EARLY DETECTION OF INSECTICIDE RESISTANCE IN MOSQUITOES

by

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ABSTRACT

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IN MOSQUITOES

Milagros Solá

Vector-borne diseases and those with intermediate hosts are among the major causes of illness. Therefore, the use of pesticides becomes indispensable to control various harmful organisms for the sake of human health. Insect resistance to chemical pesticides presents a public health challenge for the control of vector-borne diseases that make up for a large fraction of the global burden of disease. Will early detection of insecticide resistance effectively manage vector control and insecticide application? Early detection of resistance is essential to prevent both unnecessary insecticide application and disease transmission. Early detection is of major importance in the control of diseases with high mortality levels or in this research case insecticide resistance in mosquitoes. Are molecular biology methods the best avenue to detect and manage resistance? At this point of time a combination of well managed and developed bioassays and molecular methods is the most beneficial and cost effective strategy to detect insecticide resistance. Detecting resistance early is one part of the management strategy; the other is to have alternative treatments available when the previous are no longer effective. Using sound Integrated Pest Management (IPM), insecticide rotations and thoroughness in treating will ensure positive results in insecticide resistance management programs.

INTRODUCTION

Millions of humans are killed or disabled annually from insect-borne diseases and world losses from insect diseases, weeds and rats are estimated at more than $100 billion annually. Many kinds of human disease are caused by organisms carried by insects. In the 19th century, the Panama Canal was abandoned by the French because more than 30,000 laborers died from yellow fever and malaria. As late as 1955, malaria (transmitted from person to person only by female mosquitoes belonging to the genus *Anopheles*) infected more than 200 million persons throughout the world [1]. About 76% of households in the U.S. treated their homes themselves for insects, while 20% hired a commercial applicator to treat for pests such as fleas, roaches or ants. Therefore, the use of pesticides becomes indispensable to control various harmful organisms for the sake of human health.

Insect resistance to chemical pesticides presents a public health challenge for the control of vector-borne diseases that make up for a large fraction of the global burden of disease. Presently, insecticides are applied under the assumption that mosquitoes are susceptible. However, mosquitoes can develop resistance to insecticides through adaptive genetic mutations. Surveillance is necessary to ensure the continuous efficacy of pesticides, and to help us understand the insecticide resistance mechanisms in disease vectors.

Early detection of resistance is therefore essential to prevent both unnecessary insecticide application and disease transmission. Detection of resistance requires that a method is sensitive enough to detect a very small number of resistant mosquitoes. Are molecular biology methods the best avenue to detect and manage resistance? Molecular biology techniques offer a way to detect resistance that is based on mechanisms that involve changes at the level of nucleic acids. They are sensitive, and applicable to infectious agents that cannot be detected by cell culture. Molecular techniques are rapid, therefore, can be performed in a few hours. Bioassays, Biochemical and Immunological are methods also used to detect and monitor resistance. Bioassay tests are susceptibility tests, which are used to measure resistance, but are time consuming. Both Biochemical

and Bioassays tests are more time consuming than Molecular techniques, but they are less expensive and easier to perform.

Will early detection of insecticide resistance effectively manage vector control and insecticide application? Early detection of resistance is essential to prevent both unnecessary insecticide application and disease transmission. Early detection is of major importance in the control of diseases with high mortality levels or in this research case insecticide resistance in mosquitoes.

This thesis intends to visit different aspects of insecticide resistance in mosquitoes, highlighting mosquito-borne disease as a major public health, resistance mechanisms, and the methods available to detect and monitor resistance. A glossary and appendices at the end of the thesis are provided for additional information. Chapter one, Vector-borne Disease and Public Health, describes the importance of mosquitoes as vectors for diseases and how affects human health. The geographical regions were different species of mosquitoes are found, and the most significant mosquito groups are also discussed in detail. The mosquito species relevant to public health are depicted including the efforts on how to control transmission of mosquito associated diseases with the application of insecticides. The methods used by pest controllers give an idea about the effort employed to improve the efficacy of these insecticides. In addition, the toxicity and mechanisms of action of these chemicals are explored in details for a better understanding of the effectiveness of insecticides. Lastly, cases studies are discussed to expand on the implications of insecticide use and developing of resistance on mosquito populations.

The second chapter, Molecular Markers of Insecticide Resistance in Mosquito Populations, goes into details on how pesticides work. Insecticides effects at the molecular level (body cells' mechanisms) and any modification in insect behavior are described in detail.

On the last chapter, Methods of Detecting and Monitoring Resistance, the methods of detection of insecticide resistance, the way they detect changes in the susceptibility of a population of vectors, and how insecticide resistance is monitored through these methods are discussed in detail. A cost comparison between methods of detection is provided to increase awareness on cost effectiveness while performing these

tests. In addition, the criteria considered for the use of different methods is showed to explore advantages and disadvantages of the methods discussed in this section.

At this point in time a combination of well-managed and developed bioassays and molecular methods is the most beneficial strategy to detect insecticide resistance. Detecting resistance early is one part of the management strategy; the other is to have alternative treatments available when the previous are no longer effective. Using sound Integrated Pest Management (IPM), insecticide rotations and thoroughness in treating will ensure positive results in insecticide resistance management programs. Therefore it is imperative to keep studying pesticides and their modes of action, thus, we can create and use the less toxic chemical that would cause less damage to our environment.

CHAPTER 1

Vector-Borne Disease And Public Health

Vector-borne diseases and those with intermediate hosts are among the major causes of illness and death in many tropical and subtropical countries; and account for around 17% of the estimated global burden of infectious diseases [2]. Vector-borne diseases consist simply of a triad that includes an arthropod vector, a vertebrate host, and a parasite. A vector is an arthropod responsible for the transmission of parasites among vertebrate hosts, so vectors transmit parasites, not diseases. Disease is the response of the host to invasion by or infection with a parasite. A parasite is any organism, including viruses, bacteria protozoa, and helminthes, that is dependent upon the host for its survival. Parasites may or may not cause disease, and when a parasite injures its host and causes disease, it is referred to as a pathogen or disease agent. A vector-borne disease, therefore, is an illness caused by a pathogen that is transmitted by an arthropod. Arthropod vectors transmit many reemerging diseases. The spread of pathogens by arthropods is especially complex, because in addition to interactions between the vertebrate host and the parasite, an arthropod is required for transmission of the parasite to uninfected hosts. Environmental factors such as temperature and rainfall impact these processes by affecting the rate of parasite maturation within the arthropod host as well arthropod abundance in time and space [3].

The same chemicals used in control of vector insects that harbor viruses and parasites detrimental to human health are used in the agriculture. Imagine a future world population of more than 10 billion, and the amount of land available to grow food will continue to diminish. In addition to diminishing land, pest infestations threaten the success of the crop and require artificial control measures to prevent economic losses to the crop. Plants on which humans and other animals depend for life are susceptible to some 100,000 diseases caused by viruses, microorganisms or other plants [1]. Ware and Whitacre state that approximately 1800 species of weeds are responsible for serious crops economic losses; and about 10,000 of known insects contribute to the devastating loss of crops worldwide.

There is no doubt that pest resistance to chemical control measures can have devastating implications for a grower's ability to produce a crop. A serious consequence of depending exclusively on chemical control is the effects of chemical pesticides on non-target organisms. For example, insecticides are applied to an agricultural crop for only a few pest insects. In most cases these few key species require this artificial control measure to prevent economic losses to the crop.

1.1 Mosquitoes as vectors of diseases

This chapter describes the importance of mosquitoes as vectors for diseases and how affects human health. The geographical regions were different species of mosquitoes are found, and the most significant mosquito groups are also discussed in detail. The mosquito species relevant to public health are depicted including the efforts on how to control transmission of mosquito associated diseases with the application of insecticides. The methods used by pest controllers give an idea about the effort employed to improve the efficacy of these insecticides. In addition, the toxicity and mechanisms of action of these chemicals are explored in details for a better understanding of the effectiveness of insecticides. Lastly, cases studies are discussed to expand on the implications of insecticide use and developing of resistance on mosquito populations.

According to Mullen & Durden, in 1878, mosquitoes were the first arthropods formally incriminated as intermediate hosts of vertebrate parasites, and are the most important arthropods affecting human health. Mosquitoes transmit numerous diseases such as malaria; St. Louis, Eastern, Western, West Nile and LaCrosse encephalitis; dengue-dengue hemorrhagic fever; yellow fever, filariasis. Such diseases significantly impede economic and social development. In the 19^{th} century, the Panama Canal was abandoned by the French because more than 30,000 laborers died from yellow fever and malaria. As late as 1955, malaria (transmitted from person to person only by female mosquitoes belonging to the genus *Anopheles*) infected more than 200 million persons throughout the world. The Centers for Disease Control and Prevention in Atlanta estimates that about 1,000 cases of malaria are imported into the U.S. annually, usually

from travelers to Africa, Southeast Asia and South America [1]. The West Nile Virus (WNV) first appeared in New York State in 1999 and now has spread over large portions of the U.S. In 2006, there were 4269 U.S. human cases of WNV which accounted for 177 deaths [4].

Mosquito-borne diseases are especially severe in developing regions of the tropics, but they also persist in industrialized temperate countries. Mosquitoes occur in practically every region of every continent in the world except Antarctica. Arctic tundra, boreal forests, high mountains, plains, deserts, tropical forests, salt marshes, and ocean tidal zones are examples of biotic communities where mosquitoes are developed.

Mosquitoes belong to the family Culicidae, derived from *culex*, the Latin name for "gnat". Culicidae consists of about 3,200 recognized species, and is classified in three subfamilies: Anophelinae, Culicinae, and Toxorhynchitinae [3]. There are 38 genera of mosquitoes, 34 of which belong to the subfamily Culicinae. Culicines are organized into 10 tribes, where Aedini and Sabethini are the most diverse in terms of numbers of genera and species worldwide. The other 8 tribes are Aedeomyiini, Culicini, Culisetini, Ficalbiini, Hodgesiini, Mansoniini, Orthopodomyiini, and Uranotaeniini. The 14 genera in North America north of Mexico, and the number of species in each (parenthesis), are *Anopheles* (16), *Aedes* (7), *Ochlerotatus* (69), *Psorophora* (15), *Haemagogus* (1), *Culex* (29), *Deinocerites* (3), *Culiseta* (8), *Coquillettidia* (1), *Mansonia* (2), *Orthopodomyia* (3), *Wyeomyia* (4), *Uranotaenia* (4), and *Toxorhynchites* has one species. *Anopheles gambiae*, *Culex pipiens* complexes and the *Aedes* subgenus *Stegomyia* are the three important groups of mosquitoes worldwide. The *Anopheles gambiae* complex of Africa consists of six species. Two of these, *An. gambiae* and *An. arabiensis*, are important vectors of malaria and lymphatic filariasis. *An. arabiensis* tends to occur in drier regions than does *An. gambiae*. Both prefer to bite humans, but *An. gambiae* is more anthropophilic[1], endophilic, and endophagic; therefore it is the more important vector [5].

The *Cx. Pipiens* complex is a group of closely related domestic species. The medically most important taxa worldwide are the temperate species *Cx. pipiens*, the northern house mosquito, and the tropical and subtropical *Cx. quinquefasciatus* (or *fatigans*), the southern house mosquito. Their ranges are overlapping in the central

[1] The definition and explanation of terms are available in the glossary.

latitudes of the United States, where they commonly hybridize. In the Southeastern USA, *Culex quinquefasciatus* (Say) is moderately competent as a vector of West Nile virus (WNV) and is a primary vector of Saint Louis encephalitis virus (SLEV) in many urban settings [6]. *Cx. molestus* is a name sometimes applied to a variant of *Cx. pipiens*, which is facultatively autogenous and often breeds in subterranean water. *Cx. pallens*, apparently a stable hybrid of *Cx. pipiens* and *Cx. quinquefasciatusI*, occurs in temperate China and Japan, whereas *Cx. globocoxitus* and *Cx. australicus* inhabit Australia.

