Journal of the American Mosquito Control Association, 32(2):000–000, 2016

EVALUATION OF ALTERNATIVE METHODS FOR TESTING THE BIOEFFICACY OF HOUSEHOLD AMBIENT INSECTICIDE PRODUCTS AGAINST *AEDES ALBOPICTUS*

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1

ABSTRACT. Ambient insecticides are receiving increasing attention in many developed countries because of their value in reducing mosquito nuisance. As required by the European Union Biocidal Products Regulation 528/2012, these devices require appropriate testing of their efficacy, which is based on estimating the knockdown and mortality rates of free-flying (free) mosquitoes in a test room. However, evaluations using free mosquitoes present many complexities. The performances of 6 alternative methods with mosquitoes held in 2 different cage designs (steel wire and gauze/plastic) with and without an operating fan for air circulation were monitored in a test room through a closed-circuit television system and were compared with the currently recommended method using free mosquitoes. Results for caged mosquitoes without a fan showed a clearly delayed knockdown effect, whereas outcomes for caged mosquitoes with a fan recorded higher mortality at 24 h, compared to free mosquitoes. Among the 6 methods, cages made of gauze and plastic operating with fan wind speed at 2.5–2.8 m/sec was the only method without a significant difference in results for free mosquitoes, and therefore appears as the best alternative to assess knockdown by ambient insecticides accurately.

KEY WORDS *Aedes albopictus*, bioefficacy, insecticide, mosquitoes, pyrethroid

INTRODUCTION

Ambient insecticides are dispersed by a variety of devices (mosquito coils, liquid vaporizers, vaporizer mats, and emanators) into the air to kill mosquitoes that are already present, or to repel mosquitoes from a treated area. These devices have been receiving increasing interest recently, both in developed countries and in tropical areas, as a way to reduce mosquito nuisance and as a means of disease control in integrated vector management programs (Ogoma et al. 2012). Vaporizer mats are particularly advantageous in indoor environments, because they contain embedded active ingredients that are volatilized slowly with an electric heating element without producing undesirable amounts of smoke, as may occur with the burning of mosquito coils.

The recent adoption by the European Union (EU) of Biocidal Products Regulation (BPR) No. 528/2012, which aims to improve the functioning of biocidal products while ensuring a high level of protection for humans and the environment (European Chemical Association [ECHA] 2012), has pushed the research and production of new insecticidal formulations of active ingredients to be effective at progressively lower concentrations and longer durations. As biocidal products, ambient insecticides need to demonstrate their efficacy against target organisms through appropriate BPR testing and still meet the public's demand for cost-effective mosquito control. Active ingredients of household ambient insecticidal products operate in the vapor phase and mostly belong to the pyrethroid chemical class (product type 18—insecticides and acaricides; European Commission [EC] 2012). Although no officially recognized guidelines are available for efficacy studies with vaporized products, the general requirements are outlined in the "Technical Notes for Guidance on Product Evaluation," paragraph 14.2.2.3.3, and updated appendices to Chapter 7 (EC 2012). The suggested method to verify their bioefficacy is the "large room test," which includes releasing free-flying (free) mosquitoes in a 20–60-m^3 room and estimating knockdown percentages and mortality (EC 2012). Similarly, the World Health Organization (WHO) guidelines recommend simulating "real conditions" with free arthropods and suggest using a minimum-sized 30-m^3 test room to verify indoor insecticide efficacy for aerosol applications and ambient insecticides (WHO 2009). For either recommendation, estimation of average percentage of knocked-down mosquitoes is to be taken after 60 min and the average percentage mortality after 24 h.

Unfortunately, the use of free mosquitoes is time consuming and presents many complexities, such as the difficulty to recover all insects at the end of exposure time and the necessity for a specially constructed room. During free-mosquito experiments, procedures typically require having personnel outfitted with safety equipment and staying continuously inside the test room to record knockdown numbers at fixed time intervals.

