A New Methodology to Evaluate Entomopathogenic Fungi and Formulated Insecticides to Control Adults of Aedes aegypti (Diptera: Culicidae)

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A new methodology to evaluate entomopathogenic fungi and formulated insecticides to control adults of *Aedes aegypti* (Diptera: Culicidae)

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Traditionally, insecticide susceptibility tests in mosquito populations use the bioassay methodologies recommended by the World Health Organization (WHO), where mosquitoes are exposed to impregnated papers with known concentrations of an insecticide during a given time (WHO 2016). However, these papers are difficult to acquire. Also, the availability of insecticides and concentrations are limited (Perea et al. 2009). Brogdon and McAllister (1998) proposed the bottle bioassay, which has resulted in a practical methodology to estimate insecticide resistance. However, it requires the use of technical grade compounds (Perea et al. 2009). Today, these methodologies constitute the tools to estimate insecticide resistance in mosquito populations (Owusu et al. 2015; WHO 2016). The use of non-conventional pesticides such us botanical products, insecticides mixed with synergists, and entomopathogenic fungi to control adult mosquitoes is increasing.

For entomopathogenic fungi, there are several methodologies to evaluate them to control mosquitoes, which consist of the impregnation of conidia on surfaces such as filter papers, cardboards, or cloths, by using sprayers (Mnyone et al. 2010; Darbro et al. 2011; Blanford et al. 2012) or K-bars (Farenhorts & Knols 2010), or by immersion (Paula et al. 2008). Subsequently, these surfaces undergo a drying period, and finally the mosquitoes are exposed to the entomopathogenic fungi during a given time. Another way consists of exposing mosquitoes to the culture medium where the fungus grows (Leles et al. 2010; García-Munguía et al. 2011).

A method is needed to evaluate formulated as well as non-traditional insecticides. The Potter tower is a standardized system that guarantees uniform and continuous depositions of toxic substances (Hoskins & Craig 1962). It has been used to evaluate technical grade (Liang et al. 2007), formulated (Tucuch-Haas et al. 2010), non-conventional insecticides (Liu & Stansly 1995), and entomopathogenic fungi (Cabanillas & Jones 2009). Also, the equipment can be cleaned with high efficiency to avoid cross contamination. As a disadvantage, the Potter tower has a relatively high cost (Mascarin et al. 2013), typically only affordable by governmental agencies conducting studies to monitor insecticide resistance management, not by independent researchers, who usually are the ones conducting such studies. The objective of this study is to design a bioassay methodology with the Potter tower to determine the susceptibility of adult mosquitoes to commercial formulations, and botanical and entomopathogenic insecticides.

A 9 × 1.5 cm diam plastic Petri dish (Fig. 1a) is covered with 15 × 15 cm tulle fabric (Tulle #15, 100% Nylon, Modatelas S.A. de C.V., Mexico, Distrito Federal, Mexico). A circular perforation is cut in the central part of the fabric (5 mm in diam) (Fig. 1b) and the fabric is secured to the dish with a natural rubber band (90 mm × 6 mm × 10 mm) (Hercules No. 64 B, Iberoamérica de Elásticos, S.A. de C.V., Mexico, Distrito Federal, Mexico) (Fig. 1d). The rubber band is sized to hold the fabric tightly over the dish. Adult mosquitoes are introduced through that perforation. Subsequently, the hole is blocked using a 2 cm long piece of plastic drinking straw containing a piece of cotton covering the outer end (Fig. 1c). The straw simply serves as a plug to prevent escape of the mosquitoes. Each Petri dish plate covered with the tulle fabric has an estimated cost of $3.00 pesos (equivalent to 16 US cents).

To carry out the tests we used adult female (24–48 h old) of the New Orleans laboratory strain of *Aedes aegypti* L. (Diptera: Culicidae), which is susceptible to insecticides. Both the rearing and the experimental units were kept at 27 ± 2 °C, 70 ± 5% RH, and 12:12 h (L:D) photoperiod.