Aedes aegypti and *Ae. albopictus* belong in the large subgenus *Stegomyia*, and are also medically important. *Ae. aegypti*, the yellow fever mosquito, has a worldwide distribution in the tropics and subtropics. It is the primary vector of both dengue and urban yellow fever viruses. It exists in at least two forms, *aegypti* and *formosus*, considered to be subspecies or separate species. *Ae. aegypti formosus* is the original feral form and is found in large parts of interior Africa. *Ae. a. aegypti* occurs mainly in coastal regions of Africa and is distributed throughout much of southern Asia and most warmer parts of the New World, including the southern United States. *Aedes albopictus*, the Asian tiger mosquito, also transmits dengue virus. It was largely confined to Asia, where it occurs in tropical and subtropical rural settings. A cold-hardy, egg diapausing strain of this mosquito has been carried from northern Japan to other parts of the world by the trade in used automobile and truck tires. The first established population was detected in Texas in 1985. It has since spread through much of the southern, central, and eastern United States, including the upper Midwest, much farther north than the nondiapausing *Ae. aegypti*. Other important members of the subgenus *Stegomyia* include *Ae. africanus*, *Ae. bromeliaei*, and *Ae. luteoceophalus*, which transmit yellow fever virus in parts of Africa, and *Ae. polynesiensis* and *Ae. pseudoscutellaris*, which transmit lymphatic filariasis in South pacific islands [3].

1.2 Insecticides as a control strategy

The primary approach used to control transmission of mosquito associated diseases has mainly relied on the application of insecticides. However, two factors affect the effectiveness of this approach. First, mosquito-borne diseases are now resurgent mainly due to the difficulty in controlling vectors that have developed resistance to insecticides. Second, adverse effects of chemical application to human health and the environment pose a concern. Thus non-chemical control methods, reducing or limiting pesticide application, or a combination are receiving more attention.

Mosquito control in the United States has evolved from reliance on insecticide application for control of adult mosquitoes (adulticide) to integrated pest management (IPM) programs that include surveillance, source reduction, larvicide, and biological control, as well as public relations and education [7]. Kline (2006) stated that the IPM approach that Rose (2001) uses has worked well for control of the immature stages of mosquitoes because there are many options to choose from, but few options exist for use against adult mosquitoes. Besides adulticides, available options consist of personal protection (contact repellents and protective clothing) and public education (e.g., stay indoors and avoid exposure to mosquitoes during peak biting activity time) [8].

Surveillance programs track diseases harbored by wild birds and sentinel chicken flocks; vector-borne pathogens in mosquitoes; adult and larval mosquitoes and larval habitats (by aerial photographs, topographic maps); mosquito traps; biting counts; and follow-up on complaints and reports by the public. Source reduction consists of elimination of larval habitats or rendering of such habitats unsuitable for larval development. Public education is an important component of source reduction. Many county or state mosquito control agencies have public school education programs that teach children what they and their families can do to prevent mosquito proliferation. Other forms of source reduction include open marsh water management, in which mosquito-producing areas on the marsh are connected by shallow ditches to deep water habitats to allow drainage or fish access; and rotational impoundment management, in

which the marsh is minimally flooded during summer but is flap-gated to reintegrate impoundments to the estuary for the rest of the year.

Biological control includes use of many predators (dragonfly nymphs and other indigenous aquatic invertebrate predators such as *Toxorhynchites* spp. predacious mosquitoes) that eat larvae and pupae; nevertheless, the most commonly used biological control adjuncts are mosquito fish, *Gambusia affinis* and *G. holbrooki*. Naturally occurring *Fundulus* spp. and possibly *Rivulus* spp., killifish, also play an important role in mosquito control in open marsh water management and rotational impoundment management.

Mosquito traps (such as the New Jersey and the Centers for Disease Control and Prevention designs) have been used for monitoring mosquito populations for yearsThey are designed to use compressed carbon dioxide, burning propane, and octenol to attact mosquitoes and fans to control air flow. These traps may cost over $1,000 each [7]. Electric hig-voltage insect traps ("bug zappers") with "black" or ultraviolet light sources are also available.

Methods for general pest control can be classified into the following categories: chemical, the control of pests by the use of pesticides; biological (biocontrol), the reduction of pest numbers by predators, parasites, or pathogens; biorational, the use of the most sophisticated biochemical and microbial tools emerging from biotechnology; growth or reproduction of pest species; physical and mechanical, the application of direct or indirect measures that kill the pest other than by chemical means; and regulatory control, the prevention of the entry and establishment of undesirable plant and animal pests in a country or area and the eradication , containment or suppression of pests already established in limited areas.

In the development of insecticides there has been a succession of different chemicals, each representing an effort to improve the efficacy and reduce the limitations. The first-generation insecticides were stomach poisons, such as the arsenicals, heavy metals and fluorine compounds. The second generation included the familiar contact insecticides: organochlorines, organophosphates, carbamates, formamidines and pyrethroids. Biorationals are the third generation of insecticides. These agents and organisms are generally environmentally sound, comprise natural constituents of insects

or plants or are natural organisms. Characteristics that distinguish biorational pesticides or biopesticides from conventional ones include: very low orders of toxicity to non-target species, pest targets are specific, generally low use rates, rapid decomposition in the environment, and reduce reliance on conventional pesticide products. Environmental protection Agency (EPA) places biopesticides into three categories: microbial pesticides (bacteria, fungi, viruses or protozoa); biochemicals (natural substances that control pests by non-toxic mechanisms, e.g., insect pheromones); and plant-incorporated protectants (primarily transgenic plants, e.g., *Bt* corn). Biorationals then are the ideal pesticides, affecting only the target pest and having few if any side effects. In 1994, the Biopesticides and Pollution Prevention Division (BPPD) was established by the U.S. EPA. Although EPA requires careful review of biopesticide safety data (chemical composition, toxicity, degradation, etc.) prior to registration, these lower risk products are usually registered much more quickly than conventional pesticides.

1.3 Insecticide toxicity

Application of ineffective insecticides is highly undesirable because of their toxicity. They are inherently toxic because they affect biological processes that are conserved among species including humans. In 2001, 9,285 human cases out of 19,495 persons reported to have been treated in emergency room facilities for pesticides-related incidents in the United States, were exposed to insecticides [1]. Insecticides can be absorbed by humans through dermal, oral, respiratory, or ocular exposure. The type and severity of injury or poisoning depends on the toxicity and mode of action of the pesticide, the amount absorbed by the body, how fast it is absorbed, and how fast the body is able to break it down and excrete it. Poisoning symptoms vary between classes of pesticides and pesticides within a class. The presence and severity of symptoms usually is proportional to the amount of pesticide (dosage) entering the tissues of the exposed person. Common symptoms include skin rashes, headaches, or irritation of the eyes, nose, and throat; these types of symptoms may go away within a short period of time. Other symptoms, which might be due to higher levels of pesticides exposure, include burred

vision, dizziness, heavy sweating, weakness, nausea, stomach pain, vomiting, diarrhea, extreme thirst, and blistered skin. Poisoning may also result in apprehension, restlessness, anxiety, unusual behavior, shaking, convulsions, or unconsciousness.

For example, organophosphates poison insects and mammals primarily by phosphorylation of the acetylcholinesterase enzyme (AChE) at nerve endings. The enzyme is critical to normal control of nerve impulse transmission from nerve fibers to smooth and skeletal muscle cells, glandular cells, and autonomic ganglia, as well as within the central nervous system (CNS) [9]. In the CNS, high Ach concentrations cause sensory and behavioral disturbances, incoordination, depressed motor function, and respiratory depression. Increased pulmonary secretions coupled with respiratory failure are the usual causes of death from organophosphate poisoning.

N-methyl carbamates esters share with organophosphates the capacity to inhibit cholinesterase enzymes and therefore share similar symptomatology during acute and chronic exposures. N-methyl carbamates are absorbed by inhalation and ingestion and somewhat by skin penetration, although the later tend to be the less toxic route. For example, Reigart & Roberts explain that carbufuran has a rat oral LD_{50} of 5 mg/kg, compared to a rat dermal LD_{50} of 120 mg/kg, which makes the oral route approximately 24 times more toxic when ingested.

If there are strong clinical indications of insecticide poisoning, and/or a history of the insecticide exposure, the patient needs treatment immediately. First aid in the event of a chemical poisoning should be as follows: first, see if the victim is breathing (if not give artificial respiration); second, decontaminate the victim immediately by washing off any skin residues (speed is essential); and third, call the physician [1]. For other specific exposures to pesticides in an emergency you should call toll-free the Poison Control Center or your local Poison Control center, or Call 911 Emergency Hot line.

The potential effects of pesticides on humans and the environment are managed under several Federal Acts and regulated through a combination of Federal, State, and Tribal responsibilities. The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Federal Food, Drug and Cosmetic Act (FFDCA), the Food Quality Protection Act (FQPA), the Safe Drinking Water Act (SDWA), and the Clean Water Act (CWA)—all of which are administered by USEPA and partner agencies—provide the regulatory

framework that affects the assessment and control of pesticides and their degradation products in water resources (http://npic.orst.edu/reg.htm).

Some pesticides are much more toxic than others, and severe illness may result from ingestion of only a small amount of a certain chemical, while with other compounds no serious effects result even from ingesting large quantities. Factors such as the toxic potency of the chemical, the dose of the chemical, length of exposure, and the route of entry or absorption by the body, influence the effects of ingestion. The Environmental protection Agency (EPA) requires collecting toxicity data on the pure toxicant in the early stages of the development of a pesticide for further experiments and exploration. These tests are conducted on test animals that are easy to work with and whose physiology is similar to humans'; for example, white mice, white rats, white rabbits, guinea pigs and beagle dogs. Pesticide toxicologists use rather simple animal toxicity tests to rank pesticides according to their toxicity. Before a pesticide is registered with the EPA and eventually released for public use, the manufacturer must disclose the acute toxicity of the pesticide to the test animal. This toxicity is define by the LD_{50}, the dose that kills 50 % of the test animals to which it is administered under experimental conditions, expressed as milligrams of toxicant per kilogram of body weight. The amount of pesticide to kill a human being can be correlated with the LD_{50} of the material to rats in the laboratory. **Table 1** shows common, trade and chemical names, general use patterns and oral and dermal LD_{50} of some insecticides. This adapted table compares the LD_{50} values of some insecticides.

Table 1. Insecticides and Lethal Doses in test animals.
(Source: Adapted from Ware, 2004).

Common name	Chemical name	General Use Pattern	ORAL LD_{50} (rats)	DERMAL LD_{50} (rabbits)
abamectin, avermectin	macrocyclic lactone glycosides	mosquito larvicide	10	2,000
Agnique®	alcohol-ethxylate	Wetting agent, dispersant use as mosquito larvicide	Non-toxic	
Bacillus sphaericus, Vectolex®		Microbial insecticide for mosquito larvae	Non-toxic	
Bacillus thuringiensis spp. israelensis, Bactimos®	crystalline delta endotoxin from Serotype H-14	larvicide for mosquitoes, aquatic midges, black fly,& fungus gnats in greenhouses	>5,000	>2,000
DDT	1,1,1-trichloro-2,2-bis (p-chlorophenyl)ethane	Not used in U.S. Some agricultural use in other countries, mostly in malaria eradication programs.	87	1,931
deet, Delphene®, Off®	N,N-diethyl-m-toluamide	Repellent for almost all biting arthropods.	2000	
diazinon, Knox-Out®, Spectracide®	O,O-diethyl- (2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate	Broadly used insecticide against soil insects, pest of fruits, vegs, feld crops, ornamentals. Being completely phased out by 12/2003.	300	379
fenthion, Baytex®	O,O-dimethylO-[4-(methylthio)-m-tolyl] phosphorothioate	Mosquitoes, flies, ornamentals; livestock insect pests.	255	330
parathion (discontinued)	O,O-diethyl O- (4-nitrophenyl) phosphorothioate	Broad-spectrum insecticide used on wide variety of crops. Cancelled effective 12/2005.	3	6.8
methoprene, Altosid®	isopropyl (E,E)-11-methoxy-3,7,11-trimethyl- 2,4-dodecadienoate	Insect growth regulator, used as mosquito larvicide.	>34,600	>3,000

Human toxicity is the reason that some effective pesticides are banned or restricted to only few uses, thus reducing the pool of available options further. EPA cancelled all uses of **D**ichloro-**D**iphenyl-**T**richloroethane (DDT) in 1973 (**Figure 1**). It can be considered the pesticide of greater historical significance, as it affected human health, agriculture and the environment. DDT was used during World War II against

body lice in Naples, during the typhus outbreak and in the Pacific against mosquitoes known to vector malaria. The greatest agricultural benefits from DDT have been in the control of the Colorado potato beetle, and several other potato insects, the codling moth on apples, corn earworm, cotton bollworm, tobacco budworm, pink bollworm on cotton and the worm complex on vegetables. A federal ban of the use of DDT, declared by the EPA in 1973, named DDT an environmental hazard due to its long residual life and to its accumulation, along with the metabolite DDE, in food chains, where it proved to be detrimental to certain forms of wildlife [1].

