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Evaluation of the efficacy of pyrethroid pesticides against selected strains of *Aedes* mosquitoes using the who susceptibility test and by thermal fogging

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Abstract
Tests on the efficacy of pyrethroid insecticides in fogging were conducted. These were done in two approaches: 1. A laboratory-based series of the WHO susceptibility tests on F1- generation female adult *Aedes* mosquitoes, and 2. A series of actual field thermal fogging using caged sentinel F1-generation *Aedes* mosquitoes. Based on the 98% WHO susceptibility test criterion, strains of *Ae. Aegypti* have shown to have incipient resistance to pyrethroids, making them less susceptible to laboratory and field tests. *Ae. Albopictus* was found susceptible to pyrethroids, but it requires further confirmatory tests based on field test results. The study recommends molecular tests on mechanisms involved, nationwide surveillance of susceptibility/resistance of dengue vector mosquitoes, and a review of the chemical control strategies used by authorities.

Keywords: surveillance, pyrethroid, fogging, susceptibility

Introduction
Pyrethroid insecticides are neurotoxins that interact with voltage-gated sensitive sodium channels (VSSC). Insect sodium channels are more susceptible to pyrethroids than mammalian sodium channels [1]. Pyrethroids exert their toxic effect by prolonging the opening of the voltage-sensitive sodium channel, thereby paralyzing and eventually killing the insect, an effect known as "knockdown" [2, 3, 4].

Pyrethroids are the predominant chemicals used for controlling adult *Ae. Aegypti* and *Ae. Albopictus* (Skuse), the vectors of dengue viruses in the Philippines. The annual procurement plan (APP) for insecticides of the DOH Central Office alone in FY 2019 was PhP ₱192,879,074.47, and in 2020 the budget was raised to ₱265,557,829.58 ($4,917,000). But only two insecticide efficacy tests on *Aedes* dengue mosquito vectors in the Philippines have been published. One was conducted 55 years ago [5], and the other test was conducted23 years ago in Cebu City [6]. The 1967 study was a susceptibility test on *Aedes* and other Culicine mosquitoes using the WHO Susceptibility test against long-banned 1960s kinds of insecticides around Clarke Air Base, Pampanga. The study in Cebu City established that the permethrin-treated curtains effectively against dengue vector mosquitoes [6].

The WHO susceptibility bioassay is a direct response-to-exposure test [7]. It measures mosquito mortality to a known standard concentration of a given insecticide, either with a discriminating or intensity concentrations. Discriminating insecticide concentrations are concentrations of insecticide that, in a standard period of exposure, are used to distinguish the proportions of susceptible and resistant phenotypes in a sample of the mosquito population. Monitoring insecticide susceptibility of important disease vectors is significant to determine the efficacy level of chemical controls exercised in a particular location. More importantly, it may shed light on any developing resistance of vectors to insecticides used against vector population and enable vector control authorities to choose the correct insecticide to control vectors of interest.

Insecticide resistance is one of the most important issues facing mosquito control agencies and public health services. The loss of pyrethroid insecticide efficacy may lead to the failure of

mosquito vector control measures, resulting in increased disease transmission [2]. This loss will cause even more considerable capital investment into mosquito control programs by the government, e.g., the development or procurement of new insecticides, which may cause a direct socio-economic impact on a country in terms of labor and/or costs.

In the Philippines, many entomologic and dengue control researches have been conducted [8], but publications about evaluations on insecticide interventions are wanting. Without a doubt, monitoring of insecticide efficacy is necessary because

(1) It initiates the determination of insecticide resistance,

(2) Checks the appropriateness of control methods, and

(3) Determines potential causes of control failures, should they occur [7]. Since the Philippines' National Dengue Prevention and Control Programme, dengue vector interventions have been launched [9]. But has there been any evaluation of these interventions? According to literature and case studies on dengue vector-control services, none of the countries studied have a regular monitoring and evaluation process; no plans exist, and no budget is allocated for monitoring interventions [10].

This study aimed to conduct efficacy testing of pyrethroids on dengue mosquito vectors, *Ae. Aegypti* and *Ae. Albopictus*, under laboratory and field conditions. The null hypothesis tested here was that both mosquito species¸ *Ae. aegypti* and *Ae. Albopictus,* are susceptible to the insecticides used for dengue mosquito vector control.