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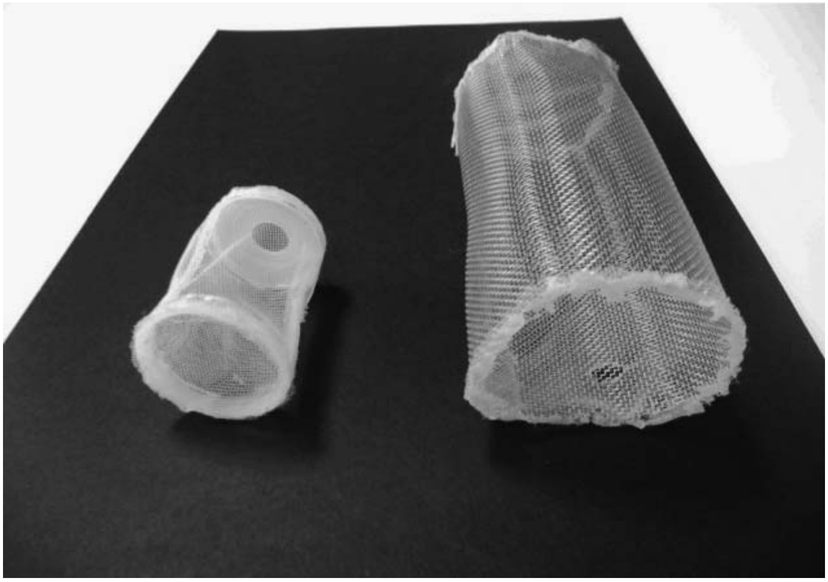

Fig. 1. Image of the steel wire net cage (right) and of the gauze cage (left).

Furthermore, no room ingress or egress is allowed to prevent mosquitoes from escaping and dilution of insecticide from the exchange of air. As a more practical approach to measure the efficacy of ambient insecticides on mosquitoes, an original monitoring system based on the use of small cages with a closed-circuit television (CCTV) system was developed for this study. The aims of this investigation were to test alternative methods and to assess the coherence of results obtained by each new method with results based on the release of free mosquitoes in a large space, which presently is considered the standard method for evaluation.

MATERIALS AND METHODS

Mosquitoes: The present study reports on test results for *Aedes albopictus* (Skuse) an easily reared, highly pestiferous, and important disease vector in Italy (Busani et al. 2012). The colony of mosquitoes used in this study originated from eggs collected in the field, in the Province of Padova (northeastern Italy). *Aedes albopictus* females were reared under standardized insectary conditions (25 ± 1°C, 60 ± 5% relative humidity (RH) and a light-dark cycle of 14L/10D with light intensity of 300 lux at 6,000 K), and supplied with 10% sucrose solution and fed with defibrinated ram blood twice a week with the use of an Hemotek® 96 device (Hemotek Ltd., Great Harwood, Lancashire, UK). Mosquitoes were kept in 50 × 50–cm metallic wire cages with bottom and front Plexiglas sides. Interior cage access was through a 30-cm-long gauze arm opening in the front for insect handling purposes. Only 3–5-day-old, non-blood-fed females were used in the experiments.

Room and cages: The test room was rectangular, measuring 3.02 × 3.34 × 3.15 m (31.8 m^3), with a tiled floor and washable white-painted walls, and was equipped with an air conditioner and extractor fan. Illumination was provided by a 6,000 K solar-spectrum artificial light of 300 lux. In order to evaluate tests with caged mosquitoes, the room was provided with a CCTV system made of 3 removable video cameras used for observing mosquito behavior to assess knockdown percentages. Each experiment was video recorded, allowing subsequent observation of prerecorded tests.

Two kinds of cages were tested: 1) Gauze and plastic cages, each measuring 5 cm high × 3 cm diameter, made of gauze (mesh size: 1 mm) over a plastic frame obtained from an old camera film container and 2) steel cages, each 10 cm high × 6 cm diameter, made of steel wire net (mesh size: 1 mm) (Fig. 1). Cages, containing 10 individuals each, were mounted on tripods at 3 different heights along the room diagonally. Among the different test methods, 4 were conducted with the use of an 18-cm-radius, 3-speed electric stand fan (Imtron GmbH, Germany) rotating at 45° and positioned at the room's rear corner; the fan was operated at the lowest setting, which generated wind speeds of 2.5–2.8 m/sec, as determined by a H2303.0 anemometer (DeltaOHM®, Italy) positioned 40 cm from the fan. In 2 methods, the stand fan was screened with tulle netting in order to decrease air speed; this screening produced wind speeds with anemometer readings ranging from 1.7 to 2.5 m/sec (Fig. 2).