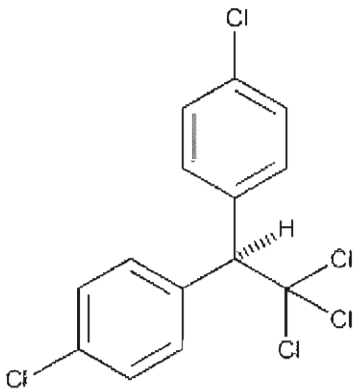

***Figure 1**. DDT-Dichloro-Diphenyl-Trichloroethane molecule.*

Diazinon (with a oral LD_{50} of 300 and a Dermal LD_{50} of 379) was the first insecticide in the heterocyclic group that appeared in 1952. It was a relatively safe OP that had an amazingly good track record around the home. It has been effective for practically every conceivable use: insects in the home, lawn, garden, ornamentals, around pets and for fly control in stables and pet quarters. It is one of the oldest classes of Ops, that affects the nervous central system, derived from the same family of chemicals as the sarin nerve class agent developed during World War II. EPA targeted this group of pesticides for review because they pose the greatest potential health risk to children. Diazinon's use on lawn poses a risk to birds, and it is one of the most commonly found pesticides in air, rain, and drinking and surface water. In December 31, 2004, EPA and diazinon registrants agreed to phase out and eliminate all residential uses of the insecticide diazinon; therefore, it is unlawful to sell diazinon outdoor, non-agricultural products in the United States [10].

Another insecticide cancelled by EPA is parathion. Parathion (ethyl) is the most familiar of the phenyl OPs, and was introduced into agriculture in 1947. Ethyl parathion was the first phenyl derivative used commercially, and, because of its toxicity, EPA cancelled most of its uses in 1991.

1.4 Resistance to insecticides as a problem in environmental health

Resistance is a condition in which pests become to a pesticide that once controlled them. In other words, after several generations exposed to the same pesticide, it becomes an inheritable decrease in sensibility of a pest organism to the toxic effects of a pesticide that results in reduced field performance of that or related pesticides [1]. At first, higher rates or more applications of a certain pesticide are necessary to achieve the same amount of control. Finally this pesticide has little effect, no matter how much is used. One strategy used is switching to a different pesticide, but sometimes when pests develop resistance to one chemical they also become resistance to others, even from a different chemical class. Resistance involves a change in the genetic characteristics of pest populations and is inherited from one generation to the next. Initially a pest population may posses a few individuals that are able to break down or chemically modify a pesticide and these individuals survive when that pesticide is used. When the resistant individuals reproduce, most of their offspring are also resistant. Biological factors such as the life span of the pest; the number of offspring it produces over a period of time; its ability to move large distances; and its food requirements influence the development of resistance.

The operational criterion of resistance has usually been taken as the survival of 20% of the individuals tested at the currently known diagnostic concentrations of commonly available pesticides, using WHO test kits in the field. These tests have been developed to compare the insect population suspected of resistance with normal or base-line populations or strains (i.e., with untreated populations elsewhere or with the population before it was exposed to insecticide treatments). The resistance ratio tells us how great a difference the suspected population must show from the normal or standard

strains for resistance to be confirmed or for the word to be applied in the first place. Brown and Pal (1971) refers to a study made by Keiding (1956) where the latest reported that a 10-fold increase in the LC_{50} (median lethal concentration) levels is sufficient to render organophosphorus compounds useless for housefly control. The resistance ratio is evidently a function of the test method employed. For houseflies, the test of which Keiding was speaking was topical application. For mosquito larvae, a 10-fold increase in LC_{50} is necessary to indicate resistance, whereas for adult mosquitoes a 4-fold increase is sufficient. Brown & Pal (1971) suggests the word "tolerance" be used in cases where the increase in LC_{50} is less than these indicated minima for the tests, but is nevertheless statistically significant [11]. This usually corresponds with a degree of change in susceptibility level that has not resulted in a detectable loss of control by the insecticide.

Depending on the test method, the estimated resistance ratios vary. Typically, when rates of insecticide are used in jar tests, the resistance ratios will be much smaller than by topical application. This is because in jar tests there is a continuous exposure of the test insects to the insecticide and this leads to much higher levels of mortality than a single exposure would, because the test insects are constantly absorbing insecticide into their bodies. In contrast, with topical applications there is one exposure at the time of the treatment, which is not sufficient to cause mortality but instead can trigger resistance mechanisms. The most important factor that this difference highlights is that topical application resistance ratios generally need to be adjusted higher (10 to 20 times) to reflect more realistically a potential loss of control. With the jar test, resistance ratios of two to five times will indicate a potential for losses of control.

Resistance to insecticides is an important human-induced pressure on the mosquito population. Several models have been developed to enable us to understand and manage the evolution of insecticide resistance, and nearly all of them assume that resistance is controlled by two alleles at one locus. According to Brown & Pal simulations [11], they distinguish three kinds of mosquitoes, namely, susceptible, moderately-resistant, and resistant individuals, taking them as three classes of individual sensitivity to insecticides. The assumption is that a certain dose of insecticide reduces fitness, whereby it is assumed that the same dose would have a more pronounced impact on susceptible mosquitoes than on (moderately) resistant ones.

Genomics will play an increasingly important part in the development of new malaria control tools. Comparing the genomes of the malaria vector *Anopheles gambiae* and of the fruit fly *Drosophila melanogaster* will not only yield new hormonal, neuronal, and regulatory molecular targets for the development of new classes of insecticides, but will also allow us to attack existing insecticide resistance and to boost the life-span of currently available insecticides.

1.5 Case studies of insecticide use and resistance

World Health Organization's studies

The introduction of DDT for control of the mosquito vector of malaria in the late 1940s, and the early eradication of malaria from the periphery of its transmission range by residual house spraying with this insecticide, led directly to the malaria eradication campaign of the 1960s backed by the World Health Organization. At the end of the 1960s, the concept of eradication was formally dropped in favor of sustainable control, largely because insecticide resistance was being selected for among the mosquito species that transmit malaria. There has recently been resurgence in antimalarial activities with the Roll Back Malaria initiative and Global Fund for Health, which supports extensive use of pyrethroid-impregnated bed nets for mosquito control campaigns in Africa and other malaria-endemic regions. It is not clear how much the current large-scale pyrethroid resistance of mosquitoes in West Africa will affect these efforts, and what will replace the pyrethroid-treated nets if selection of multiresistance mechanisms results in widespread failure of this strategy [12].

The World Health Organization summarizes in their report available literature on DDT, dieldrin and pyrethroid insecticide resistance in *An. gambiae* and *An. arabiensis* from the 1950s to the present and they discussed the implications for malaria control programs. A number of countries in southern Africa have malaria control programs based on vector control in combination with rapid case detection and effective treatment. While all components of the program are important for control of the disease, its success is dependent on the reduction of transmission brought about by the control of vector

mosquitoes. This in turn is dependent on the availability of an effective and safe insecticide that can be used in close association with humans at risk. For many years the insecticide of choice was, and in some cases still is, DDT. In countries with unstable malaria transmission that carried out malaria vector control in the 1950s, for instance in the Madagascar highlands and Swaziland, the cessation of indoor house-spraying in later years is considered to be the most important cause of the increase in malaria incidence. Both countries reintroduced extensive DDT house-spraying, which has once again reduced malaria transmission significantly.

The South African control programs, however, have moved away from DDT for various reasons, resistance in the vectors not being one of them. There was considerable social resistance to house-spraying with DDT because of the marks left on walls, the build-up of resistant bedbug populations and political pressure brought to bear because of emerging evidence of DDT's damage to the environment and its long-term presence in the tissues of exposed people. Pyrethroids are seen as being eco-friendly, have low mammalian toxicity, do not leave marks on walls and, in some instances, the excito-repellancy effect is not as marked as in DDT. Evaluation of the efficacy of four different pyrethroids on mud surfaces was carried out by the South African Medical Research Council. The tests showed that pyrethroids were as effective and long-lasting as DDT for residual house-spraying, and thus the decision was made to phase out DDT.

When considering the case in which mosquitoes and parasites do not adapt to the use of insecticides and drugs, it is possible to calculate the new equilibrium given that constant levels of insecticides and/or drugs used. The control program will lower the rate of infection as a result of (a) rendering the mosquitoes and/or parasites less fit, and (b) the decrease in the percentage of infected persons. The percentage of immune persons will likewise decrease, resulting in an increase in the size of the fraction of susceptible humans.

The incidence of malaria decreased in regions of low endemicity, as a consequence of the control programs. In regions of high endemicity an increase of malaria may occur if the control programs are not stringent enough, the effect being a steeper increase in susceptible humans (immune persons lose their immunity) relative to the decrease in the infection rate. As a result of the ability of vector and parasite to adapt

to the control programs, such programs' effectiveness decreases in such a manner that the new equilibria are similar to those obtained in the absence of control programs. Not unexpectedly, adaptation may eventually lead to higher incidence rates than those obtained in the absence of adaptation. In regions of low endemicity the adaptive vectorial capacity first decreases, but due to adaptation among mosquitoes, subsequently increases, albeit to a level that lies somewhat below the initial level. The result is a similar pattern in the incidence of malaria, although the level continues to fall (gradually). It is, thus, evident that a combination of both drugs and insecticides at low levels is more efficient than high level use of only one of the two, a finding that reflects the enhanced development of resistance at higher doses.

In regions of high endemicity, the decrease in adaptive vectorial capacity exhibits a similar pattern to that obtained in regions of low endemicity. Resistance development differs in the two regions due to a difference in the gene pool. Due to the difference in the profiles of the populations, the patterns of incidence of malaria are quite dissimilar [12]. Following a reduction in incidence at the outset of the control programs, incidence subsequently shows an increase due to the lower effectiveness of the control measures. Due to the high fraction of susceptible humans after a successful period of control, again as a result of the flow of immune persons due to the increased rate of immunity loss, incidence may even rise to surpass the initial level. In the long run, a combination of two low levels of control does not achieve a better performance than control by a single method. Indeed, incidence peaks at a level even higher than the initial (precontrol) level due to the higher number of susceptible humans who become reinfected.

Resistance in the three main malaria vectors, *Anopheles arabiensis*, *A. gambiae* and *A. funestus*, have developed widespread resistance to dieldrin and HCH, while the *A. gambiae* complex has developed more focal resistance to DDT, and these results have been detected using discriminating-dosage bioassays transmitted to World health Organization (WHO). Resistance in *Culex quinquefasciatus*, one of the vectors of filariasis, is found mainly in urban areas of the African Region and has developed resistance to many types of organochlorines, organophosphorus compounds and carbamates. *A. gambiae*, *A. melas* and *A. funestus* are also important filariasis vectors in the African Region. For example, *A. gambiae* shows resistance to DDT in Cameroon,

Central Africa Republic, Congo and Ghana; while, *A. funestus* has became resistant to DDT in Sudan [12].