We used 5 formulated insecticides: Biflex® Pluss (bifenthrin, 81.37 g ai per L, aqueous suspension, FMC Agroquímica de México S. de R. L de C.V., Mexico); Cielo® (imidacloprid 3.0% + pralethrin 0.75%, oily solution, Clarke Mosquito Control Products Inc., Mexico); Aqua Reslin® SUPER (permethrin 108.7 g ai per L + sbioalethrin 1.5 g ai per L, aqueous suspension, Bayer de Mexico S.A. de C.V., Mexico); MOSQUITOCIDA UNO U.L.V. (chlorpyrifos 122.8 g ai per L, liquid insecticide in mineral oil, Public Health Supply and Equipment de Mexico, S.A. de C.V., Mexico); and Green Control® ULV (Extract of natural pyrethrins I and II at 17.5%, cinnamon extract oil 9.35%, and neem extract oil 9.35%, oily solution, Distribuidores Agrícolas Salamez S. de R.L. de C.V., Mexico).

Four fungal isolates were used in this study: (a) GC03 (*Beauveria pseudobassiana* (Balsamo) Vuillemin (Cordycipitaceae), isolated from *Phyllophaga polyphylla* (Bates) (Coleoptera: Scarabeidae); (b) Bb88 (*B. bassiana* (Bals.) Vuill., isolated from *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae); and GHA (c) with and (d) without pass through *A. aegypti* (*B. bassiana*, Mycotrol, Laverlam Int. Corp., Mexico) were

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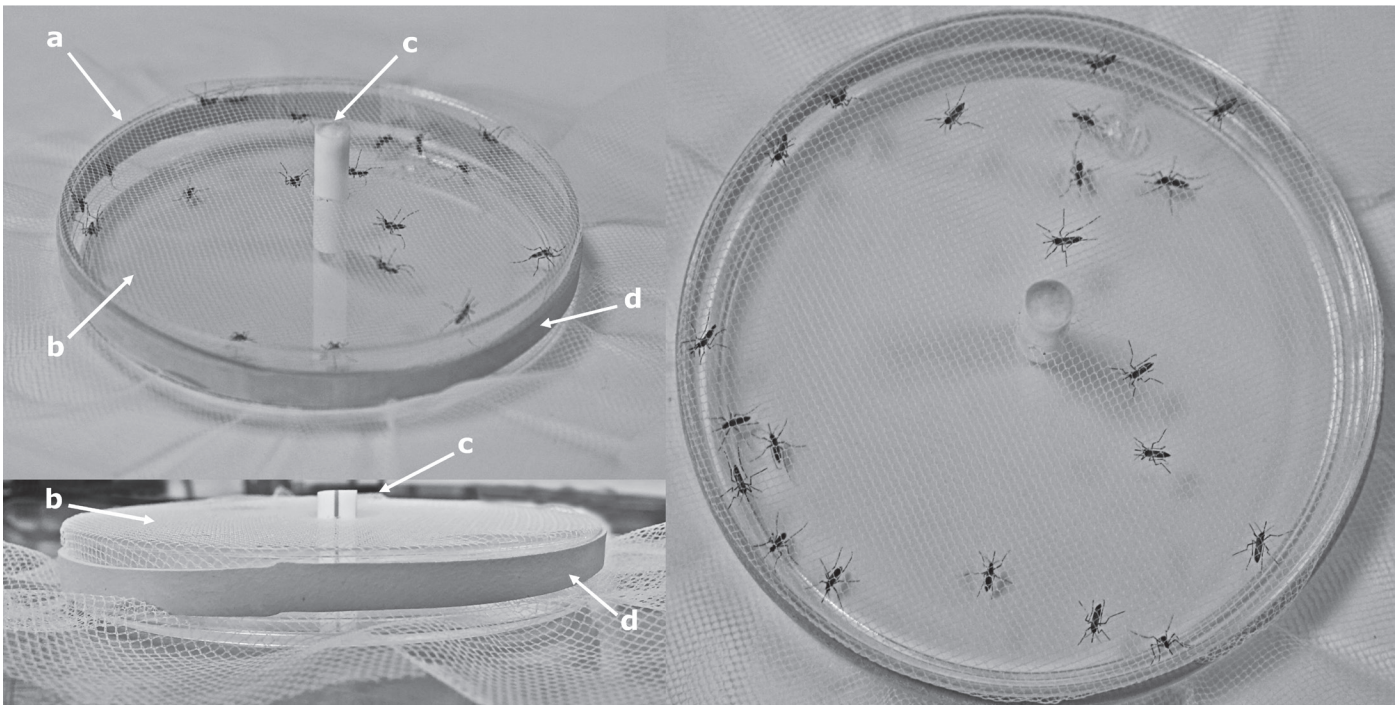

