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A Study of Insecticide Resistance in West Nile Virus Vectors: Culex pipiens and Culex quinquefasciatus in Shelby County, Tennessee

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A STUDY OF INSECTICIDE RESISTANCE IN WEST NILE VIRUS VECTORS: CULEX PIPIENS AND CULEX QUINQUEFASCIATUS IN SHELBY COUNTY, TENNESSEE

by

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Abstract

West Nile Virus (WNV) is a vector-borne virus transmitted to humans through mosquitoes. Shelby County, Tennessee had 138 cases of WNV and 11 fatalities from 2002 to 2014. Genus *Culex* mosquitos are an important vector for the disease. Since there is no treatment available, a WNV outbreak is a growing public health concern in Shelby County. The purpose of this study was to assess the efficacy of commonly used insecticides in Shelby County and evaluate the presence of resistance. Partial resistance was found for permethrin, deltamethrin, chlorpyrifos, and bendiocarb. Permethrin performed the poorest in comparison to deltamethrin, chlorpyrifos, and bendiocarb. Deltamethrin and bendiocarb were found to be the most effective insecticides against the *Culex* mosquito population in Shelby County. The results of the study can be expected to support future consideration and planning towards handling insecticide resistance in hope of preventing a West Nile Virus epidemic in the community.

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Chapter 1

Background

West Nile Virus (WNV) is a virus transmitted to humans by mosquitoes with potential to transition into life threatening outcomes such as aseptic meningitis or encephalitis (CDC, 2015). Treatment for WNV disease is limited to supportive therapy therefore causing it to be a public health concern, especially during an outbreak.

Shelby County, Tennessee had its first human case of WNV in September of 2002. In the following decade, a total of 138 cases of WNV and 11 fatalities were reported (Chakraverty et al., 2013). Since WNV is transmitted by mosquitoes, monitoring mosquito resistance to insecticides is important.

To monitor and manage the viral activity, the Shelby County Health Department Vector Control performs an array of services for the community including surveillance work, breeding site source reduction, mosquito larvaciding and adulticiding, and community outreach and public education (Chakraverty et al., 2013).

Since 1979, the Environmental Protection Agency (EPA) registered and permitted the use of permethrin for insecticide use (EPA, 2009). The Shelby County Health Department Vector Control has been utilizing permethrin for mosquito control since then. However, no data on resistance to permethrin has been recorded. This study is the first to evaluate the existence of resistance to permethrin in *Culex* species in Shelby County. This study will also assess resistance to other commonly used insecticides including bendiocarb, delamethrin, and chlorpyrifos, and compare the resistance status of permethrin to that of these other insecticides.

Insecticides are important tools for vector control. When resistance to insecticide develops among mosquitoes, their populations grow rapidly increasing the risk of vector-borne outbreaks in the community. For this reason, detecting and monitoring mosquito resistance is important.

The Overall Objectives

1. To examine whether there is resistance to permethrin insecticide in Shelby County in *Culex* species

2. To examine whether there is resistance to other commonly used insecticides (bendiocarb, chlorpyrifos, and deltamethrin) in *Culex* species and how each compares to the resistance of permethrin

Specific Aims

Aim 1: Assess the efficacy of permethrin in *Culex pipiens* and *Culex quinquefasciatus* mosquitoes from Shelby County

The working hypothesis: There is no difference in death rates between treated and control groups (resistance exists)

The null hypothesis: Death rates in treated and control groups are different (no resistance)

Aim 2: Assess the efficacy of bendiocarb, chlorpyrifos, or deltamethrin *Culex pipiens* and *Culex quinquefasciatus* mosquitos from Shelby County

The working hypothesis: There is no difference in death rates in treated and control groups for each of the three tested insecticides (resistance exists)

The null hypothesis: Death rates in treated and control groups are different (not resistance)

Aim 3: Compare the efficacy of permethrin vs. bendiocarb, chlorpyrifos, and deltamethrin

The working hypothesis: Death rates attributable to permethrin treatment are different from those attributable to the other three tested insecticides.