Resistance to permethrin and increased tolerance to deltamethrin in *An. gambiae* were reported from two localities in the Ivory Coast while increased tolerance to permethrin in *An. gambiae* was reported from Nigeria and Togo, and Kenya. Recent work in the Ivory Coast and Burkina Faso has revealed extensive pyrethroid resistance in wild populations of *An. gambiae*. Tsetse flies, the trypanosomiasis vector, have shown slightly increases in the tolerance to DDT, dieldrin and endosulfan in Kenya and Nigeria [12].

In the Americas Region, the malaria vectors *Anopheles pseudopunctipennis* and *A. albimanus* are resistant to DDT all along the Pacific coast but remain susceptible on the Atlantic coast. Another vector of human public concern is *Aedes aegypti*, the dengue vector. This vector displays low to moderate resistance to several insecticides in the Caribbean and in North, Central and South America, and *Ae. albopictus* is resistant to malathion and fenitrothion in North America. Resistance among Triatominae has been found focally in Venezuela, where the principal vector for Chagas disease, *Rhodnius prolixus*, is highly resistant to dieldrin and some organophosphorus compounds and carbamates [12].

Brogdon and McAllister studies

Growth regulators, ivermectins, and other microbial agents have been introduced to vector control programs as well. The initial mechanisms that conferred resistance to insect growth regulators were oxidase-based. Resistance to ivermectins has resulted from a number of factors, including oxidation, conjugation, and altered target-site mechanisms. Vectors have not yet demonstrated resistance to these compounds in the field [13].

The potential of resistance to interfere with emergency use of insecticides first became apparent in 1993 when flooding in nine midwestern states increased the threat over the next 2 years of arboviral disease transmission. Most of the nine states affected had no public health entomologic or vector control resources, and none had susceptibility data for their vector mosquitoes. Preliminary data showed that resistance to the insecticides proposed for emergency use was widespread throughout the Midwest. As a result of these findings, a resistance surveillance laboratory was established at the Centers

for Disease Control and Prevention (CDC), Atlanta, Georgia. Data collected by this laboratory in the last 3 years confirm that states vary enormously in their resources to deal with insecticide resistance. At present, 26 states participate in the Emerging Infectious Disease insecticide resistance surveillance project.

Brogdon and McCallister (CDC) provide an update on resistance of disease vectors to insecticides, use specific instances of emerging resistance to illustrate this complex, worldwide problem, and offer strategic priorities for combating it. Their study showed that insecticide resistance mechanisms (as opposed to insecticide avoidance behaviors important in the control of malaria vectors) have a biochemical basis. They discuss in their paper the two major forms of biochemical resistance. The first is the target-site resistance, which occurs when the insecticide no longer binds to its target, and the second, detoxification enzyme-based resistance occurs when enhanced levels or modified activities of esterases, oxidases, or glutathione S-transferases (GST) prevent the insecticide from reaching its site of action [13].

Alterations of amino acids responsible for insecticide binding at its site of action cause the insecticide to be less effective or even ineffective. The target of organophosphorus (OPs) (e.g., malathion, fenitrothion) and carbamate (e.g., propoxur, sevin) insecticides is acetylcholinesterase in nerve synapses, and the target of organochlorines (DDT) and synthetic pyrethroids are the sodium channels of the nerve sheath. DDT-pyrethroid cross-resistance may be produced by single amino acid changes (one or both of two known sites) in the axonal sodium channel insecticide-binding site. This cross-resistance appears to produce a shift in the sodium current activation curve and cause low sensitivity to pyrethroids. Similarly, cyclodiene (dieldrin) resistance is conferred by single nucleotide changes within the same codon of a gene for a gamma-aminobutyric acid (GABA) receptor. At least five point mutations in the acetylcholinesterase insecticide-binding site have been identified that singly or in concert cause varying degrees of reduced sensitivity to OPs and carbamate insecticides.

The enzymes responsible for detoxification of xenobiotics in living organisms are transcribed by members of large multigene families of esterases, oxidases, and GST. Perhaps the most common resistance mechanisms in insects are modified levels or activities of esterase detoxification enzymes that metabolize (hydrolyze ester linkages) a

wide range of insecticides. These esterases comprise six families of proteins belonging to the alpha/beta hydrolase superfamily. In Diptera, they occur as a gene cluster on the same chromosome. Individual members of the gene cluster may be modified in instances of insecticide resistance, for example, by changing a single amino acid that converts the specificity of an esterase to an insecticide hydrolase or by existing as multiple-gene copies that are amplified in resistant insects (the best studied examples are the B1 and A2-B2 amplicons in *Culex pipiens* and *C. quinquefasciatus*).

The cytochrome P450 oxidases (also termed oxygenases) metabolize insecticides through O-, S-, and N-alkyl hydroxylation, aliphatic hydroxylation and epoxidation, aromatic hydroxylation, ester oxidation, and nitrogen and thioether oxidation . The cytochrome P450s belong to a vast superfamily. Of the 62 families of P450s recognized in animals and plants, at least four have been isolated from insects. The insect P450 oxidases responsible for resistance have belonged to family 6, which, like the esterases, occur in Diptera as a cluster of genes. Members of the cluster may be expressed as multiple (up to five) alleles. Enhanced levels of oxidases in resistant insects result from constitutive overexpression rather than amplification. The mechanisms of oxidase overproduction in resistance are under extensive investigation and appear to result from both cis- and trans-acting factors, perhaps associated with the phenomenon of induction.

Most organisms possess multiple GST from two or more classes. GST implicated in DDT insecticide resistance exist as clusters of genes that have been further shuffled through the genome by recombination. A number of resistance GST genes, including multiple forms in the same insect, have been characterized in insect vectors.

Brown and Pal studies

According to Brown & Pal simulations [11], they distinguish three kinds of mosquitoes, namely, susceptible, moderately-resistant, and resistant individuals, taking them as three classes of individual sensitivity to insecticides. The assumption is that a certain dose of insecticide reduces fitness, whereby it is assumed that the same dose would have a more pronounced impact on susceptible mosquitoes than on (moderately) resistant ones. The fitness function expresses the notion that the fitness of the three classes drops in a decreasing rate with higher doses of insecticides. The higher the

resistance the lower the decrease in fitness. Obviously, if alternative insecticides are applied that affect the three categories differently, the alternative insecticides would be less dependent on a single mechanism of resistance.

Depending on the test method, the estimated resistance ratios vary. Typically, when rates of insecticide are used in jar tests, the resistance ratios will be much smaller than by topical application. This is because in jar tests there is a continuous exposure of the test insects to the insecticide and this leads to much higher levels of mortality than a single exposure would, because the test insects are constantly absorbing insecticide into their bodies. In contrast, with topical applications there is one exposure at the time of the treatment, which is not sufficient to cause mortality but instead can trigger resistance mechanisms. The most important factor that this difference highlights is that topical application resistance ratios generally need to be adjusted higher (10 to 20 times) to reflect more realistically a potential loss of control. With the jar test, resistance ratios of two to five times will indicate a potential for losses of control.

It would seem self-evident that, depending on landscape and infrastructure, mosquitoes are more or less able to migrate from place to place, and that mosquitoes susceptible to insecticides may, thus, enter a treated area. Moreover, parasites susceptible to antimalarial drugs can also migrate, whether they are carried by mosquitoes or humans. Migration is modeled by assuming that during each time step (0.1 year intervals) a fraction of the new population is bred under the initial conditions, that is, not yet adapted to the changed conditions.

Insecticides are sprayed on specific areas so that 100% coverage is seldom achieved. Drugs are not taken (sufficiently) by all humans, so that a fraction of the parasites escape from it. This phenomenon of refugees is modeled by assuming that during each time step, a part of the population, the size of which is randomly selected, has not been treated despite the control programs that have been implemented. This means that susceptible populations are constantly mixed with the resistant ones and this way the resistant genes are diluted in the population and are not as effective in providing full resistance.

The experiments deal with the consequences of the use of insecticides and antimalarial drugs, together with a temperature change, on the occurrence of malaria for a

time horizon of one decade, using time steps of 0.1 year. Although malaria situations are extremely heterogeneous with respect to resistance to change, the two types of regions distinguished are a region of low endemicity and a region of high endemicity [2]. Although the real generational longevity among the parasites and mosquitoes is not specified, the time horizon is based on observed time elapsed in acquiring resistance. Furthermore, it was assumed that the initial rate of infection is 2.0 per annum in highly endemic regions and 0.1 year in areas of lower endemicity. These values were chosen because they lie within the range of the values reported in several studies on the force of infection among young children. Areas of lower endemicity can be characterized as exhibiting low vectorial capacity resulting in a high percentage of susceptible persons (approximately equal to 80%), and low percentages of infected (approximately equal to 8%) and immune persons (approximately equal to 12%). The early phases of the host response to infection depend on innate immunity in which the innate resistance mechanism recognizes and responds to the presence of the pathogen. Innate immunity is present in all individuals at all times, does not increase with repeated exposure to a given pathogen, and discriminates between a group of related pathogens. Only if a pathogen can breach this early mechanism of defense will an adaptive immune response develop, with the generation of antigen-specific cels that specifically target the pathogen, and memory cells that can prevent reinfection with the same microorganism.

Areas of low endemicity vis-a-vis *Plasmodium. falciparum* can be found in Southeast Asia and South America. Regions of high endemicity are characterized by a relatively high vectorial capacity. In the first study there is a high percentage of immune (approximately equal to 68%) and infected persons (approximately equal to 27%). The younger age class especially suffers from a high percentage of infected (approximately equal to 45%). Highly endemic regions are mainly found in tropical Africa.

The scientists proposed to report a set of results that they have derived using the complex adaptive systems approach. In the starting year, the situation is assumed to be near equilibrium. This assumption about an equilibrium state is made for analytical purposes, namely, to render the impact of control policies and temperature change on the

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[2] Endemicity- Prevalent or belonging exclusively or confined to a particular place 14. *Webster's New Universal Unabridge Dictionary*. 1996, Barnes & Noble, Inc.: New York.

occurrence of malaria transparent, thereby including the adaptation of mosquitoes and parasites. Therefore, they had assumed a steady-state situation in demographic, social, and economic development, although they recognized that these factors may influence future developments of malaria.

The results are presented as time series covering a period of 10 years. In view of the stochastic elements of the model, they elected to use a large number of runs (100) and determine the mean and the extremes of important indicators. This procedure yields ranges of uncertainty, whereby the uncertainty does not lie in the different parameter values of the model, but rather in the stochastic characteristics and the complexity of the system.

In the interest of analytical lucidity, two broad control levels for both insecticides and antimalarial drugs are distinguished, namely, the low and the high dose. In the case of a low dose, they adopted a value of ui (units of infection) equal to 0.002, which represents a 50% deterioration in the fitness of susceptible mosquitoes or parasites. The high dose ui is assumed to be equal to 0.05, such that the fitness of the moderately resistant mosquito or parasite decreases by 50%.

Although on average the use of a low dose of insecticides leads to an increase in the incidence of malaria in the long run, it might also lead to a slow decrease of the incidence if evolutionary adaptation among mosquitoes proceeds very slowly.

Other studies

Hemingway et al. reported 8.5% survival rate in insecticide bioassays with permethrin at a dose of 0.25% permethrin for 1 hour [15]. However, if the mosquitoes (*Anopheles. gambiae, An. arabiensis and An. melas*) were exposed for 2 hours, there was 100% mortality [15]. In Culex mosquitoes, the most common organophosphate insecticide resistance is caused by co-amplification of two esterases (Estβ2' is produced 3 times more than Estα2'). In mosquitoes, esterase-based resistance is the primary mechanism for organophophorous (OPs), and in some cases a secondary mechanism for carbamate resistance [16]. Susceptibility test results revealed that adults of *Culex quinquefasciatus* from Baan Suan community, Nonthaburi, Thailand were highly resistant to DDT, deltamethrin, fenitrothion and permethrin with the percentage mortality

of 0%, 11.0%, 21.2% and 10.1%, respectively; while a 100% mortality was obtained for malathion showing that this strain was susceptible to malathion [17]. Pesticides such as DDT, pyrethroids (deltamethrin and pelmethrin) are chemical groups in which the resistance is due to sodium channel modulators. As supposed to fenitrothion and malathion that are OPs which resistance mechanism is due to acetylcholinesterase inhibitors.