The principal objective of this study was to determine the level of susceptibility of laboratory-reared F1-generation adults of *Ae. Aegypti* and *Ae. Albopictus* to pyrethroid insecticides. In finding out *Aedes* susceptibility to pyrethroids, first, thermal fogging was done by employing the WHO protocol of susceptibility test of *Aedes* dengue mosquitoes using diagnostic concentrations of pyrethroids. The second method was entomological field evaluation of thermal fogging using sentinel cages containing adult *Aedes* dengue vector mosquitoes. Field evaluation was done every time the Sanitary Health Inspectors of the City Health Office were on scheduled fogging activities. The evaluations were based on percent knockdown and mortality every 24-72 hours of exposure to the pyrethroid pesticides.

Materials and Methods

Establishment of Insectary

Field-caught *Ae. Aegypti* and *Aedes albopictus* larvae from tires and bamboo cuttings were reared to adulthood in shallow, white plastic pans at an insectary. Confirmation of correct test species was done during larval and adult stages by subsampling and microscopic examination of identifying characters. As adults, females of *Ae. Aegypti* and *Ae. Albopictus* emerged from the rearing pans, were segregated from males, and transferred to separate 1ft x 1ft insect cages. The males were sustained by 10% sucrose and maintained in individual cages inside large styroboxes for breeding purposes until they reached the limits of their lifespan. The adult female *Aedes mosquitoes* were secured in separate, insulated 1 ft. x 1 ft. cages until used for the WHO insecticide susceptibility test.

WHO Insecticide Susceptibility test procedure

The WHO Susceptibility test kit consists of six (6) pairs of clear, plastic tubes with specialized caps on each end and sets of insecticide-impregnated papers. The insecticide-impregnated papers used in this study contained discriminating concentrations of permethrin 0.75%, etofenprox 0.5%, bifenthrin 0.2%, and deltamethrin 0.05%. Each run of the WHO insecticide susceptibility test required hundred-fifty individual mosquitoes sampled from a vector population of interest. The whole procedure required four (4) batches of twenty-five (25) individual mosquitoes as treatment or experimental groups and two (2) other groups of twenty-five (25) individual mosquitoes each to serve as the control. Sufficient time was followed for monitoring and recording mosquito knockdowns and mortalities. The insecticide susceptibility test was done in sequences of two experimental batches and one control group at a time. The subsequent two other test batches of experimental and control groups of mosquitoes were tested a day after within the same period as the previous run (6:00-8:00 a.m. at a range of 26-28°C room temperature and relative humidity range of 60-70%). The insecticide-impregnated papers in-between tests were sealed and stored in a refrigerator.

The 2-5 days old adult female mosquitoes were transferred to smaller 5-in x 5-in cages during the test. Subsamples of 10 *Aedes* mosquitoes were first aspirated and examined morphologically to confirm the species. Scutal markings were used to ascertain the species of the test mosquitoes.

The susceptibility kit's mosquito holding tubes were first prepared by inserting and clipping rolled clean, white 12cm x 15cm papers to the interior wall of the holding tubes. The papers were labeled correctly on the outside so that no mix-up of test mosquito batches happened when they were transferred to their respective holding containers for 24-78-hrs observation.

Groups of twenty-five (25) *Aedes* mosquito individuals were aspirated from the small 5-in x 5-in cages and transferred to three (3) green-dotted holding tubes through the filling hole in the slide, preparing three (3) replicates of 25 mosquitoes per tube. The mosquitoes were secured in the holding tube by closing the sliding port and set upright for 1 hour. The number of mosquitoes immobilized (knockdown) within the hours of exposure at 10-minute intervals was recorded. After an hour, any moribund or dead were removed from the tubes using a handheld bulb aspirator. A cotton ball plug was used to prevent live mosquitoes from escaping.

The treatment and control tubes were also prepared in a different but parallel procedure. One oil-treated paper (as control) was inserted into yellow-dotted tubes, and two pyrethroid-impregnated papers (as treatment) were inserted into two red-dotted exposure tubes, with all labels of the papers facing the exterior of each tube. Wire clips were used to fasten the papers against the walls of the tubes and secured with screened screw caps.