The null hypothesis: There is no difference in attributable death rates among the four insecticides

Chapter 2

Introduction & Literature Review

West Nile Virus Epidemiology

Female mosquitoes have been identified as a major vector for numerous human diseases (Hoi & Roiberg, 2014). West Nile Virus (WNV) is a neurotropic flavivirus that is acquired from bird hosts and transmitted from female *Culex* mosquitoes to humans, one of two “dead-end” hosts in the lifecycle of the virus (Barrett, 2014). The first case of West Nile Virus in the Western hemisphere was reported 17 years ago in New York. It is unknown how WNV arrived in the United States (Johnston & Conly, 2000), but one of the first two human cases in the United States had recently returned from a visit to Africa in June of 1999 where WNV was presently endemic (Johnson & Conly, 2000). Between 1999 and 2010, there were nearly three million reported infections of WNV with 39,400 of them being clinical cases across the United States (Barrett, 2014). WNV cases were found throughout the north, south, east and west of the United States (Hayes et al., 2005). *Culex pipiens* is the most important vector of WNV in Northern US, while *Culex quinquefasciatus* is the important one in Southern US (Hayes et al., 2005). The WNV infections have been more prevalent between the months of July and August following the pattern of the birds and mosquito presence in an area (Hayes et al., 2005). However, cases of WNV illness in the United States have appeared as early as April and have extended through December (Hayes et al., 2005).

The severe consequences of WNV disease, such as paralysis and brain damage, have affected nearly 17,367 people and killed 1,654 people (Barrett, 2014). Presently, there is no vaccine nor specific antiviral treatments for WNV (CDC, 2015).

Insecticides

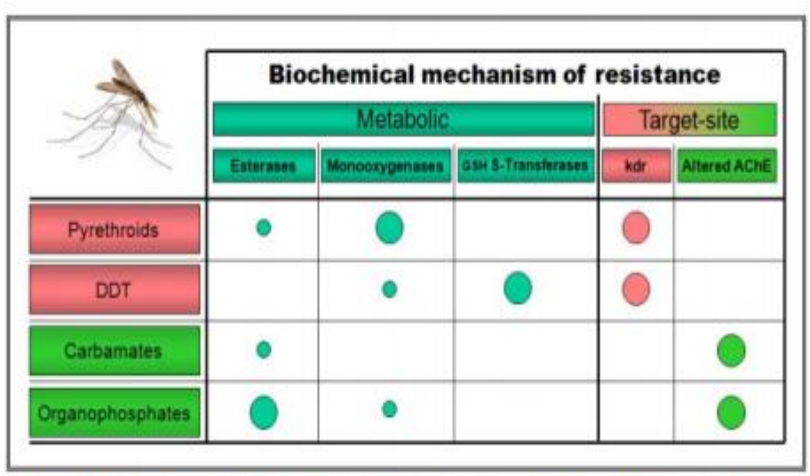
Mosquito control depends greatly on the use of insecticides. The insecticides are chemical products utilized to kill adult mosquitoes on contact and are dispersed by use of Ultra Low Volume (ULV) spraying or fogging contrivances (CDC, 2015).

There are many different types of insecticides on the market. Pyrethroids, a widely-used class of insecticides, are man-made with both peripheral and central nervous system effects on the mosquitoes (Davies et al, 2007). Upon contact, pyrethroids cause tremors, convulsions, and paralysis in the mosquito (Coats, 1990). The mechanism of action occurs as a result of interference on the voltage-gated sodium channel causing prolonged opening of the sodium channel in the nerve membrane (Coats, 1990). This causes the nerve signal to continue and overwork the central nervous system of the mosquito resulting in tremors and loss of coordination (Coats, 1990). A common pyrethroid insecticide is permethrin; it has been registered by the EPA since 1979 and is the most popular insecticide on the market as a result of its cheap price and efficiency (EPA, 2015). Deltamethrin is another common pyrethroid insecticide on the market with a similar mechanism of action (Casida, Gammon, Glickman, & Lawrence, 1983).