Bioassays were carried out to determine the level of malathion resistance in the Sri Lankan mosquito populations [18]. No mortalities occurred for any insect species after exposure to control papers or insecticide-impregnated papers, prepared by standard WHO recommended methods. A high level of resistance to malathion occurred in *Culex. quinquefasciatus* (78% survival on the WHO malathion discriminating dosage), *A. culicifacies* (70%) and *C. tritaeniorhynchus* (65%). The level of malathion resistance was lower in *A. subpictus* (15%). Populations of *C. gelidus*, *A. aegypti* and *A. albopictus* were fully susceptible to malathion at this dose.

When malathion was first introduced in Sri Lanka in 1977, a 20-min exposure to 5% malathion produced 100% mortality in *A. culicifacies*. The first *A. culicifacies* survivors at this species-specific discriminating dosage were detected in Sri lanka in 1979, after two years of malathion spraying. Resistance to the WHO standard *Anopheles* discriminating dosage (exposure to 5% malathion for 1 h) was first observed in 1982, with increased malathion carboxylesterase activity being the major underlying mechanism. In contrast, monooxygenases played the major role in malathion resistance in *A. subpictus* in 1987, with no evidence of a malathion carboxylesterase mechanism. The oxidase mechanism produced broad-spectrum resistance to organophosphorus compounds, which included a low level of resistance to malathion, and was still the only major mechanism of resistance to these compounds detected in 1991.

Molecular characterization of pyrethroid knockdown resistance (*kdr*) has been done in the major malaria vector *Anopheles gambiae s.s.* in West Africa. A PCR-based diagnostic test was developed for the rapid identification of the *kdr*-like allele found in the domain II region of the *para*-type sodium channel from pyrethroids susceptible and resistant strains of *A. gambiae* [19]. In insects, it has been reported a single mutation (leucine (Leu) to phenylalanine (Phe)) in the S6 transmembrane segment of domain II in

th sodium channel sequence is associated with *kdr* and DDT in *Musca domestica*, and the German cockroach. Also, a different mutation (leucine to histidine) at this same position has been found in pyrethroids-resistant population of the tobacco budworm, *Heliothis virescens*. In *super-kdr* houseflies, this mutation is associated with a second substitution further upstream in the same domain which replaces a methionine with a threonine. This study identified in the resistant strain of *A.gambiae*, the same Leu (TTA) to Phe (TTT) point mutation as described for houseflies and cockroaches.

Research into insecticide resistance is ripe for the move from the static genome map to the functional genomics approach, which will help to understand the evolution of resistance in these complex organisms through modulation of gene expression. Material from East Africa has already been subjected to standard genetic quantitative trait loci (QTL) mapping, which has defined a polytene[3] chromosome region within which the regulator of P450 gene expression must be encoded. The availability of the *A. gambiae* genome sequence now allows us to use new molecular micro satellite markers from the sequence to narrow down this control region to a few kilobases of DNA. Open reading frames can then be identified and candidate genes analyzed for function with recently developed anopheline transformation technology. Regulatory genes controlling the expression of glutathione transferases (enzyme families that are important for protecting insect cells from insecticides) will be similarly defined from QTLs that are already mapped to the *A. gambiae* polytene chromosomes [15].

In *Culex* mosquitoes and aphids, elevated esterases confer organophosphate resistance through gene amplification, with multiple copies of DNA amplicons of about 30 kb being integrated stably into the insect genome, either contiguously or, in the case of aphids, sometimes disparately. The resistant phenotype results from a complex tissue-specific interplay of differential up-regulation of these amplified genes and in the case of aphids involve changes in DNA methylation. The sequenced *A. gambiae* genome is from an insecticide-susceptible strain, and there are obvious orthologs[4] for the amplified *Culex* esterases. To date, there is no evidence of esterase gene amplification-based resistance in any *Anopheles* species. However, as new resistant strains of *A. gambiae* are investigated,

[3] Polytene- of multi-stranded chromosome (see glossary for more details).
[4] Orthologs- genes in different species that have evolved from a common ancestral gene by speciation and generally retain a similar function in the course of evolution.

amplifications may well be found, and analysis of the genome sequence surrounding the amplicons will allow us to expose the size of the units and may shed light on the amplification mechanisms.

The availability of the complete *A. gambiae* genome sequence should stimulate a rapid shift in research aimed at improving the management of insecticide resistance. If the problem of resistance is subdivided into three stages—detection, monitoring, and management—then the benefits of this genome sequence become obvious. The genome sequence will enable access to the major regulatory genes involved in resistance, particularly if orthologous regulators control metabolically based resistance in insects generally. For example, management of resistance in practice currently involves basic rotations and mixtures or mosaics of different insecticides. Access to insect-specific metabolic enzyme regulators will provide a target for "add-ons" to current insecticides, which should expand their natural life-span by blocking common resistance pathways while leaving mammalian toxicity unaffected.

The Anopheles genome will provide information on the target site genes, facilitating cloning and mutagenesis studies to determine the precise nature of the mutations and to aid in predicting interactions between insect proteins and insecticides. In the longer term, this could lead to new insecticidal molecular targets. This approach may be especially important for AChE as there is increasing evidence for multiple AChE genes from the Anopheles and Drosophila genome databases [20]. Two AChE genes are apparent in the *A. gambiae* genome, and to date no resistance-linked mutations have been identified in mosquitoes predominantly in studies on the sex-linked AChE gene [20]. The Anopheles genome in conjunction with that of Drosophila also provides sequences of nicotinic acetylcholine receptor subunits, which will facilitate their cloning from other insect species.

Furthermore, some fruit flies are showing the GABA (gamma-aminobutyric acid) receptor mutation, which consists of a single change in the chain of nucleotide bases. This genetic mutation that makes the insects resistance to cyclodiene insecticides may be responsible for up to 80 percent of all insecticide resistance, says Richard H. French-Constant, an entomologist at the university of Wisconsin-Madison [21]. However, Richard T. Roush, an entomologist at Cornell University asserts that there are insufficient

data to determine whether the resistance mutation has one origin [21]. In addition, the GABA receptor probably has a limited number of ways it can mutate to become resistant without also killing the insect, making the possibility of multiple origins more likely. Regardless of the mutation's beginnings, its identification should help in insecticide development, the report notes. Since insecticides were first used in the 1940s, over 600 insect species have developed resistance, leading chemists to constantly search for new products. Insecticide resistance now costs an estimated $1.4 billion a year in crop losses in the United States alone.

Today very little is known about how the present products lead to the types of resistance that researchers observe. These products could be used incorrectly, so a single product will lead to resistance to that product (and others closely related). In addition, two often overlooked facts also should be considered in this regard. First, when a product that was initially used to select for resistance is not used on a population for an extended period of time, the population will again become susceptible to that product. Second, even though an insecticide does not completely eliminate a population following its use, the survivors will still be "sub-lethally" affected. These two facts tell us that using sound Integrated Pest Management (IPM), insecticide rotations and thoroughness in treating will ensure positive results in insecticide resistance management programs.

In addition, there are very few new active ingredients on the horizon to replace the ones that presently are available. If these active ingredients are lost to resistance on a wide scale, the topic of resistance may become more negative, and the image of the industry will suffer.

At any rate, all discussions of pest control ultimately lead to this topic of resistance. What then, does resistance in mosquitoes mean to the everyday business of pest management professionals? Studies had shown that the over-reliance on a single product leads to resistance towards that and similar products.

As a result, university researchers can offer recommendations for managing resistance. They can confidently recommend preventative IPM measures based on information that has been obtained from many decades of research on the biology and behavior of insects.

Resistance can generally be considered as a loss of control; and can be categorized in many ways, one of which is by referring to it as natural or induced. If resistance is natural, it is the result of natural genetic processes that make individuals in populations different from one another. Generally, natural resistance doesn't lead to control failures. Resistance that has been caused by previous insecticide exposure in a population can be referred to as induced, which, will almost certainly lead to control failures.

It is very difficult to keep up with pest problems, because of resistance, persistence hazards, and environmental complications. Therefore, alternative methods should be used where possible so that we preserve the chemical methods for the pests that cannot be controlled with other methods and thus preserve the effectiveness in the species where they are necessary.

CHAPTER 2

Molecular Markers of Insecticide Resistance in Mosquito Population

2.1 Pesticides modes of actions

This chapter goes into details on how pesticides work. Insecticides effects at the molecular level (body cells' mechanisms) and any modification in insect behavior are described in details. Pesticides main purpose is to impede essential metabolic process in the organism. How they accomplish this,-that is, their mode of action- is very difficult to determine. Mode of action consists of the sum of anatomical, physiological and biochemical interactions and responses that result in toxic action of a chemical, as well as the physical (location) and molecular (degradation) fate of the chemical in the organism [1]. The term mechanism of action is limited to the biochemical and biophysical responses of the organism that are associated with the pesticidal action. The modes of actions of insecticides are presented into eight classes: physical toxicants, protoplasmic poisons, metabolic inhibitors, cytolitic toxins, muscle poisons, alkylating agents and disruptors of molting, metamorphosis and cuticle formation, and nerve poisons. Appendices A through E show United States agricultural products by modes of action.

- **Physical toxicants** are those materials that block any physiological process by a mechanical reaction such as, oils and abrasive dusts. Oils are used to control mosquito larvae by blocking or clogging the respiratory openings or the gills. Heavier oils applied to fruit trees during the dormant season control scales by clogging their spiracles. When using other physical toxicants such as boric acid, diatomaceous earth, silica and aerosilica gels, insects are killed by absorbing waxes from their cuticle, affecting the continuous loss of water from the insect body.

- **Protoplasmic poisons.** Mercury and its salts, all strong acids and several of the

heavy metals, including cadmium and lead are included in the second class, which attack multiple enzymes in the insects' system, apparently leading to their precipitation.

- **Metabolic inhibitors.** Mitochondrial electron transport, polysubstrate monooxygenase, and glycolysis inhibitors are examples of this group. The electron transport chain contains the series of cytochromes in the mitochondria involved in the production of energy from the oxidation of carbohydrate, lipid and protein molecules. Pesticides that acting on the electron transport chain are rotenone, fumigants that work through the cyanide ion (CN-), dinitrophenols, organotin acaricides and fungicides.

Polysubstrate monooxygenase inhibitors are also metabolic inhibitors that act synergistically. The synergistic mode of action is the inhibition of cytochrome P-450 dependent polysubstrate monooxygenases (PSMOs), enzymes produced by microsomes, subcellular units found in the liver of mammals and in some insect tissues (e.g., fat bodies). The earlier name for these enzymes was mixed-function oxidases (MFO). PSMOs oxidize insecticides and can lead either to detoxification or activation of an insecticide. If the enzyme normally detoxifies the insecticide, PSMO inhibitors act synergistically as the insecticide is left intact to exert its action. If, however, the enzyme normally activates the insecticide, as with some phosphorothioates, PSMO inhibitors act as antagonists as the insecticide is not activated.