When all these were in place, these exposure tubes and control tubes were securely screwed to the bottom of the holding tubes. The sliding units were momentarily slid open, and the mosquitoes were mouth-blown into the exposure and control tubes, then closed the sliding elements between the tubes again. A duct tape secured the sliding elements. The mosquitoes were kept in the exposure tubes and control tubes for an hour. A timer and handheld counter counted and recorded the number of mosquitoes knocked down by the insecticide at 10 minutes intervals.

After an hour, the mosquitoes that remained alive were transferred back to the holding tubes by reversing the earlier

procedure of mosquito transfer. The exposure tubes and control tubes were detached, and the dead mosquitoes in each tube were collected and preserved. The holding tubes with the live mosquitoes were stored and sustained with 10% sucrose in small screen-topped containers for 24 hours.
After 24 hours, the number of dead and live mosquitoes was counted and recorded for the final time. The mosquitoes were terminated two days after. An adult mosquito was considered alive during data gathering when it could fly, regardless of the number of legs remaining. A mosquito was deemed to be dead if it were immobile or knocked down, regardless of having complete legs or not.
The following day, the test was repeated for the second batch of two (2) experimental and one (1) control group of twenty-five (25) individual mosquitoes to complete one run of the WHO insecticide susceptibility test. Two more complete runs of the test were done in subsequent months to confirm the findings. To avoid the effects of confounding meteorological variables, no tests were done after a day of rain nor on a day with forecasted rain.

Determination of susceptibility and resistance
The assessment of mortality, i.e., the count of dead mosquitoes in both exposure and control tubes, was made during the test and 24 hours post-exposure period. Mortality was calculated by adding the number of mosquitoes killed across all four exposure and expressing this as a percentage of the total number of exposed mosquitoes:

The criterion for insecticide resistance used here was based on the WHO guidelines (WHO, 2013), which consider that a population is (1) fully susceptible if the final percentage mortality is >98%, (2) incipient resistant if 80-97 percent mortality is observed, and (3) considered resistant if mortality is <80%.

Field entomological evaluation of fogging
The schedules and locations of fogging activity by the vector control personnel of the City Health Sanitary Inspectors were regularly monitored. Upon notification by the sanitary inspector's team, preparations were done immediately to evaluate fogging on site. The sentinel mosquitoes were the most important preparation, composed of adult female *Ae. Aegypti*, and *Ae. Albopictus.* A mixture of adult, female *Ae. Aegypti* (75%) and *Ae. albopictus* (25%) in thirty (30) sentinel cages (15 indoor, 15 outdoor) were used as sentinel populations in the field. The sentinel mosquitoes were brought to the planned fogging site 1.5-2 hours ahead of the scheduled activity. They were transported in 1ft x 1ft cages draped with wet towels to prevent the desiccation of the insects during transit (Fig. 1).