Organophosphates and carbamates are also common insecticides. They are cholinesterase inhibitors that interfere with nerve impulse transmission at the synapse in the Central Nervous System (Fukuto, 1990). At the active site, the organophosphate and/or carbamate block acetylcholine from attaching to its binding site (Fukuto, 1990). As a result, the mosquito undergoes tremors, convulsions, and paralysis (Fukuto, 1990). Bendiocarb is a common carbamate, and chlorpyrifos is a common organophosphate used in vector control.

Mechanism of Resistance

According to the Insecticide Resistance Action Committee (IRAC), there are four types of resistance mechanisms: metabolic resistance, target site resistance, reduced penetration, and behavioral resistance (IRAC, 2011). Metabolic resistance is based on enzyme systems that include elevating esterase enzymes to "sequester insecticides" (IRAC, 2011). The majority of the insecticides are affected by this mechanism. The next common mechanism is the target site resistance. Here, "the site of action can be modified in resistant strains of insects such that the insecticide no longer binds effectively" (IRAC, 2011). This causes the site of action to continue performing as normal and leaving the site essentially unaffected (IRAC, 2011). The third type is the reduced penetration where the "insect cuticle or digestive tract linings prevent the absorption or penetration of the insecticide" (IRAC, 2011). The fourth type is the behavioral resistance where the insect's conduct affects its exposure to the insecticide; the insect's behavior has an effect on the resistance, despite that it is not a physiological reaction like the previous three mechanisms of resistance (IRAC, 2011). Figure 1 from the Insecticide Resistance Action Committee depicts four types of insecticides: pyrethroids, DDT, carbamates, and organophosphates and their biochemical mechanism of resistance (IRAC, 2011). All four insecticides show metabolic mechanisms but the monooxygenase enzyme has a relatively larger impact on pyrethroids, and the esterase have a relatively large impact on the organophosphates. Additionally, in target site resistance, the pyrethroids and DDT site has relatively equal impact on resistance as that of carbamates and organophosphates on altered acetylcholinesterase (AChE) (IRAC, 2011).



(Circle size reflects the relative impact of the mechanism on resistance)

Figure 1From the Insecticide Resistance Action Committee depicts four types of insecticides: pyrethroids, DDT, carbamates, and organophosphates and their biochemical mechanism of resistance.

Insecticide Resistance Action Committee. (2011). Prevention and management of insecticide resistance in vectors of public health importance. *CropLife International*, 70.

Mosquito control greatly depends on the effectiveness of insecticides. According to IRAC, insecticide resistance is defined as “the selection of a heritable characteristic in an insect population that results in the repeated failure of an insecticide product to provide the intended level of control when used as recommended” (IRAC, 2011). Although resistance is not immediate, frequent reliance on and insecticide over time leads to development of resistance, especially physiologic and behavioral resistance (Liu, 2006).

Globally, pyrethroid resistance in *Culex pipiens* and *Culex quinquefasciatus* have been found in Benin, Burkina Faso, Cuba, Côte d'Ivoire, Israel, Malaysia, Tunisia, Tanzania, Saudi Arabia, Mauritius, Martinique, Zambia, Ghana, and Egypt. (Scott, Yoshimizu, & Kasai, 2015). Since pyrethroid chemical products have been commonly utilized in mosquito control, evidence of resistance has developed across the United States as well. Pyrethroid resistance in *Culex pipiens* was found in adult mosquitoes in California and in Ohio (Brogden & McAllister, 1998; McAbee et al., 2004). These studies in the published literature provide evidence for growing resistance to insecticides.

Chapter 3

Methodology

Study Area and Sampling Plan

Laboratory work was performed at the Vector Control Office at the Shelby County Health Department in Memphis, Tennessee, between May – October 2015. Mosquito samples were caught using Gravid traps located in 20 fixed sites in Shelby County. The mosquitoes were fed 10% sucrose solution and left to rest overnight in a mosquito rearing cage before testing the following day. The *Culex pipiens* and *Culex quinquefasciatus* mosquitoes were identified with the help of Vector Control entomologists. Approximately, a total of 100-150 female mosquitoes were used for testing each insecticide. Per treated and control bottles, 15-20 mosquitoes were subjected to respective insecticide exposures.