A third class of metabolic inhibitors is the glycolysis inhibitors. Fluorines and arsenicals belong to this group. In mammals, organofluorine compounds (fluoroacetate) cause symptoms only after 20 to 60-minute delay, when convulsions begin. Fluoroacetate is not a direct enzyme inhibitor, but is a latent inhibitor, requiring conversion to a derivative, fluorocitic acid, which is a potent enzyme inhibitor. In the presence of fluorine compounds the heartbeat increases and the body temperature drops. In addition, the result of fluoroacetate poisoning in insects and mammals is the accumulation of citric acid. The arsenicals of interest are lead and calcium arsenate. Calcium is by far the more toxic to both insects and mammals. Arsenicals kill primarily by inhibiting mitochondrial respiratory enzymes. These enzymes are localized in membrane-enclosed organelles called mitochondria where the process of respiration and oxidative phosphorylation (a mechanism of ATP formation) occurs [22]. Arsenic chemically resembles phosphorus

and substitutes partially for phosphorus in some reactions. The best example is arsenolysis, which takes place instead of phosphorylysis. Phosphorylysis is essential in forming the high energy bond of ATP and oxidative phosphorylation is the major energy producing step of the cell, but arsenolysis undo phosphorylation.

- **Cytolytic toxins.** These are not chemical insecticides but biological toxins that cause cells to rupture and disintegrate. Biological toxins are products of genes in the organisms that produce them. The toxins in spider and some snake venoms are cytolytic. This same cytolytic effect is caused by the toxins of *Bacillus thuringiensis* in the guts of insects that have ingested these bacteria. *Bt var. israelencis* and *var. sphaericus* are effective against mosquito and blackfly larvae, while *Bt var. kurstaki* is used for the control of various lepidopteran (caterpillar) pests. The toxicity of these bacterial varieties is due to proteins present in crystalline parasporal inclusions within the bacteria. When ingested by insects these inclusions are dissolved in the alkaline midgut by proteases and the proteins delta-endotoxins and beta-exotoxins (thuringiensin) released. The toxins then disrupt the peritrophic and midgut cell membranes resulting in gut paralysis and a cessation of larval feeding. Upon further action the toxins also affect the muscular and nervous system. At the cellular level, the toxins bind to unsaturated phospholipids on cell membranes and subsequently rupture the cell. It is notable that these insecticidal crystalline proteins are not only effective when susceptible species consume foliage on which the bioinsecticide has been sprayed, but they are as (or more) effective when the gene that codes for them is incorporated into the plant tissues genetically, as in the transgenic crops, Bt Cotton and Bt Corn.

- **Muscle poisons** such as ryania and sabadilla act as a membrane disruptor, and when insects are poisoned with these agents, show flaccid paralysis and death. Ryania contains an alkaloid, ryanodine, the active principle of *Ryania speciosa*, grown in South America. Ryanodine is at least 20 times more toxic to mammals than to most insects. The mode of action of ryanodine is that of membrane disruption and its effect is specific for the excitable membrane of muscle. Poisoned insects show tremendous increased in oxygen consumption, as much as tenfold, followed by flaccid paralysis and death.

Sabadilla comes from the powdered seeds of the lily *Schoenocaulon officinale* and contains two insecticidal alkaloids, cevadine and veratridine. Houseflies, household insects and the Hemiptera and Homoptera, which include the true bugs, are especially susceptible. In mammals veratridine produces a prolonged rigor in skeletal muscle following the initial twitch, accompanied by repetitive impulse discharge in muscle fibers. Oxygen consumption increases, but not to the extent note in ryanodine poisoning. Sabadilla appears to have the same general mode of action in insects as ryanodine, resulting in flaccid paralysis and death.

- **Alkylating agents** react directly with cell chromosomes by attacking one or more of the reactive loci on nucleic acid molecules (DNA and RNA), and proteins thus deactivating essential enzymes, which subsequently cannot execute normal functions in the synthesis of nucleic acids. Notable among these are the early World War I gases, mustard and nitrogen mustard and a more recent group of experimental chemosterilants, particularly the aziridines. Several of the halogen-containing compounds, mostly fumigants, are also identified as alkylating agents. Methyl bromide and ethylene dibromide are the most prominent of these.

- **Disruptors of molting, metamorphosis, and cuticle formation**. The progression of various orders of insects through their various stages leading to adulthood is not only essential for their growth and development; it also exposes processes that are sensitive to attack by several insecticidal groups. Tebufenozide, methoxyfenozide, and Chromafenozide are disruptors that inhibit the molting process in insects. In some insects, environmental factors such as temperature and food availability control molting; in others, the number of molts is fixed and is controlled by hormones. These hormones are released when an insect's growth reaches the physical limits of its exoskeleton. Each molt represents the end of one growth stage (instar) and the beginning of another [23].

Insect growth, maturation and molting processes are under endocrine control from hormones. Molting hormones (MH), or ecdysones (Ecy), are steroid hormones that act in arthropods much as the vertebrate steroid hormones act, through a nuclear receptor system that binds to DNA [24]. The ecdysones themselves have a toxic effect on insects. They can interfere with cuticle development. When pupae are treated with ecdysones

they may molt to form another pupa rather than molt to the adult form [25]. The insect quickly secretes a new cuticle in response to the hormone; consequently, the epidermal cells do not have sufficient time to perform the DNA replication necessary for the synthesis of an adult cuticle, and a second pupa is formed

Molecules that mimic the juvenile hormone (JH) of insects can disrupt metamorphosis when applied to immature stages. Such analogues of JH are Fenoxycarb, Hydroprene, Methoprene, and Pyriproxifen. Ecdysone, a natural hormone of insects that initiates the molting process (shedding of the exoskeleton, typically to let the organism grow), is also a target of insecticides that acts as mimics or inhibitors. Lastly, the inhibitors of insect cuticle formation are the benzoylurea insecticides (diflubenzuron), the thiadiazines that afeect Homopteran insects (buprofesin) and triazines (cyromazine) that affect Diptera.

- **Nerve poisons** is the significant class for this thesis research. The insecticides mechanism of action discussed are restricted to the biochemical and biophysical responses of the organism that are associated with a specific chemical. They include two categories: axonic poisons (sodium channel blockers); and synaptic poisons (chloride channel blockers).

a) **Axonic poisons,** which are sodium channel blockers, are the first group under the nerve poisons**.** Axonic chemicals are those that affect the electrical impulse transmission in the axon. The axon of a nerve cell or neuron is an elongated extension of the cell body and is especially important in the transmission of nerve impulses from the region of the cell body to other cells (**Figure 2**). The nerve cell may be divided on the basis of its structure and function into three main functional parts: the cell *body*, also called the soma; numerous short processes of the *soma*, called the *dendrites*; and, the single long nerve fiber, the *axon.* The cell body is the biosynthetic center of the cell. This is where cellular metabolism occurs, as well as the production of proteins and membrane. This production machinery, consisting of free ribosomes and rough endoplasmic reticulum (rER), is the most active and best developed of any cell in the body. The ribosomes and rER are the cellular organelles responsible for protein production and packaging. The

dendrites are responsible for receiving signals and conducting them up the cell to the cell body and on to the axon. The axon is the portion of the neuron that is responsible for the passing of the cellular message from the neuron to either other neurons, or neural receptors.

The body of a nerve cell is similar to that of all other cells and includes the nucleus, mitochondria, endoplasmic reticulum, ribosomes, and other organelles. Nerve cells are about 70 - 80% water; the dry material is about 80% protein and 20% lipid. The cell volume varies between 600 and 70,000 μm^3.

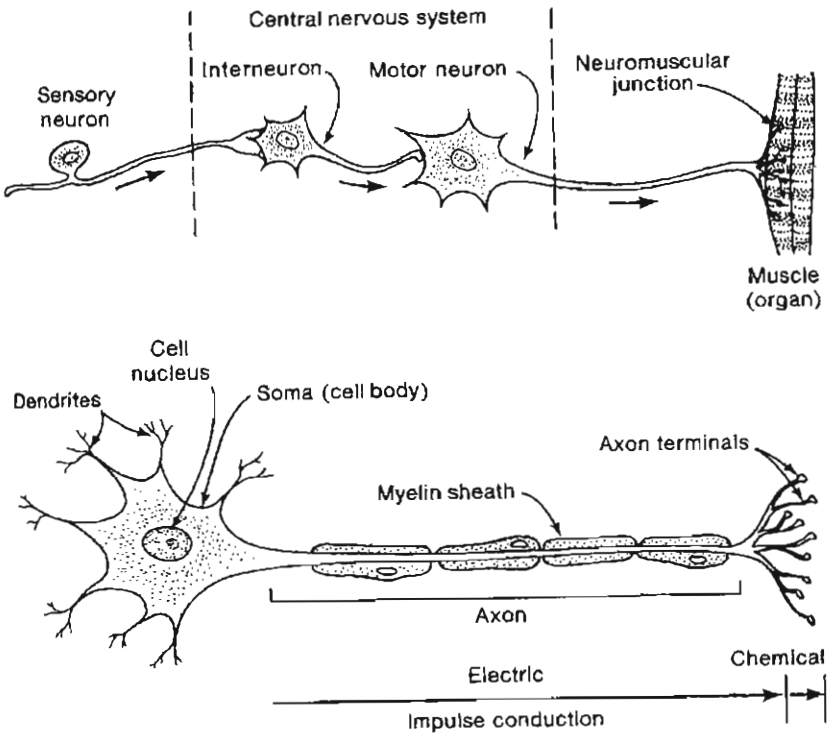

Figure 2. *Mammalian Nerve cells (Source: Ware, 2004).*

The short processes of the cell body, the dendrites, receive impulses from other cells and transfer them to the cell body (*afferent signals*). The effect of these impulses may be *excitatory* or *inhibitory*. A *cortical neuron* may receive impulses from tens or even hundreds of thousands of neurons [26]. The transmission of the impulse is a result of ion transport inside and outside the cell membrane. The plasma membrane of neurons has an unequal distribution of ions and electrical charges between the two sides of the

membrane. The outside of the membrane has a positive charge, inside has a negative charge. This charge difference is a resting potential and is measured in millivolts. Passage of ions across the cell membrane passes the electrical charge along the cell. The voltage potential is -65mV (millivolts) of a cell at rest (resting potential). Resting potential results from differences between sodium and potassium positively charged ions and negatively charged ions in the cytoplasm. Sodium ions are more concentrated outside the membrane, while potassium ions are more concentrated inside the membrane. This imbalance is maintained by the active transport of ions to reset the membrane known as the sodium potassium pump. The sodium potassium pump maintains this unequal concentration by actively transporting ions against their concentration gradients.

Changed polarity of the membrane (the action potential) results in propagation of the nerve impulse along the membrane. Then, the sodium gates and potassium gates open in the membrane to allow their respective ions to cross. Sodium and potassium ions reverse positions by passing through membrane protein channel gates that can be opened or closed to control ion passage. Eventually enough potassium ions pass to the outside to restore the membrane charges to those of the original resting potential. The cell begins then to pump the ions back to their original sides of the membrane. The action potential begins at one spot on the membrane, but spreads to adjacent areas of the membrane, propagating the message along the length of the cell membrane. After passage of the action potential, there is a brief period, the refractory period, during which the membrane cannot be stimulated. This prevents the message from being transmitted backward along the membrane.

Across the junction of a neuron with other cells, synaptic transmission occurs. A synapse is the junction of a neuron with other cells, including the junction between neuron and muscle or neuromuscular junction **(Figure 3)**.

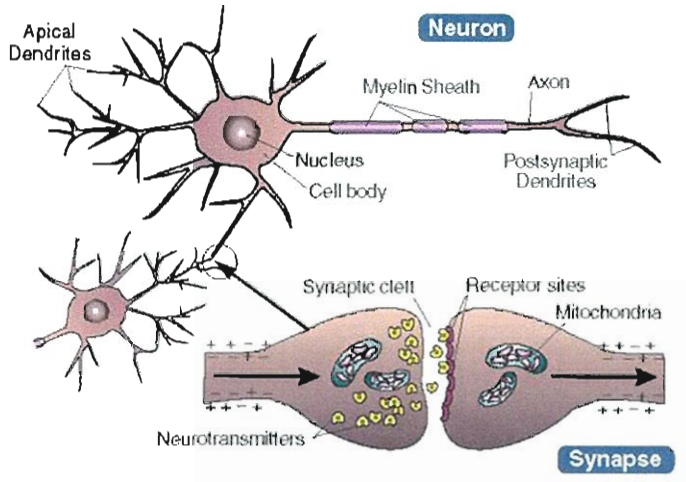

Figure 3. *Chemical and electrical synapses (Source: www.cyberlepsy.com)*

When an impulse, traveling along an axon, reaches a synapse, the impulse dies out, while causing to be released from the end of the axon a small charge of a chemical transmitter substance. This substance moves across the synapse (gap) and sets off another impulse if the synapse is between a neuron and a muscle or gland [27]. There are two well known chemical transmitters: acetylcholine and norepinephrine. Synapses that utilize acetylcholine are referred to as cholinergic, while those that use norepinephrine are called adrenergic.