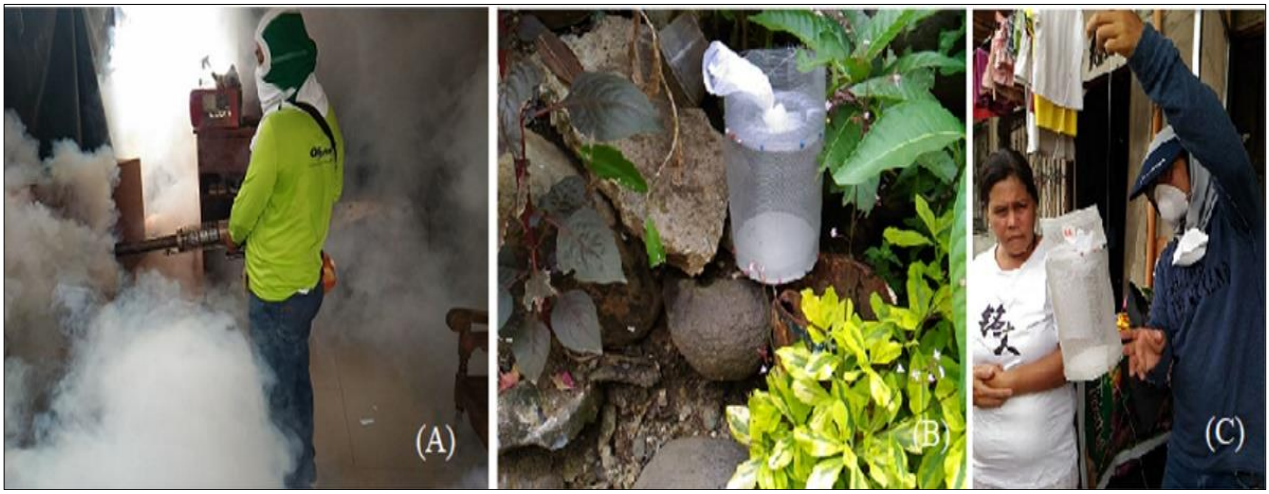

Fig 1: Indoor thermal fogging (A); an outdoor sentinel cage (B); Observing knockdown and mortality of sentinel mosquitoes in an indoor sentinel cage (C).

The mosquitoes were then transferred to 1ft (height) x 0.5ft (diameter) cylindrical sentinel cages constructed mainly from stiff polyethylene net rolled into a cylinder and a base made of circular white sliced foam. The top of the cylindrical sentinel cage was topped by a narrower cylinder of soft tulle netting to act as a "chimney" for easy access and transfer of sentinel mosquitoes (Fig 1).
Before the fogging activity and with homeowners' consent, 15 sentinel cages were hung indoors, and another set of 15 cages was placed outdoors at fifteen (15) different households. 15-30 minutes after fogging, the number of dead and moribund test mosquitoes in the sentinel cages were counted *in situ* and recorded for indoor and outdoor locations. Control setup for each experimental village was set upwind roughly a kilometer away from the fogging zone, where only crude oil mixture was used minus the insecticide.
The sentinel cages with live and dead mosquitoes were immediately taken back to their respective boxes in the transport vehicle, draped in water-soaked towels, and then brought back to the laboratory and transferred to small screen-topped containers. Surviving *Aedes* mosquitoes in sentinel cages were sustained with 10% sucrose for 24 hours. Twenty-four hours later, the number of live, moribund, and additional dead Aedes mosquitoes was counted and added to the field record for each species. The mosquitoes were further held and sustained in their respective containers for 48 hours before being terminated.

Results and Discussion
3.1. WHO Susceptibility test results
After 1-hour exposure to four pyrethroid insecticides following WHO susceptibility test methods, the total percent knockdown and mortality in sampled *Aedes aegypti* population was 85.17% and 85.33%, respectively, during the

sampled *Ae. Albopictus* population showed percent knockdown and mortality of 98.10% and 98.32%, respectively (Table 1, Fig. 2 &3). The observed 85.33% mortality in *Aedes aegypti* was too low for the acceptable range of WHO standard, 98-100% mortality. It was observed that among *Aedes aegypti* individuals that were initially knocked down during the 1-hour exposure, some 15% of these recovered.

Table 1: Average percent knockdowns and mortalities of Cagayan de Oro strains of *Aedes* dengue mosquito vectors to pyrethroid insecticides.

	Aedes aegypti		*Aedes albopictus*	
Pyrethroid	**Knockdown**	**Mortality**	**Knockdown**	**Mortality**
Permithrin 0.75%	81.67	83	98.33	98.96
Etofenprox 0.5%	86.67	86	98	98
Bifenthrin 0.2%	84.67	84	98.33	98.67
Deltamethrin 0.05%	87.67	88.33	97.67	97.67
Average	85.17	85.33	98.08	98.32