CDC Bottle Bioassay

Bioassays were performed to test resistance for permethrin, bendiocarb, deltamethrin, and chlorpyrifos insecticides according to the protocol approved by the Centers for Disease Control and Prevention (CDC) (Brogdon & Chan, 2010). Per trial, four 250 mL Wheaton bottles were labeled and coated with one milliliter of the diagnostic dose of the insecticide. An additional bottle was coated with pure acetone and served as the control. The bottles were let to dry for two hours after coating. Meanwhile, an aspirator was used to collect 15-40 female *Culex* mosquitoes and transfer them into the coated bottles. After all mosquitoes were transferred, the stop watch was started. Therefore, time 0 began after the last group of mosquitoes was introduced to the final Wheaton bottle per trial. The number of dead and alive mosquitoes were counted and recorded at 15 min intervals from 0 to 120 min when the test ended. Mosquitoes were considered dead if they could no longer stand or were immobilized (Brogdon & Chan, 2010). Three trials were run for deltamethrin, bendiocarb, and chlorpyrifos. Five trials were run for permethrin. Data were analyzed by SAS statistical software. Three trials were run for deltamethrin, bendiocarb, and chlorpyrifos. Five trials were run for permethrin. The resistance status of the mosquito population was evaluated by the World Health Organization (WHO) criteria, with 98-100% mortality suggesting susceptibility, 80-97% mortality indicating possible resistance needing confirmation, and <80% mortality suggesting resistance and indicating the need for surveillance.

Statistical Analysis

Fisher's exact test or the Chi-square test was performed to test the association between 120-min mortality of each insecticide versus their respective control. 95% Confidence Intervals (CI) for the proportion of deaths with each group were calculated using the Agresti-Coull method or the Exact method (for differences in proportions). For consistency, Fisher's exact test was used for all single experiment comparisons, since some of the tables had expected cell counts less than 5. The Chi-square test was utilized for the overall comparisons, because all expected cell counts were sufficiently large. The proportion of death attributable to each insecticide was calculated by subtracting the proportion of mortality in the control group from the proportion of mortality in the corresponding insecticide group at each time point. Experimental data was compiled in Microsoft Excel and transferred into a statistical database suitable for statistical analysis. Analyses were conducted using SAS Version 9.4 (Cary, NC). The acceptable Type-I error level was set at 5%.

Chapter 4

Results

A total of 849 female *Culex* mosquitoes collected from Shelby County, Tennessee were tested for resistance against permethrin, bendiocarb, chlorpyrifos, and deltamethrin Each insecticide was measured for its effectiveness to kill mosquitoes in comparison to its control exposure. For Aim 1, the permethrin exposure and control exposure were compared for each trial and in aggregate (Table 1). A statistically significant difference (p-value<0.0001) was found in the aggregate mortality between the control and the permethrin exposure (Table 1). The significant difference in the proportion of death between groups indicates that resistance to permethrin in this mosquito population was not 100%.

Table 1

Comparison of 120-minute Mortality between Permethrin and Control

	Total sample	**% Dead**	**Total Control**	**% Dead**	**Fisher's P-Value**	**Chi Square P-Value**
Permethrin Trial 1	34	47	11	18	0.1563	
Permethrin Trial 2	47	78	10	0	0.0000	
Permethrin Trial 3	53	32	15	7	0.0549	
Permethrin Trial 4	50	82	12	67	0.2559	
Permethrin Trial 5	65	29	13	0	0.0306	
Total	249	53	61	18		<.0001

Bendiocarb exposure was compared to its control exposure. The Chi Square P-value indicated a statistically significant difference (p-value<0.0001) in the collective mortality between the control and the Bendiocarb exposure (Table 2), indicating that resistance to bendiocarb was not 100%.

Table 2

Comparison of Percentage Mortality between Bendiocarb and Control

	Total sample	% Dead	Total Control	% Dead	Fisher's P-value	Chi Square P-Value
Bendiocarb Trial 1	56	79	18	0	<.0001	
Bendiocarb Trial 2	59	73	13	0	<.0001	
Bendiocarb Trial 3	56	80	11	0	<.0001	
Total	171	77	42	0		<.0001

Additionally, chlorpyrifos exposure was compared to its control exposure, and the Chi Square value indicated a statistically significant difference (p-value<0.0001) in aggregate mortality between the control and the chlorpyrifos exposure (Table 3). This suggests there is not 100% resistance to chlorpyrifos.