All DDT-type chlorinated insecticides and the pyrethroids are considered axonic poisons. DDT as well as pyrethrin and some pyrethroids have a negative temperature coefficient; in other words, they have a greater insecticidal effect when the temperature is lowered. The complexity of DDT's mode of action is based on the manner it destroys the delicate balance of sodium and potassium within the neuron, in that way preventing it from conducting impulses normally. This is the result of "ion leakage", which produces prolonged impulses expressed as muscle tremors.

Pyrethroids are sodium channel modulators, which act as an axonic poison by interfering with sodium channels of both the peripheral and central nervous system, thus

stimulating repetitive nerve discharges, leading to paralysis and death. This action on the sodium channel, a tiny hole through which sodium ions are permitted to enter the axon to cause excitation, are produced in the insect nerve cord, which contains ganglia and synapses, as well as in giant nerve fiber axons. The stimulating effect of pyrethroids is much more pronounce than that of DDT [1]. The exact site of pyrethroids at synapses is not known. It is possible that the toxic action of pyrethroids is primarily due to its blocking action on the nerve axon since this action shows a negative temperature coefficient [1].

Oxadiazines also acts via blockage of the sodium channels, but appears to do so uniquely in a voltage dependent manner. Indoxacarb, the sole member of the oxadiazines, is active via ingestion and dermal routes.

b) **Synaptic poisons,** the second category included in the nerve poisons group, are chloride channel blockers. These chloride channel blockers impair the normal nerve impulse transmission in the nervous system at the synaptic site.

Cyclodienes act as chloride channel blockers. Unlike DDT and the pyrethroids, the cyclodienes have a positive temperature correlation; that is, their toxicity increases with increases in the surrounding temperature. The mechanism of cyclodiene poisoning also involves changes in ion permeability at axonic membrane levels. Cyclodienes have two electron-rich sites positioned opposite to each other along the line of symmetry and they fit into a particular biological site in the nervous system, blocking its normal physiological function. This group of chemicals acts on the inhibitory mechanism that acts as an internal off-switch and is naturally active in the nervous system, the GABA (gamma-aminobutyric acid) receptor, which operates by increasing chloride ion permeability into neurons. Cyclodienes prevent chloride ions from entering the neurons and thereby antagonize the inhibitory effects of GABA. They are thus known as GABA-gated chloride channel antagonists.

Cholinesterase inhibitors are another example of synaptic transmission inhibitors. The organophosphates (OP's) and carbamates use their toxic action by tying up or inhibiting cholinesterases (ChE). Acetylcholine (ACh) transmits an impulse at the synapse, and then Ach is destroyed by the ChE enzyme so the synapse will be cleared for

another transmission. These chemical reactions happen within microseconds and continue constantly, as needed, under normal conditions. However the OP's attach to the enzyme ChE in a way that prevents it from clearing away the Ach transmitter; in effect, the electric transmission circuits jam because of the accumulation of Ach. In mammals the accumulation of Ach interferes with the neuromuscular junction, producing rapid twitching of voluntary muscles, finally resulting in paralysis and death due to respiratory failure. Symptoms in insects follow the pattern of nerve poisoning; restlessness, hyperexcitability, tremors, convulsions, and paralysis.

Carbamates inhibit cholinesterase as OP's do and they behave in almost identical manner in biological systems, but with two main differences. First, some carbamates are potent inhibitors of aliesterase (miscellaneous aliphatic esterases); and second, unlike OPs, ChE inhibition by carbamates is apparently reversible. When ChE is inhibited by a carbamate, it is said to be carbamylated, while an organophosphate results in the enzyme being phosphorylated.

In insects, the effects of organophosphates and carbamates are primarily those of poisoning of the central nervous system and not at the neuromuscular synapses, since the insect neuromuscular junction is not cholinergic, as in mammals. The only cholinergic synapses known in insects are in the central nervous system. The chemical neuromuscular junction transmitter in insects is glutamic acid.

2.2 Resistance mechanisms and efficacy of the insecticides

Insecticide resistance mechanisms have a biochemical basis. The various mechanisms that enable insects to resist the action of insecticides can be grouped into four distinct categories: metabolic resistance, target-site resistance, reduced penetration, and behavioral resistance.

Metabolic resistance is the most common resistance mechanism that occurs in insects. This mechanism is based on the enzyme systems which all insects possess to help them detoxify naturally occurring foreign materials. Three categories of enzymes

typically fulfill this function, namely esterases, monooxygenases and glutathione-S-transferases. These enzymes systems are often enhanced in resistant insect strains enabling them to metabolize or degrade insecticides before they are able to exert a toxic effect. One of the most common metabolic resistance mechanisms is that of elevated levels or activities of esterases, enzymes known to hydrolyze ester bonds or sequester insecticides. Nearly all of the strains of *Culex quinquefasciatus* which resist a broad range of organophosphate insecticides have been found to possess multiple copies of a gene for esterases, enabling them to over produce this type of enzyme [28]. In contrast strains of malathion-resistant *Anopheles* have been found with non-elevated levels of an altered form of esterase that specifically metabolizes the OP malathion at a much faster rate than the normal form. Metabolic resistance can therefore range from compound-specific resistances to very general resistances, affecting a broad range of compounds. In the same way, the level of resistance conferred can vary from low to very high and may differ from compound to compound. Metabolic resistance mechanisms have been identified in vectors populations foe al major classes of insecticides including organophosphates, carbamates, pyrethroids, and DDT [29].

Target-site resistance occurs when the insecticide no longer binds to its target. Insecticides generally act at a specific site within the insect, typically within the nervous system (for OP, carbamates, and pyrethroids insecticides). The site of action can be modified in resistant strains of insects such that the insecticide no longer binds effectively at that site. The result is that these insects are unaffected, or are less affected, by the insecticide than are susceptible insects. For example, the target site for OP and carbamates insecticides is acetylcholinesterase (AChE) in the nerve cell synapses. Several mutated forms of AChE (also called MACE, modified acetylcholinesterase) have been found which result in reduced sensitivity to inhibition by these insecticides-resistance to OPs in *Culex* spp. e.g. typically results from this mechanism. Similarly, a mutation (known as *kdr*) in the amino acid sequence in the voltage gated sodium channels of nerve cell membranes leads to a reduction in the sensitivity of the channels to the binding of DDT and pyrethroids insecticides. Resistance to pyrethroids conferred by *kdr*

mutations has for example been confirmed in *An. gambiae* in West, Central and East Africa [30].

Reduced penetration can affect a broad range of insecticides (benzoylorea, thiadiazines, and triazines). Modifications in the insect cuticle or digestive tract linings that prevent or slow the absorption or penetration of insecticides can be found in some strains of resistant insects. Reduced-penetration mechanisms have been identified in houseflies, and are often considered a contributing factor rather than a powerful mechanism of resistance of its own.

Behavioral resistance describes any modification in insect behavior that helps to avoid the lethal effects of insecticides. Insecticide resistance in mosquitoes is not always based on biochemical mechanisms such as metabolic detoxification or target-site mutations, but may also be conferred by behavioral changes in response to prolonged spraying programs. Behavioral resistance does not have the same importance as physiological resistance but might be considered to be a contributing factor, leading to the avoidance of lethal doses of an insecticide. A behavioral response is either dependent or independent on a stimulus. If mosquitoes avoid a treated place due to sensing the insecticide it is considered to be a behavioral change dependent on a stimulus, whereas the selective and sustained occupation of an untreated area can be considered as stimulus independent response.

CHAPTER 3

Methods of Detecting and Monitoring Resistance

The main purpose of this chapter is to explore methods of detection of insecticide resistance, the way they detect changes in the susceptibility of a population of vectors, and how insecticide resistance is monitored through these methods. A cost comparison between methods of detection is provided to increase awareness on cost effectiveness while performing these tests. In addition, the criteria considered for the use of different methods is showed to explore advantages and disadvantages of the methods discussed in this section.

Appropriate monitoring of vector resistance to insecticides is an integral component of planning and evaluation of insecticides uses in vector-borne control programs. The initial step in identifying a potential problem with the effectiveness of vector control programs is to detect changes in the susceptibility of a population of vectors. Identification of resistance mechanisms helps determine the cross-resistance spectrum, facilitates the choice of alternative insecticides, and allows detailed mapping of areas with resistant populations. Detection of resistance development can be done through bioassay, biochemical assay, or molecular assay.

3.1 Bioassay method

The World Health Organization (WHO) has developed bioassay tests for mosquitoes, lice bedbugs, reduviid bugs, cockroaches, blackflies, houseflies, ticks, and fleas [31]. Bioassays tests are susceptibility tests which are used to measure resistance. There are two methods for performing bioassays. One method is designed to work on mosquito larvae while the other is designed for mature adult mosquitoes. Both assays require their own separate materials and preparations. The purpose of the bioassays is to detect insecticide resistance in individual insects by measuring changes in the time

required for an insecticide to reach its target and exert a toxic effect. In the presence of a resistance mechanism, this time interval increases.

The bottle bioassay allows resistance levels to be established for populations of adult mosquitoes reared in an insectary or collected in the field. The major advantages of the bottle or larval assays are that any concentration of any insecticide may be evaluated at the one time. This means that insecticide resistance is detected in individual insects by measuring changes in the time required for an insecticide to reach its target and exert a toxic effect. In the presence of a resistance mechanism, this time interval increases. Secondly, the technique is simple and rapid. The goal of the bioassay is to measure the time it takes for a given insecticide to kill the adult mosquito or the larvae. The larval bioassay allows resistance levels to be established for populations of larvae reared in breading pan or collected in the field. Both tests can be performed in less than 48 hours. The cost of the bioassay kit is $42.00 [31]. According to Brogdon (1998), time-mortality bioassays were more sensitive than dose-mortality bioassays in detecting changes in susceptibility, and they had better correlation with micro-plate based biochemical assays for resistance mechanisms [13].

Bioassays require the use of relatively large numbers of insects and insecticide impregnated test papers which may be difficult to prepare and store reproducibly. On the other hand, biochemical and molecular methods can detect resistance mechanisms in individual insects; consequently, they can confirm resistance with the use of only a small number of insects. Although the bioassay approach has been the best resistance detection technology available, it has some limitations. There are some scientists that argue that resistance cannot be detected at low frequency using bioassay, especially where susceptible insects survive due to deterioration of papers or resting on netting of exposure chambers [32]. Another disadvantage is that viral growth using cell cultures is required in most cases taking up to a week for the isolation, delaying reporting for up to several weeks.

3.2 Biochemical assays

Biochemical assays/techniques may be used to establish the mechanism involved in resistance. When a population is well characterized some of the biochemical assays can be used to measure changes in resistance gene frequencies of metabolic enzymes in field populations under different selection pressures. These assays detect altered enzyme activities for acetylcholinesterase (cannot be used with mosquito larvae), elevated esterase, glutathione-S-transferase and protein. To date biochemical assays have successfully been used in mosquitoes (*Culex*, *Anopheles*, and *Aedes*), sand flies, cockroaches, houseflies and blackflies as well as some agricultural pests [33]. Two main variants of the assays are in use. One variant of the assays uses filter paper or another support media; the second variant is run in microtiter plates. The filter paper or nitrocellulose membrane assays generally use one mosquito per assay and are quantified visually or using a densitometer, but provide a permanent record which can be rechecked in the future. The microtitre tests allow the same insect to be used for all assays and are quantified visually. The formation of a colored end product allows direct observation of a reaction or automated spectrophotometric reading [34]. A permanent record can be made on paper by simply using a transfer plate, but this is not an automatic result of the test.