Fig. 1. Adults of *Aedes aegypti* L. contained inside a Petri dish covered with tulle: (a) Petri dish; (b) tulle; (c) straw; (d) natural rubber band.

seeded in Sabouraud Dextrose Agar culture medium (SDA, BD BIOXON®, Becton Dickinson de Mexico S.A. de C.V., Mexico, Distrito Federal), and incubated in darkness for a period of 15 to 20 d at 25 ± 2 °C. Conidia were suspended in Tween 80 (0.02% in distilled water). The concentration of conidia was estimated using a Neubauer hemocytometer and adjusted to 1×10^8 conidia per mL. In all fungi tested, the viability was ≥ 90%.

Evaluations were conducted to determine the usefulness of Petri dishes covered with tulle for the application of entomopathogenic fungi and commercial insecticide formulations. Groups of 20 to 25 adult females were confined to the Petri dish covered with tulle and were sprayed with an appropriate volume of the material being evaluated.

For assessment of entomopathogenic fungi, we applied a volume of 1.5 mL, which contained 1×10^8 conidia per mL, using a Potter tower (Burkard Manufacturing Co., Rickmansworth, Herts, United Kingdom) with a nozzle of 0.275 mm diameter at a pressure of 0.703 kg per cm^2 (10 lb per in^2). The control was treated with the diluent used in all treatments (1.5 mL of Tween 80 at 0.02%). Three replicates of the experiment were performed on different d, and each replicate included an untreated control. Treated insects were placed in entomological cages (30 × 30 × 40 cm) containing cotton soaked with a 10% sugar solution to feed the adults. For 30 d, the number of individuals dying was recorded. To verify if dead individuals had been infected with the entomopathogenic fungi, the cadavers were placed in moist chambers and incubated at 27 ± 2 °C for 7 d, after which they were checked for mycelial growth using a stereoscopic microscope.

For assessment of commercial insecticide formulations, 1.5 mL of the recommended concentration of each insecticide was sprayed via the Potter tower, as described above. The control was treated with distilled water. The treated insects were confined as mentioned previously. The percent mortality was evaluated at 15 min after application. Four replicates were performed, and each replication included an untreated control.

Logistic regression was used to analyze the mortality data produced by the entomopathogenic fungi at 20 d after the application. An analysis was performed comparing the mortality in the control against all treatments and subsequently between treatments. The analyses were conducted using the GenStat v 8.0 statistical package (Payne et al. 2005). Mortality data from formulated insecticides were subjected to an analysis of variance ($P \leq 0.05$) and the means comparison test (Tukey test, $P \leq 0.05$) using the SAS statistical software, version 9.4 (SAS Institute 2016). Data were arcsine transformed prior to analysis.

In the tests with entomopathogenic fungi, there were significant differences in the proportion of adult female *A. aegypti* killed when comparing the control against the different evaluated isolates of *Beauveria* spp. ($F = 23.0$, df = 1, 28; $P < 0.001$). When comparing isolates, significant differences were found ($F = 4.48$; df = 3, 28; $P = 0.011$), where the highest mortality at 20 d post application was produced by isolate GC03 followed by GHA (with pass and without pass for *A. aegypti*) and Bb88 (Fig. 2). Likewise, the highest proportion of cadavers with sporulation was obtained by isolate GC03 with 50%, whereas in the control (no fungus), no sporulation was observed.

In the tests with formulated commercial insecticides, the New Orleans population was susceptible to insecticides evaluated at the rec-

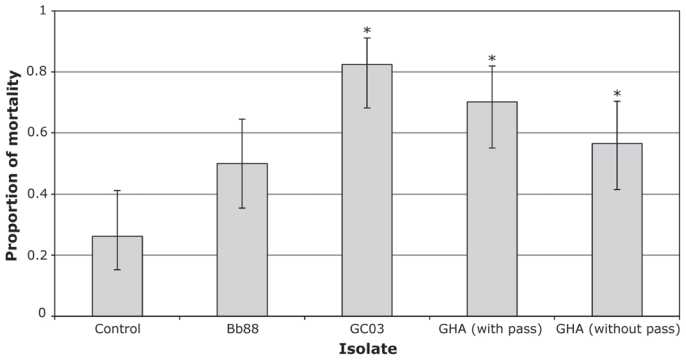

Fig. 2. The mortality proportion of *Aedes aegypti* females caused by isolates of *Beauveria* spp. at 20 d after application. Error bars represent 95% confidence intervals back-transformed from the logistic scale. An asterisk (*) indicates that the treatment was significantly different from the control.

ommended doses, demonstrating a high efficacy by direct application to adults of *A. aegypti*, where 100% mortality was achieved 15 min after application ($P < 0.0001$), while the control (no insecticide) showed 0% mortality.