Table 3

Comparison of Percentage Mortality between Chlorpyrifos and Control

	Total sample	% Dead	Total Control	% Dead	Fisher's P-Value	Chi Square P-Value
Chlorpyrifos Trial 1	49	65	11	0	0.0001	
Chlorpyrifos Trial 2	51	45	17	18	0.0499	
Chlorpyrifos Trial 3	49	69	7	0	0.0007	
Total	149	60	35	9		<.0001

Finally, the deltamethrin exposure was compared to its control exposure, and the Chi Square value indicated a statistically significant difference (p-value<0.0001) in the total mortality between the control and the deltamethrin exposure (Table 4). Again suggesting there was not 100% resistance to the insecticide.

Table 4

Comparison of Percentage Mortality between Deltamethrin and Control

	Total sample	% Dead	Total Control	% Dead	Fisher's P-Value	Chi Square P-Value
Deltamethrin Trial 1	39	100	10	0	<.0001	
Deltamethrin Trial 2	36	61	11	27	0.0832	
Deltamethrin Trial 3	33	100	13	8	<.0001	
Total	108	87	34	12		<.0001

Head-to-head comparison between permethrin, deltamethrin, bendiocarb, and chlorpyrifos was addressed by comparing proportions of death with 95% CIs (Table 5). Upon comparison, deltamethrin and bendiocarb display the highest levels of insecticide efficacy with 87% (95% CI: 79%, 92%) and 77% (95% CI: 70%, 82%) mortality. Permethrin shows the lowest performance of efficiency at 53% in this study population.

Table 5

Head to Head Comparison of Tested Insecticides

	Treatment Group			
	Deaths	**Total**		**95% CI**
Permethrin	132	249	0.53	(0.47, 0.59)
Deltamethrin	94	108	0.87	(0.79, 0.92)
Bendiocarb	131	171	0.77	(0.70, 0.82)
Chlorpyrifos	89	149	0.60	(0.52, 0.67)

Insecticides were further compared based the proportion of death attributable to the insecticide in each group (adjusted for control group mortality). The highest attributable mortality was in the deltamethrin and bendiocarb groups, with 75% (95%CI: 59%, 87%) and 77% (95% CI: 62%, 88%) mortality, respectively (Table 6). The percentage mortality from Table 6 indicate possible resistance to all four insecticides based on the WHO criteria.

Table 6

Head-to-Head Comparison of tested insecticides with attributable deaths

	Treatment Group				Control Group				
	Deaths	**Total**		**95% CI**	**Deaths**	**Total**		**Attributable Deaths**	**95% CI**
Permethrin	132	249	0.53	(0.47, 0.59)	11	61	0.18	0.35	(0.21, 0.48)
Deltamethrin	94	108	0.87	(0.79, 0.92)	4	34	0.12	0.75	(0.59, 0.87)
Bendiocarb	131	171	0.77	(0.70, 0.82)	0	42	0.00	0.77	(0.62, 0.88)
Chlorpyrifos	89	149	0.60	(0.52, 0.67)	3	35	0.09	0.51	(0.34, 0.67)

Figure 2 exhibits aggregate mortality data for each insecticide over time for the 120-minute study. Figure 3 displays the attributable mortality over time, for each insecticide. In both figures, every 15-minute interval marks a count point denoting the total number of mosquitoes that were determined to be dead. All four insecticides produced lines with positive slopes, implying increasing mortality over the 120-minute period. Both figures demonstrated deltamethrin and bendiocarb to be superior in performance compared to permethrin and chlorpyrifos. Both insecticides operated quickly, within the first 30 minutes of testing. Furthermore, deltamethrin resulted in 34% mortality beginning from time 0. This suggests high potency of the insecticide at onset of exposure. It is interesting to note the plateau effect of bendiocarb with a gradual positive increase after 15 minutes.