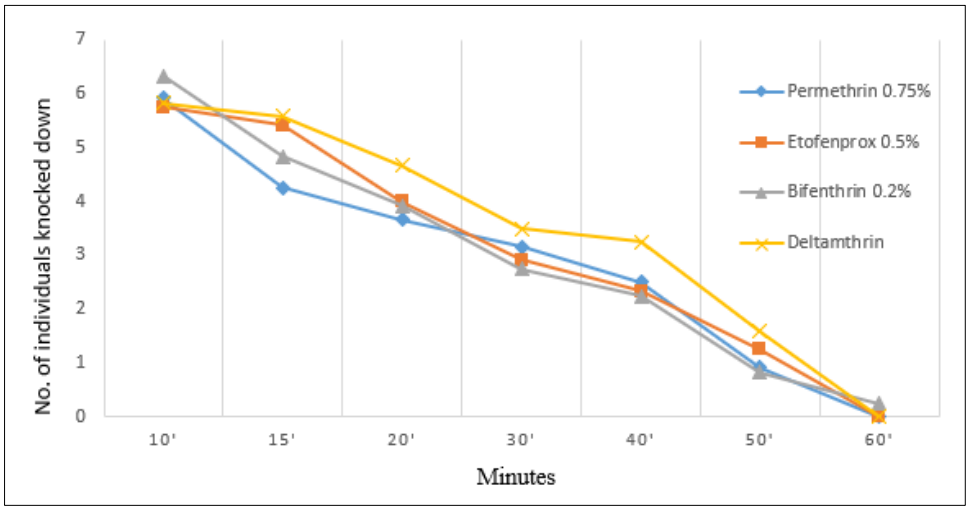

Fig 2: *Ae. Aegypti* knockdown rates in 1-hour exposure to Pyrethroids at 30-mins interval.

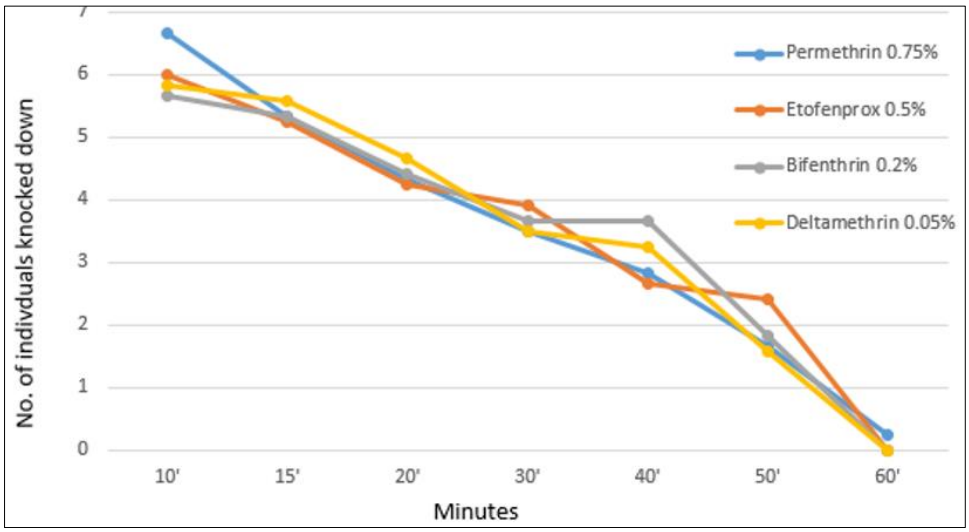

Fig 3: *Ae. Albopictus* knockdown rates in 1-hour exposure to Pyrethroids at 30-mins intervals.

The sample population of *Ae. Albopictus*, on the other hand, showed a percent knockdown and mortality at 98.02% and 98.32%. These rates lie within the lower limit of the 98-100% standard range of WHO for species susceptible to insecticides. (See Table 1). No significant differences in knockdown rates for all pyrethroids within *Ae. Aegypti* or *Ae. Albopictus* sample populations were detected, but knockdown scores between species showed very significant differences ($p=6.7x10^{-05}<0.05$) (Fig 4).