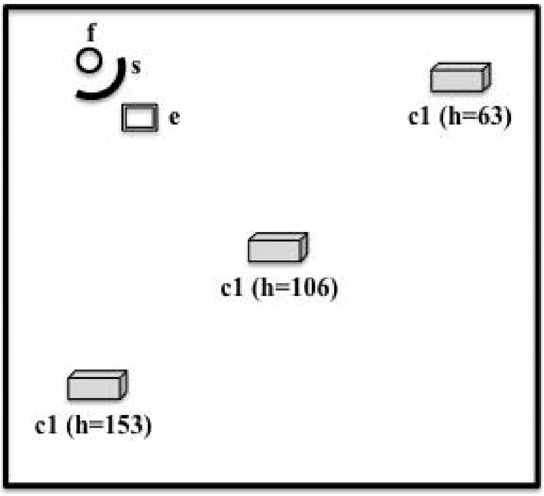

Fig. 2. Experimental set: fan (f), screen (s), emanator (e), and cages (c1–c3) arrangement inside the test room (height in centimeters).

PROCEDURES

A commercially available product (VAPE®, Guaber, Italy) consisting of vaporizing mats (each containing 12 mg of prallethrin 91%, exclusive excipients, and cellulose up to 0.72 g) for electric diffusers was used in laboratory bioassays on caged and free mosquitoes. Mosquitoes were exposed to insecticidal vapors for 90 min with knockdown numbers recorded at 5-min intervals and mortality at 24 h.

For ambient insecticide performance on free mosquitoes, an operator wearing personal protective equipment stood inside the test room to count knockdown. The vaporizing device was switched on simultaneous to the release of mosquitoes for each trial. Overall, 5 replications with free mosquitoes were made. A minimum of 30 mosquitoes was used for each replication.

For ambient insecticide performance on caged mosquitoes, the following 6 experimental methods were used.

1. Steel wire net cages (SC), without fan
2. Gauze and plastic cages (GC), without fan
3. Steel wire net cages with operating fan (SC_F)
4. Gauze and plastic cages with operating fan (GC_F)
5. Steel wire net cages with screened fan (SC_SF)
6. Gauze and plastic cages with screened fan (GC_SF)

Procedures were replicated 4–5 times for each method. All replications were performed with 30 mosquitoes, 10 in each of the 3 cages. New cages were used for each replication, with a minimum 12-h interval between each test to replace air in the test room by the extractor fan (air flow rate: 380 m^3/h).

At the end of the 90-min exposure time, free mosquitoes and cages were moved to a confined environment in a noncontaminated room with adequate conditions of temperature, humidity, light, and food/water, per mosquito rearing method to evaluate mortality rates after 24 h. Mosquitoes were considered to be dead if they were motionless, shaking faintly, or moving slightly and not reacting to external stimuli (i.e., when touched with a pair of tweezers).

Statistical analysis: Differences in knockdown percentages among all replications of the 7 methods were assessed at each time interval with the Kruskal–Wallis test, followed by a multiple pairwise comparisons corrected by the Bonferroni method. Differences in the overall number of mosquitoes dead at 24 h, expressed as percentage of the total number of mosquitoes released for each method, were investigated by chi-square test, followed by pairwise comparison with the Marascuilo procedure and Bonferroni correction. Statistical analyses were implemented with SAS (version 9.2), under a statistical threshold value of $P < 0.05$.

RESULTS

The results of the comparison among average knockdown percentages at each 5-min interval, for all 7 methods, are reported in Table 1 and shown in Fig. 3. In experiments where a fan was used, the mosquito knockdown increased exponentially after 10 min and exceeded 90% at 20–25 min. The 2 methods without an operating fan showed a much slower rise in their percentages, requiring 80–85 min to reach similar knockdown values. The overall mortality at 24 h for each method is reported in Table 2, and significant differences are highlighted. Results for steel wire net cages showed a very high mortality rate at 24 h, significantly greater ($P < 0.05$) than the 77.0% recorded with free-flying mosquitoes. In contrast, gauze and plastic cages showed comparatively lower mortality rates, with results not significantly different from that of free mosquitoes.