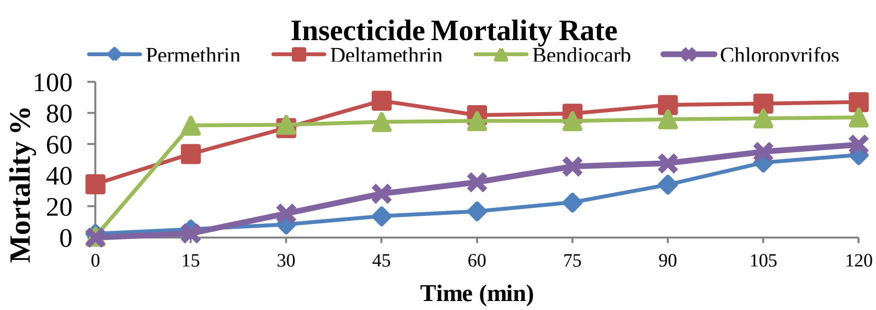


Figure 2 depicts the raw aggregate insecticide mortality over 120 minutes.

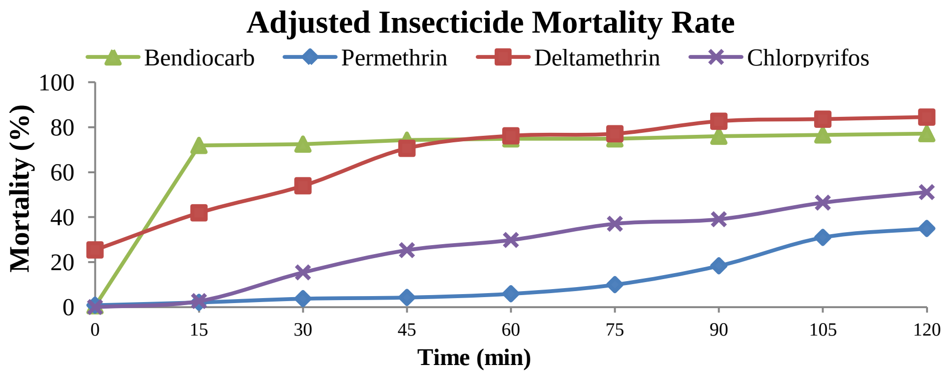


Figure 3 depicts the adjusted aggregate insecticide mortality over 120 minutes.

Chapter 5

Discussion

Mosquitoes are vectors for a number of serious illnesses including WNV, malaria, and dengue. Globally, mosquito-borne diseases are the leading cause of death, and revival of these diseases is chiefly due to insecticide resistance (Liu, 2015).

This study determined the status of resistance present in *Culex pipiens* and *Culex quinquefasciatus* to four insecticides: permethrin, bendiocarb, chlorpyrifos, and deltamethrin. Partial resistance in the mosquito samples was found for each insecticide. Permethrin performed the poorest of the four insecticides, and deltamethrin and bendiocarb performed the best.

Since the introduction of permethrin to Shelby County, this study was the first to assess the status of resistance in the *Culex* species. Complete resistance was not found in permethrin, however, it was not completely effective in killing mosquitoes. Additionally, tests of the effectiveness of bendiocarb, deltamethrin, and chlorpyrifos also indicated partial resistance in the sampled mosquito population.

Finally when comparing each insecticide head to head, permethrin performed at a 53% (95% CI: 47%, 59%) efficacy rate. As expected, it proved to be the poorest performer in contrast to the other three insecticides. Comparatively, deltamethrin and bendiocarb performed the best yielding the highest mortality rates in the samples tested.

Bendiocarb's attributable death proportion was nearly twice as high as that of permethrin (Table 6). Partial resistance to all four insecticides was evident, however, deltamethrin and bendiocarb demonstrated the least resistance compared to chlorpyrifos and permethrin (Table 6).

All four insecticides studied produced mortality rates <80% (Table 5). Bendiocarb and deltamethrin may have produced better results due to the limited exposure in the Shelby County area. A study in China found evidence of deltamethrin resistance in Chaoyang, Fuxin, and Yingkou regions, but resistance was not established in Panjin region (Li et al, 2007). This study suggests that insecticide use varies from region to region and careful monitoring may prevent the rise of insecticide resistance (Li et al., 2007).

