonly used in vector control were chosen for testing: alpha-cypermethrin (pyrethroid; BASF Corporation, Florham Park, NJ–CAS67375-30-8); bendiocarb (carbamate; Sigma-Aldrich Inc., St. Louis, MO–CAS22781-23-3) and propoxur (carbamate; Sigma-Aldrich Inc., St. Louis, MO–CAS000114-26-1); and malathion (organophosphate; Sigma-Aldrich Inc., St. Louis, MO–CAS121-75-5). Tests were performed with alpha-cypermethrin 0.05%, 0.02% and 0.002%; bendiocarb 0.1% and 0.001%; malathion 0.8%, 0.1% and 0.4%; and propoxur 0.1%, 0.01% and 0.001%.

2. Bioassay systems

HTS in the toxicity configuration is composed of an outer metal chamber (10.2-cm outside diam, 0.6-cm thick) ended by a solid cap and a gated funnel cap (Fig. 1A & B). The inner cylinder housed a nylon netting strip impregnated with the test chemical or diluent in the case of the control (275 cm²; G Street Fabrics, Rockville, MD). The bottle assay used a 250 ml glass bottle for testing (Wheaton Science, Millville, NJ).

Net strips were treated with 1.5 ml acetone-based insecticide solution and were allowed to dry for at least 15 min prior to being placed into the HTS metal test cylinder. Bottles were coated with 1.5 ml of the same solution and then stored upside down overnight in a dark place. Controls for both assays were treated using the same protocol but with acetone only.

Both assays were performed simultaneously, with laboratory temperatures ranging from 20 to 24°C and relative humidity from 35 to 50%. HTS and Bottle assay protocols slightly differed in methodology. In the HTS, Females were exposed to insecticide for 1 hr and then held for 24 hr before counting deaths. The Bottle assay could also be used to obtain a diagnostic time. Therefore, a group of 20 females, per four repetitions, were introduced into the two systems and the number of knocked-down mosquitoes was recorded every 5 min during a 1-hr observation period (1 hKD). A mosquito was recorded as knocked-down if it was lying on its back or side and was unable to maintain flight after a gentle tap on the test system. After 1 hr, mosquitoes were transferred to individual cups using mechanical aspiration and maintained with a 10% sugar pad at 28°C/80% RH for 24 hr before

recording mortality (24 hM).

3. Data Analysis

A non-parametric Wilcoxon signed-rank test (Proc NPARIWAY, SAS 1999) was used to test for differences in 1 hKD and 24 hM data between the Bottle and HTS for each insecticide and dose used. Lethal times which produced 50% knock-down after 1 hr (LT50) were obtained by probit analysis performed for each system, insecticide and dose (Proc PROBIT, SAS 1999). To further investigate differences between the two assay systems, probit curve parameters were compared by insecticide and by dose using a Chi-square test (Proc PROBIT, SAS 1999). Locations of the paired curves were compared allowing for the detection of differences in times trends here referred to as the reaction time. The slopes of the curves were also compared to detect differences in the speed of killing, here referred to as the mortality rate, between the two assay systems.

Results and Discussion

Both the HTS and Bottle assay systems gave equivalent mortality for all chemicals tested at high doses. Indeed, assays conducted with alpha-cypermethrin 0.02 and 0.05%, malathion 0.4 and 0.8% and propoxur 0.01 and 0.1% produced over 80% 1 hKD and 24 hM, confirming the susceptibility of this population to these insecticides. However, after 1-hr exposure, the two systems exhibited significant statistical differences for the three doses of malathion ($p=0.02$) (Table 1) with 100% knock-down in the Bottle and values ranging from 82.1 to 88.6% knock-down in the HTS. No difference was observed 24-hr post exposure with mortality reaching 100% in both systems. LT50, reaction time and mortality rate values were calculated by probit analysis. Bottle assay and HTS results were equivalent for alpha-cypermethrin 0.05%, propoxur 0.01% and bendiocarb 0.1%.

Stronger divergence was recorded at lower doses. Alpha-cypermethrin at a dose of 0.002% produced a significant difference between HTS and Bottle assay ($p=0.02$) with 40.2% (SE=13.6) 1 hKD and 38.8% (SE=10.6) 24 hM for HTS, compared to 97.1% (8.1) 1 hKD and 83.0% (11.2) 24 hM (Table 1) with the Bottle assay. Results from carbamate assays showed that propoxur 0.001% induced significantly lower 1 hKD in the Bottle assay (50%) than in the HTS (97.6%), as well as significantly lower 24 hM (50.91 and 100%, respectively). In addition, high variability (SE=44.94) was detected among bottles. Such variability did not occur with HTS, whose highest standard error was 17.3 (Table 1). Similar results were found using bendiocarb tested at 0.1% and 0.001% (Table 1). There were no significant differences at the highest dose (0.1%) with 100% knock-down and mortality after 1-hr and 24-hr intervals, respectively. Once again, however, the Bottle assay demonstrated high variability (SE=57.7) among bottles at the lowest dose (0.001%) (Table 1). Due to this high variability among bottles for bendiocarb and propoxur at 0.001%, these data were not included in the probit analysis. Significant differences were also observed between LT50, reaction time and the mortality rate, indicating that mosquitoes exposed in the Bottle assay are affected earlier and faster

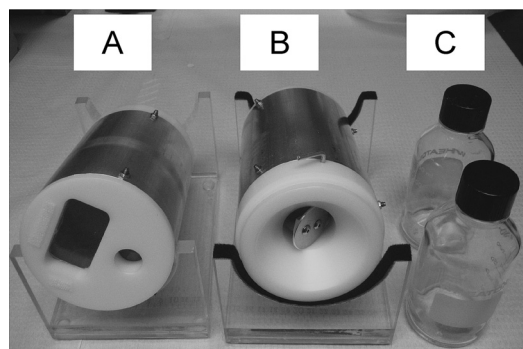


Fig. 1. Systems used for evaluating insecticide toxicity. A and B: HTS two sides, C: 250 ml Wheaton glass bottle.