Our results indicate that this bioassay is useful in order to obtain information about the susceptibility of mosquitoes towards different substances such as natural and conventional insecticides in commercial formulations, as well as for entomopathogenic fungi. When comparing the effect of entomopathogenic fungi with respect to the insecticides in commercial formulations, a slow effect was observed in causing a mortality higher than 50%. Because the fungus requires penetration of the cuticle of the insect in order to reach the hemocoel and develop, in some cases this process can cause the death of the host in a period of 3 to 14 d after the application (Gillespie & Claydon 1989). Significant differences in proportion of mortality when comparing isolates of *Beauveria* spp. could be due to natural variation in virulence (Valero-Jiménez et al. 2014). It is surprising that the isolate GC03, isolated from white grub, obtained the highest proportion of mortality, while Bb88 isolated from *H. hampei* obtained the smallest. This indicates that although they have been found to infect insects, it does not guarantee a high mortality rate in insects from other orders, so the proper selection of an isolate for mosquito control is of vital importance for the management of this vector. Similar results of the effect of entomopathogenic fungi have been observed in other studies (Scholte et al. 2007; Leles et al. 2010). In addition, the level of control was about 20%, similar to those reported for other methods of selection of entomopathogenic fungi (Scholte et al. 2007; Leles et al. 2010). The results obtained here are not different from those already reported in the literature. In the case of entomopathogenic fungi, an advantage of using application equipment such as the Potter tower allows a greater standardization of the selection method, avoiding possible biases in the results, because other methods can have a high variation in the acquisition of inoculum by mosquitoes (Leles et al. 2010; García-Munguía et al. 2011). This methodology does not require the use of impregnated substrates which can be time consuming (Paula et al. 2008; Mnyone et al. 2010; Blanford et al. 2012). Likewise, most of the selection methods require a variable time of exposure of the mosquitoes to the treated substrates, about 16 to 48 h (Paula et al. 2008; Mnyone et al. 2010; Blanford et al. 2012), which would be reduced with the proposed method.

Therefore, this bioassay could provide basic information on the effect of different insecticide formulations and entomopathogenic fungi on adult mosquitoes.

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Summary

The use of non-conventional insecticides and entomopathogenic fungi to control adult mosquitoes is increasing; however, the current methods are designed to evaluate the insecticidal action of the active ingredient. We describe the bioassay method to evaluate the effect of non-conventional insecticides and entomopathogenic fungi to control mosquitoes using the Potter tower. Twenty d after application, entomopathogenic fungi produced a proportion of mortality of 0.49 to 0.82, while non-conventional insecticides produced 100% mortality from 15 min after application. Our results showed that this bioassay could provide basic information on the effect of different non-conventional insecticides and entomopathogenic fungi on adult mosquitoes.

Key Words: mosquitoes; *Beauveria*; botanical insecticides; bioassay; Potter tower

Sumario

El uso de insecticidas no convencionales y hongos entomopatógenos, contra mosquitos está en aumento, sin embargo, las metodologías actuales de evaluación están diseñadas para evaluar la capacidad insecticida del ingrediente activo. En este estudio, describimos un método de bioensayo para evaluar el efecto de insecticidas no convencionales y hongos entomopatógenos contra mosquitos usando la torre de Potter. Los hongos entomopatógenos a los 20 días después de la aplicación produjeron una proporción de mortalidad de 0.49 a 0.82, mientras que los insecticidas no convencionales produjeron 100% de mortalidad desde los 15 minutos después de la aplicación. Nuestros resultados muestran que este bioensayo podría proveer información básica sobre el efecto de diferentes insecticidas no convencionales y hongos entomopatógenos sobre adultos de mosquitos.

Palabras Clave: Mosquitos; *Beauveria*; insecticidas botánicos; bioensayo; Torre de Potter

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