Applying this evidence to the WHO criteria, this population of *Culex* species tested in Shelby County is considered resistant to each of the tested insecticides (WHO, 2013). Similarly, a study in West Africa (Corbel et al., 2007) found resistance in the *Culex* species to all three classes of insecticides using the WHO standards. Resistance to insecticides could be the result of many factors. Resistance could stem from prolonged insecticide exposure in Shelby County from daily spraying exposure in the summer time. Furthermore, overuse of insecticides in the agricultural parts of the county could contribute to resistance as well (Diabate et al., 2002). Moreover, polluted areas with higher incidence of mosquito breeding locations may influence the transmission of insecticide resistance in future progeny (Kabula, Attah, Wilson, & Boakye, 2011).

Limitations

One limitation of this study was the lack of a controlled environment; temperature, humidity, and barometric pressure were not monitored in the laboratory setting. Because mosquitoes are fragile organisms, a cooler temperature, for example, may weaken or kill a mosquito. Therefore, without proper control of an environment, it is difficult to ascertain that the insecticides contributed to the total mortality in the laboratory setting. It was also difficult to identify the age of the adult mosquito upon testing. Natural mortality from age could have occurred impacting the overall mortality. In addition, human error may have resulted in possible misclassification of dead or alive mosquitoes during the counting process. Three trials were run per insecticide test to account for possible human error, but this may not have been sufficient to control for human error.

Additionally, sample collection depended on weather and location site of mosquito traps. On rainy days, the mosquitoes in the gravid traps were found to be dead. This limited available days to collect samples contributing to the overall small sample size. We did meet the minimal mosquitos per trial called for in the methods. However, with a larger number of live mosquito samples, more trials could be run to strengthen results for future studies.

Finally, the mosquito samples were tested only during one summer season which may not show the complete representation of effectiveness or resistance. Future studies can look into testing mosquitoes for resistance over multiple summer seasons to gain deeper insight into efficacy of the insecticides.

Recommendations

This study is the first in Shelby County, TN to assess resistance to the insecticides commonly used against mosquitoes in the community. Resistance was found in each insecticide, therefore, according to the WHO criteria, resistance surveillance measures must be taken. Precautions should be taken by the Shelby County Health Department Vector Control through active surveillance that monitors the progression of resistance in the county. According to a study, "resistance surveillance has three objectives: 1) provide baseline data for program planning and pesticide selection before the start of control operations 2) detect resistance at an early stage so that timely management can be implemented and 3) continuously monitor the effect of control strategies on resistance" (Brogdan & McAllister, 1998). Since resistance was detected at an early stage, the Shelby County Health Department should implement protocols to carefully monitor the use of insecticides during mosquito season. As suggested by Brogdan and

Chan, resistance monitoring should be conducted at the beginning and end of the mosquito season annually (Brogdan & Chan, 2010). Also, given permethrin's poor performance, the Health Department should consider discontinuing its use and replacing it with deltamethrin or bendiocarb as alternate insecticides.

Additionally, use of synergists, organic compounds that enhance the potency of insecticides, should be considered as part of the Health Department's resistance management plan (B-Bernard & Philogene, 1993). Since resistance in Shelby County was detected early, synergists may be a proactive tool to invest in better mosquito control. Finally, additional efforts to eliminate breeding areas such as locations with standing water, discarded tires, and empty planting pots should be considered.

Chapter 6

Conclusion

Insecticide resistance is an important public health issue as it can “directly affect the reemergence of vector-borne diseases” in the public (Krostad, 1996). This study identified the presence of partial resistance in *Culex* mosquitoes to all four common insecticides used in Shelby County. Moreover, none of the tested insecticides in this study met the WHO standards of an effective insecticide; this level of resistance potentially compromises the health of the citizens of Shelby County, TN. Therefore, mosquito surveillance is an essential step to towards managing resistance in the community (Brogdan & McAllister, 1998). This is an important finding for Shelby County and the future of mosquito control. The results of this study can support future consideration and planning toward managing resistance in efforts to prevent future vector-borne outbreaks in the community.

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