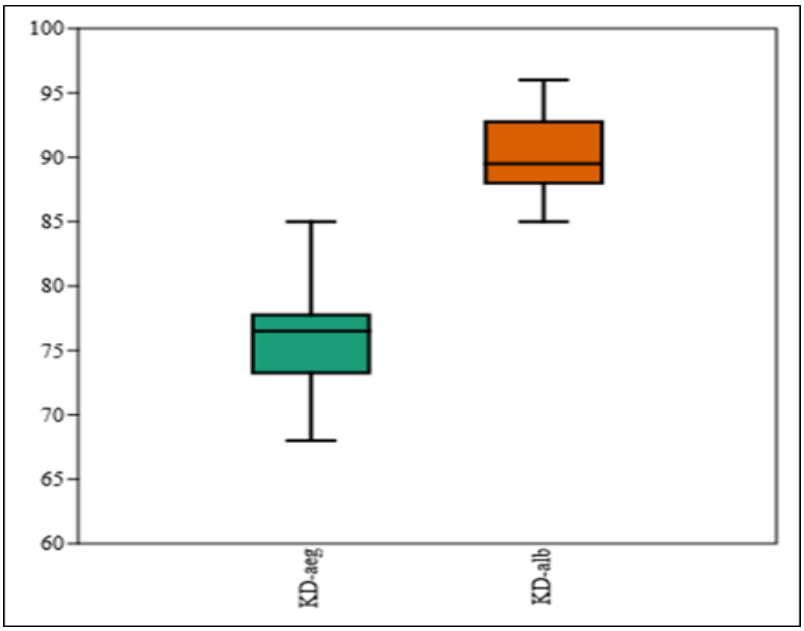

Fig 4: Boxplot of means of knockdown scores between *Ae. Aegypti* and *Ae. Albopictus*

Statistical analysis of means of mortality (dead and moribund) between *Ae. Aegypti* and *Ae. Albopictus* showed highly significant p values ($p=1.30x10^{-25}<0.05$), suggesting that the null hypothesis be rejected. According to the WHO guidelines, if observed mortality is between 90 and 97%, the presence of the resistant gene in the vector population must be confirmed by additional tests with the same insecticide on the same population, or by molecular assays for known resistance mechanisms [7]. Resistance is confirmed if at least two other tests consistently show mortality below 98%. Since molecular tests were inaccessible during this work, further rounds of susceptibility tests were the alternative to confirm the findings. Two more rounds of susceptibility tests were done following the results of the first susceptibility test a month later. The results presented here integrate the results of the follow-up susceptibility tests.

Since the first report of mosquito resistance to chlorinated-hydrocarbon insecticides, the research effort to understand insecticide resistance in mosquitoes has been intense [11]. Many studies report multiple mechanisms involved across major genera of mosquitoes, namely *Anopheles*, *Culex*, and *Aedes* [12-19]. But only two were extensively studied and widely accepted mechanisms. These mechanisms best explain how insecticide resistance develops either by increased metabolic detoxification of insecticides or target protein insensitivity [11, 26].

In recent years, insecticide resistance in *Ae, aegypti* and *Ae. Albopictus* have engaged more investigators because of the mosquitoes' global distribution and role in various disease transmission, and insecticide resistance in these species has been rampant [2, 19, 20, 21]. The increasing resistance can be due to the increased metabolic detoxification of the insecticide. The increase results in increasing detoxifying enzyme synthesis, facilitating quick biodegradation of the insecticide. The most involved metabolic detoxification mechanisms were mixed-function oxidases (p450 mediated monooxygenases), esterases, and glutathione S-transferases (GSTs). Insecticide resistance can also be attributed to target protein insensitivity. The *Vssc* (voltage-sensitive sodium channel) gene, for example, is said to undergo mutations that lead to sodium ion channel insensitivity to pyrethroids.

The contrasting pyrethroid susceptibilities of the local Cagayan de Oro strains of *Ae. Aegypti* and *Ae. Albopictus* are probably a disproportionate consequence of the city's decades-old dengue vector control strategy. During outbreaks and routine runs, the local *Aedes* vector control practice has been heavily reliant on only one approach: chemical control with thermal fogging. And this chemical control had mainly been targeting urban areas during fogging that may have exposed only the more domicile *Ae. Aegypti* than the sylvan and rural *Ae. Albopictus*. These decades of exposure may have induced more development of insecticide resistance in *Ae. Aegypti* population than in *Ae. Albopictus*. This disproportionate insecticide resistance among the major dengue vector mosquito populations has been observed elsewhere in Haiti, Thailand, New Zealand, Malaysia, Vietnam, Papua New Guinea, and Central Africa [17, 18, 22, 23 24, 25].