DISCUSSION

The ventilation of the room was the most influential factor in determining differences in knockdown percentages between free-flying mosquitoes (the currently recommended method) and caged mosquitoes. Compared to the free mosquito results, methods SC and GC (without operating fan) were significantly different ($P < 0.05$) in knockdown percentages from minute 15 onward, with an evidently longer time for the mats to be effective. A possible explanation of this aspect may depend on a filtration effect performed by

Table 1. Knockdown mean percentages at each time interval for the 7 methods (number of replications is reported in parentheses).

Time interval (min)	Methods[1,2]							P value (Kruskal–Wallis test)
	FM (5)	SC (4)	GC (4)	SC_F (5)	GC_F (5)	SC_SF (4)	GC_SF (4)	
5	0.0	0.8	0.0	1.3	0.0	1.7	0.0	0.608
10	4.7	0.8	0.8	8.0	2.0	9.2	0.8	0.135
15	57.3 a	4.2 c	1.7 c	62.7 a	45.3 a,b	35.0 b	13.3 c	<0.001
20	89.0 a,b	5.8 d	1.7 d	96.7 a	94.7 a	80.0 b	55.8 c	<0.001
25	94.7a	17.5 b	6.7 b	100.0 a	100.0 a	100.0 a	91.7 a	<0.001
30	97.3a	27.5 b	18.3 b	100.0 a	100.0 a	100.0 a	99.2 a	<0.001
35	99.3a	30.8 b	19.2 b	100.0 a	100.0 a	100.0 a	100.0 a	<0.001
40	99.3 a	39.2 b	31.7 b	100.0 a	100.0 a	100.0 a	100.0 a	<0.001
45	100.0 a	45.0 b	40.0 b	100.0 a	100.0 a	100.0 a	100.0 a	<0.001
50	100.0 a	50.8 b	55.0 b	100.0 a	100.0 a	100.0 a	100.0 a	<0.001
55	100.0 a	57.5 b	60.8 b	100.0 a	100.0 a	100.0 a	100.0 a	<0.001
60	100.0 a	71.7 b	66.7 b	100.0 a	100.0 a	100.0 a	100.0 a	<0.001
65	100.0 a	76.7 b	71.7 b	100.0 a	100.0 a	100.0 a	100.0 a	<0.001
70	100.0 a	84.2 b	76.7 b	100.0 a	100.0 a	100.0 a	100.0 a	0.002
75	100.0 a	88.3 b	80.8 b	100.0 a	100.0 a	100.0 a	100.0 a	0.008
80	100.0	92.5	87.5	100.0	100.0	100.0	100.0	0.429
85	100.0	95.8	90.0	100.0	100.0	100.0	100.0	0.429
90	100.0	100.0	100.0	100.0	100.0	100.0	100.0	

[1] FM: free-flying mosquitoes; SC: steel wire net cages without fan; GC: gauze and plastic cages without fan; SC_F: steel wire net cages with operating fan; GC_F: gauze and plastic cages with operating fan; SC_SF: steel wire net cages with screened fan; GC_SF: gauze and plastic cages with screened fan.

[2] Different letters along rows refer to significant different values ($P < 0.05$).

the cage (Bonds et al. 2010, Fritz et al. 2014). Because the exposure pathway for an insecticide is by direct impact on mosquitoes, finely screened cages, acting as a mechanical filter, can reduce the amount of active substances reaching caged mosquitoes, delaying the knockdown effect. The use of a fan was meant to compensate for this effect partially, forcing higher insecticide concentrations inside the cages. In fact, the 4 methods with caged mosquitoes together with fans were more comparable to the insecticidal knockdown effect on free mosquitoes, as clearly demonstrated by the similarities in their trends (Fig. 3). The 2 methods with the screened fan (methods SC_SF and GC_SF) showed a slight delay in reaching similar knockdown percentages, particularly in the time frame between 15 and 20 min (Table 1). Nonetheless, both methods still achieved 100% mortality within 35 min. Both steel wire net cages and gauze/plastic cages, each using a fan with