Table 1. Toxicity measured in HTS and Bottle assay for four insecticides at different doses.

	Dose %	% 1hKD (SE)			% 24hM			LT50 ^{b)}		<i>p</i> -reaction time ^{c)}	<i>p</i> -mortality rate ^{c)}
		(SE)		<i>p</i> ^{a)}	(SE)		<i>p</i> ^{a)}	[95% CI]			
		HTS	Bottle		HTS	Bottle		HTS	Bottle		
Alpha-cypermethrin	0.002	40.2 (13.6)	97.1 (8.1)	0.02	38.8 (10.6)	83.0 (11.2)	0.02	69 [63–80]	23 [22–24]	<0.001	0.010
	0.02	100 (0)	100 (0)	1	100 (0)	100 (0)	1	16 [13–19]	11 [9–13]	0.00025	0.074
	0.05	98.8 (1.7)	100 (0)	1	100 (0)	100 (0)	1	12 [10–13]	10 [9–11]	0.390	0.800
Malathion	0.1	82.10 (9.97)	100 (0)	0.02	100 (0)	100 (0)	1	46 [44–48]	19 [18–20]	<0.001	0.625
	0.4	88.62 (9.22)	100 (0)	0.02	100 (0)	100 (0)	1	44 [43–45]	12 [11–13]	<0.001	0.800
	0.8	88.49 (4.45)	100 (0)	0.02	100 (0)	100 (0)	1	42 [41–43]	11 [9–12]	<0.001	0.056
Propoxur	0.001	87.83 (17.29)	25.52 (44.94)	0.01	84.82 (15.10)	21.11 (37.62)	0.002	40 [38–43]	–	–	–
	0.01	100 (0)	100 (0)	1	100 (0)	100 (0)	1	11 [10–11]	7 [6–8]	0.069	0.070
	0.1	100 (0)	100 (0)	1	100 (0)	100 (0)	1	10 [9–11]	5 [4–6]	<0.001	0.500
Bendiocarb	0.001	97.61 (4.12)	50.00 (57.73)	0.65	100 (0)	50.91 (53.48)	0.14	23 [21–25]	–	–	–
	0.1	100 (0)	100 (0)	1	100 (0)	100 (0)	1	15 [13–17]	9 [8–10]	0.440	0.540

^{a)} *p*-value produced by the Wilcoxon signed-rank. test ^{b)} Lethal time 50% in minutes with 95% confidence interval. ^{c)} *p*-value produced by Chi-square test to compare reaction times and mortality rates between systems.

than those in HTS (Table 1).

The results allowed us to assess the HTS as equivalent to the standard Bottle Assay system in evaluating the effect of insecticides at high doses by an end-point measurement after 24 hr.

Differences between screening systems is not novel, since filter paper tests have reported resistance levels 50% lower for organophosphates and 90% lower for pyrethroids than the Bottle assay.²⁾ We assume that such differences are due to the physical structure of the two assay systems and the relative volatility of insecticides. Our study showed that mosquitoes react faster in the Bottle assay than in HTS. The HTS toxicity configuration allows ventilation due to the butterfly valve located on the funnel cap. In this regard, the HTS system is more comparable to the WHO filter paper test that has screened ends. Conversely, the bottles are sealed tight with screw caps preventing the ventilation of volatile substances. This may explain the similar 1 hKD and 24 hM between the two systems at high concentrations but differences at

lower doses. The issue of ventilation may not be a factor at the highest concentration because the saturation of treated interior surfaces is sufficient to kill all mosquitoes; however, at lower concentrations, ventilation may decrease the quantity of volatile compounds in HTS as compared to the sealed Bottle assay, leading to higher knock-down and mortality values recorded in the latter system. Moreover, the entire interior surface of the bottle is coated with chemical whereas HTS has untreated end caps on which the mosquito can rest and thus not pick up a lethal dose of compound. These assay systems are also under the influence of a number of extrinsic factors which, when combined with the structural difference of the assay devices, could result in the observed differences. Some of these factors have already been documented for different pesticides, such as the physical properties of insecticides (i.e. volatility, adsorption) combined with environmental factors (i.e. airflow, humidity, temperature) and the type of treated material (i.e. glass, netting).^{9–11)} These are just some of

the factors which, individually or in combination, are most likely to play a role in the efficacy and availability of insecticide and could result in the differences observed in the present study.

Our research program aims to evaluate novel compounds as toxicants, contact irritants and spatial repellents in order to improve vector control strategies. To limit bias and to better compare the results, HTS will be used across the three assays, under controlled temperature and humidity regimes. We will also consider the cited factors when evaluating chemical effectiveness and comparing compounds. This information will be used to guide the testing protocol for the evaluation of chemical effects on behavioral responses in various mosquito populations.

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