3.2. Caged mosquito field evaluation of thermal fogging

The CDO City Health Sanitary Inspectors used two kinds of insecticide formulations during thermal fogging activities at various Cagayan de Oro City sites: Pesguard® (consisting of σ-Tetramethrin, 40g/L + Cyphenothrin, 120g/l + solvent, 1L) and Resigen® (comprised of Bioallethrin, 7.2g/L+Permethrin,173.1g/L + Piperonyl Butoxide, 155.9g/L). These formulations compose mainly synthetic pyrethroids as mosquito-killing agents for the thermal fogging of dengue vector mosquitoes. The fogging device used was PulsFog™ K10 thermal fogging machine made by Frans Veugen Company. Data was gathered from five (5) fogging activities by the team of Sanitary Inspectors of Cagayan de Oro City Health Office, headed by Mr. Sergio Ramon R. Bautista, and another five (5) fogging activities by a private pest control team (name withheld upon request). After recording the mortality and knockdown in the field at indoor and outdoor locations, the sentinel cages were immediately transported back to the laboratory for 24-72 hours of observation and data gathering.

In the field evaluation of fogging, percent knockdown and

percent mortality of *Aedes aegypti* was 74.8% and 83.3%, respectively, while *Aedes albopictus* percent knockdown and percent mortality was 90.5% and 95.8% (See Table 2). The average percent mortality of *Aedes aegypti* calculated from indoor field evaluation was slightly lower than the laboratory findings (83.05%> 85.30%), but no significant difference was detected in their means (p=0.987>0.05). Both laboratory and field results suggest that incipient resistance is present in the population based on the WHO susceptibility test criterion. The mortality scores and average percent mortality of *Aedes albopictus* (95%) in the field were consistent with the laboratory findings.

Table 2: Percent knockdown and mortality of *Ae. Aegypti* and *Ae. Albopictus* 24 hours after fogging at various sites of Cagayan de Oro based on indoor and outdoor sentinel cages

	% Knockdown		% Mortality	
	Indoor	Outdoor	Indoor	Outdoor
Aedes aegypti	77	73	84.05	84.35
Aedes albopictus	91	90.2	96.1	95.5
Relative humidity	65	62.10	65	62.10
Temperature	29	31.90	29	31.90

When indoor and outdoor mortality scores of *Aedes* mosquitoes were compared by their means with one-way ANOVA, no significant differences were found (p=0.195<0.05). Means in mortality scores were also compared based on the insecticide brand used (Pesguard® vs. Resigen®); no significant differences were found either (p=0.750>0.05). The Resigen® formulation contains Piperonyl butoxide (PBO), a p450 inhibitor in this case, but there was no significant difference in mortalities between Pesguard® (no PBO) and Resigen® (PBO-enhanced) insecticides. This result seems to indicate insecticide resistance in *Ae. Aegypti* may be due mainly to *Vssc* gene mutations.

But when mortality scores during fogging were compared between *Ae. Aegypti* and *Ae. Albopictus*, a significant difference was found ($p=1.89x10^{-09}<0.05$).

Conclusions

The WHO susceptibility test has demonstrated low susceptibility of *Ae. Aegypti* to the currently used pyrethroids while *Ae. Albopictus* remains highly susceptible. Fogging with PesGuard® and Resigen® pesticides showed lack of significant differences in mortalities in *Ae. Aegypti* but not with *Ae. Albopictus* indicating insecticide resistance in *Ae. Aegypti*. The tests results presented in this study lend preliminary evidence that chemical control using pyrethroids is less effective for the adult *Ae. Aegypti* population than for adult *Ae. Albopictus.*

Recommendations

The evidence presented here only accounts for a small sample population of local *Ae. Aegypti* and *Ae. Albopictus* strains in Cagayan de Oro City. The results here cannot be extrapolated to a regional or country-wide situation. Therefore, it is recommended that regional or country-wide surveillance of insecticide susceptibility be done to assess the insecticide resistance in the Philippines and decide on comprehensive management of dengue vector control. It should also be important to know the distribution of dengue vector mosquito populations' insecticide resistance on a region-wide or country-wide basis.

As evidence of pyrethroid resistance in dengue mosquito vectors is mounting worldwide, this information should also be a call to re-examine the traditional dengue vector control strategies. Every strategy should always undergo evaluation regularly to monitor any resistance development.

At the local government level, instituting a systematic, evidence-based "search-and-destroy" practice against larval habitats should be implemented and strictly monitored at the local government levels. Source reduction is still the best practical alternative to chemical control.

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