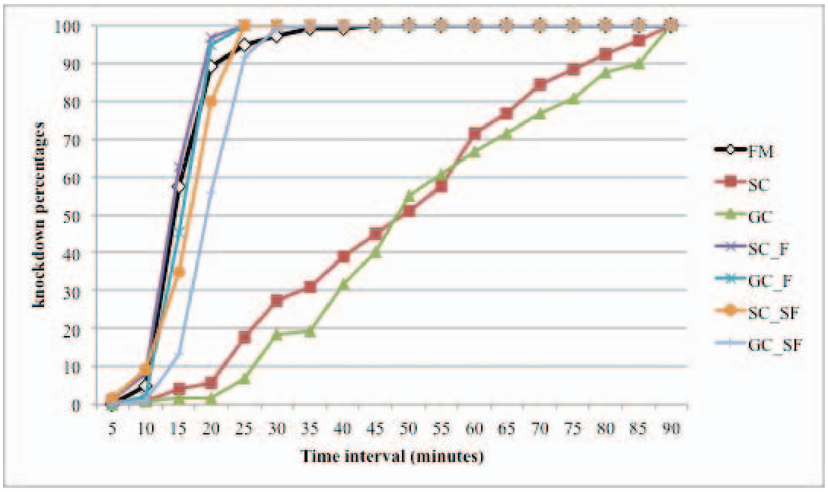

Fig. 3. Trends of knockdown percentages for the 7 methods (FM: free-flying mosquitoes; SC: steel-wire net cages without fan; GC: gauze and plastic cages without fan; SC_F: steel wire net cages with operating fan; GC_F: gauze and plastic cages with operating fan; SC_SF: steel wire net cages with screened fan; GC_SF: gauze and plastic cages with screened fan).

Table 2. Comparison among mortality values of the 7 methods, with the use of *Aedes albopictus* females.

Method[1]	*N* tested overall	*N* killed at 24 h	Mortality at 24 h (%)[2]
FM	174	134	77.0 a,b
SC	120	116	96.7 c,d
GC	120	82	68.3 a
SC_F	150	150	100.0 d
GC_F	150	135	90.0 b,c
SC_SF	120	120	100.0 d
GC_SF	120	119	99.2 c,d

[1] FM: free-flying mosquitoes; SC: steel wire net cages without fan; GC: gauze and plastic cages without fan; SC_F: steel wire net cages with operating fan; GC_F: gauze and plastic cages with operating fan; SC_SF: steel wire net cages with screened fan; GC_SF: gauze and plastic cages with screened fan.

[2] Different letters refer to significant different values ($P < 0.05$).

wind speeds of 2.5–2.8 m/sec (methods SC_F and GC_F), resulted in knockdown percentages not significantly different ($P > 0.05$) from the free mosquitoes at any time interval and therefore, constitute a suitable alternative to determine this aspect of insecticide bioefficacy.

For mosquito mortality at 24 h, methods that made use of steel cages showed the highest mortality relative to gauze/plastic cages and free mosquitoes. The high mortality in steel cages was probably due to the physical characteristics of the metal. Mosquitoes in both steel and gauze/plastic cages remained in continuous contact with the inner surface, where active ingredients of vaporized insecticide were increasingly deposited during the duration of the test. Plastic and gauze are known for their capacity to absorb, whereas steel can keep insecticide molecules on its surface for longer time. Therefore, insecticide bioavailability on a steel cage surface is higher, resulting in greater mortality. Comparatively, free mosquitoes are exposed even less once they fall to the floor after knockdown, where continuous exposure to volatizing insecticide is minimal.

In the past, many researchers investigated the differences caused by the experimental setup in knockdown effect and mortality of tested mosquitoes. Generally, it is recognized that different conditions in volume and/or ventilation during laboratory experiments can cause great data deviations from actual conditions, due to unrealistic relative insecticide aerial concentrations (Katsuda et al. 2008, Ogoma et al. 2012).

The results obtained in this study are encouraging, and contribute to the identification of the optimal ventilation conditions and of cage characteristics. The methods implemented, including caged mosquitoes monitored by a CCTV system, are practical and cost-effective. Among tested methods, the use of gauze and plastic cages with an operating fan appears to be the best substitute for the room test with free-flying mosquitoes, because the results for this method were not significantly different from the free mosquito results. However, accurate validation is requested, in order to avoid biased results, which may be difficult to confirm by actual field conditions.

ACKNOWLEDGMENTS

This work was partially funded by the European Regional Development Fund (Project code: 2105/101/4/1148/2013). We are very grateful to reviewers for their accurate revision and for the valuable suggestions.

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