The assay for altered acetylcholinesterase, in either its nitrocellulose membrane or microplate form, is based on the difference in the sensitivity of the enzyme to pesticide in the resistant mosquitoes as compared to the susceptible specimen. A carbamate insecticide is used as the inhibitor, although it may be replaced by the axon analogue (metabolic active form) of an OP insecticide under laboratory conditions. In some assays, the enzyme is preincubated with the insecticide before the addition of substrate alone is determined in both types of assays.

An increase in esterase activity is a common mechanism of resistance, especially to the OP compounds, in *Culex* mosquitoes. This type of mechanism can be detected by either filter paper or microplate assay using the general substrates 1- or 2-naphthyl acetate. Fast garnet GBC is then used as the stain with the filter paper and fast blue RR with the microplate test. When elevated esterase activity is detected, its connection with resistance should be confirmed by bioassays and the specific esterase(s) involved

determined by electrophoresis.

Some resistance to DDT and OP compounds is based on glutathione *S*-transferase. Where this mechanism is active, there is an associated increase in activity against the general substrate chlorodinitro-benzene (CDNB). A microplate assay can be used to detect this mechanism by determining the rate of conjugation of reduced glutathione and CDNB.

The biochemical assays described above differ both qualitatively and quantitatively. The minimum requirement is the ability to determine whether a resistant mechanism is present or not. This can be achieved with any of the variants of the assays. Quantification of the esterase activity is possible visually with both filter paper and microplate assays. The microplate esterase assay has quantitative efficiency; but accurate quantification is not possible when several resistance mechanisms are present in the same insect. Some of the advantages of the biochemical assays over the bioassays are: results are obtained within minutes; the results of the test are presented as a colored end product or as a number representing a spectrophotometric reading. In contrast, in diagnostic dosage bioassays, some insects may have survive as a result of inaccurate dosing, so that confirmation is required either by repetition of the test or by testing the offspring of the survivors.

3.3 Immunological methods

This method uses antiserum and is available for specific elevated esterases. An affinity purified IgG fraction from the antiserum is used in an immunoplate assay to discriminate between resistant variants of the insect population in question. The cost ranges from $190.00 through $356.00 depending on the kind of test [35]. The sensitivity of this assay is such that it gives a clearer differentiation of resistant phenotypes than the esterase microplate biochemical assay [33].

3.4 Molecular methods

Molecular information on resistance mechanisms will increasingly be incorporated into resistance diagnostic procedures. The type of resistance mechanism that molecular assays detect is based on the point mutations that cause target-site resistance or changes in detoxification enzyme specificity. Thus far, target-site mechanisms have been detected by polymerase chain reaction (PCR) amplification of specific alleles.

Polymerase chain reaction method

PCR is increasingly being applied to the detection of infectious agents. The fundamental feature of PCR is to replicate fast and exponentially a particular DNA sequence (template). The template should represent a relatively small fragment of DNA, typically 0.5 to 2.0 kilobases (kb) [36], because larger target sequences are more difficult to amplify efficiently. Only minute quantities of the template need be present; theoretically, even a single copy is detectable. DNA polymerase is the enzyme responsible to initiate the elongation at the 3' end of a short primer bound to a longer strand (target) of DNA (**Figure 4**).

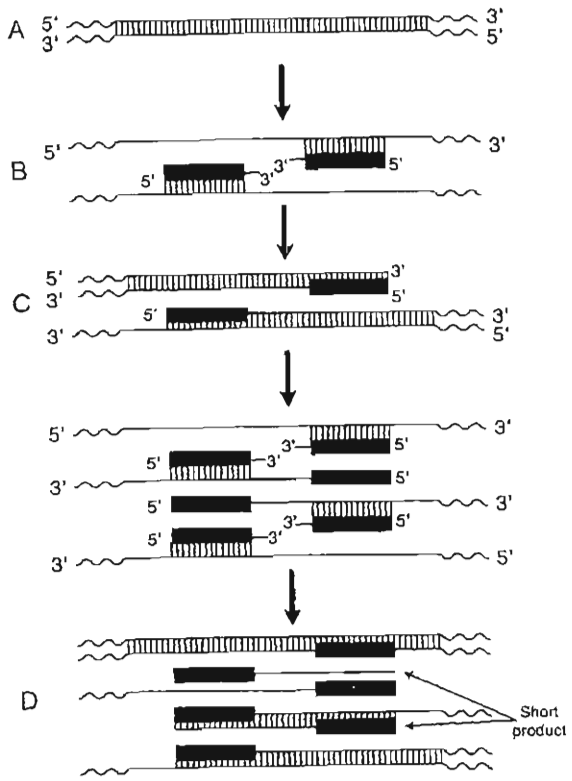

Figure 4*.PCR technique. (Source: Murray, 1999).*

In the first cycle, a double stranded DNA target sequence is used as a template (A). In step (B) two oligonucleotides (called primers), are separated by heat denaturation, and the synthetic primers (solid bars) anneal to their respective recognition sequences in the 5'→ 3' orientation. These primers should be long enough to define those sites uniquely; 18 to 20 basepairs (bp) is typically sufficient [37]. In step (C) a thermostable DNA polymerase initiates synthesis at the 3' ends of the primers. Extension of the primer via DNA synthesis results in new strands and therefore new primer-binding sites. The net result after one round of synthesis is two ragged copies of the original target of the DNA molecule. In step (D), the second cycle, each of the four DNA strands in panel C anneals to primers (present in excess) to initiate a new round of DNA synthesis. An entire procedure, consisting generally of 20 to 30 cycles, can be conducted in a small, closed container (e.g., microcentrifuge tube) and within few hours will generate sufficient product, also known as amplicon, to be visualized and sized in an agarose or polyacrylamide gel.

DNA amplification by PCR can be performed in a few hours from a specific target DNA sequence, but some prior DNA sequence information from the target sequences is required. According to Strachan (1999), the general requirement for prior target sequence information is a disadvantage of PCR [38]. This means that the DNA region of interest has been partly characterized previously, often following cell-based DNA cloning. In other words, although PCR can be applied to guarantee hole genome amplification, it does not have the advantage of cell –based DNA cloning in offering a way of separating the individual DNA clones comprising a genomic DNA library.

Real-Time Reverse-Transcriptase PCR

Rapid advances in molecular biology have facilitated new approaches to evaluate quantitative aspects of vector competence. In particular, quantitative real-time reverse-transcriptase (RT)-PCR based assays provide the sensitivity, speed, and statistical power to conduct high-throughput experiments [39]. The precise quantitation of viral RNA from a sample of only 5 μL allows for the study of viral infection, replication, and dissemination in specific tissues in individual mosquitoes on a scale that was previously impractical. Real-time PCR is the technique of collecting data during the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of fluorescent chemistries that correlate PCR product concentration to fluorescence intensity. Reactions are characterized by the point in time (or PCR cycle) where the target amplification is first detected. This value is usually referred to as cycle threshold (C_t), the time in which fluorescence intensity is greater than background fluorescence. Therefore, the greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescence signal will appear, yielding a lower C_t, allowing for quantification of the starting material with the help of a standard curve [37]. The general steps performed during a real-time PCR experiment, from RNA isolation to data analysis, are outlined in (**Figure 5**).

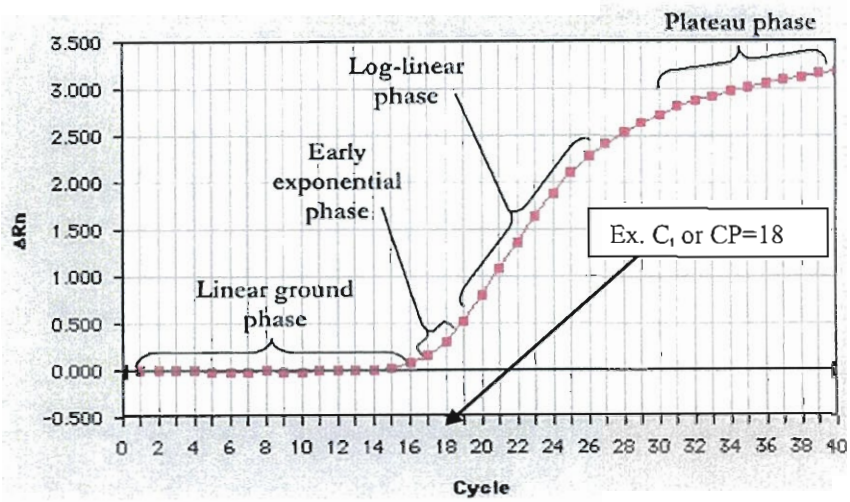

Figure 5*.Phases of the real-time PCR amplification curve (Source: Wong, 2005).*

Real-time PCR can be broken into four main phases: the linear ground phase, early exponential phase, log-linear (i.e. exponential) phase, and plateau phase. During the linear ground phase (usually the first 10-15 cycles), PCR is just beginning, and fluorescence emission at each cycle has not yet risen above background. Baseline fluorescence is calculated at this time. At the early exponential phase, the amount of fluorescence has reached a threshold where it is significantly higher than background levels. The cycle at which this occurs is known as C_t (Applied Biosystems, Inc.), or crossing point (CP) (Roche Applied Science, Inc.) [37]. During the log-linear phase, PCR reaches its optimal amplification period with the PCR product doubling after every cycle in ideal reaction conditions. Finally, the plateau stage is reached when reaction components become limited.

Real-time PCR uses two formats, SYBR Green I and TaqMan. According to Richardson, SYBR Green I based assays are less expensive, more flexible, and less susceptible to false negatives due to single nucleotide polymorphisms in the probe sequence than assays that use TaqMan [39]. While a single point mutation in the probe region can reduce target detection by 47% in TaqMan assays, SYBR Green I is a nonspecific dsDNA binding dye that only requires design of oligonucleotide primers for PCR to measure fluorescence emission. When bound to dsDNA, the fluorescence of

SYBR Green I is increased ~1,000-fold, providing a sensitivity equal to that of TaqMan. However, SYBR Green I raise a potential concern due to its lower specificity; this is because it is not sequence specific, and low-level background fluorescence can raise from primer-dimers and other nonspecific amplicons. These amplicons are small fragments of DNA that are amplified using PCR. The production of an amplicon tells that a specimen contains a pathogenic agent. However, sometimes it needs further analysis of the amplicon to completely identify or characterize the agent. These analyses include sequencing or identify the nucleotides that make up the amplicon. There are many commercial centers that can provide the sequencing services. Some are located on universities, with others privately owned. The price per sequencing varies depending on the type of amplicon and reagents used. Sequencing of an amplicon can cost between $12 and $62 ([40], [41], [42]). The price for Real-Time PCR is approximately $10.00 per sample (including the DNA extraction) [43].

Real-time PCR has some benefits over other methods to quantify gene expression. One is the production of quantitative data with an accurate dynamic range of 7 to 8 log orders of magnitude and does not require post-amplification manipulation (such as gel electrophoresis). [37]. For example, Dengue virus-2 (DENV-2) RNA was quantified from the midgut and legs of individual *Aedes aegypti* at each 14 days post infectious blood meal (dpi) in a DENV-2 susceptible strain from Chetumal, Mexico from a sample of only 5 μL. The lower detection and quantitation limits were 20 and 200 copies (1 copy = one organism) per reaction, respectively [39]. In addition, real-time PCR assays can reliably detect gene expressions (on methods testing for repeatability) differences as small as 23% between samples [44]. On the other hand, the Real-time PCR can be affected if the quality of the specimen is poor or the genetic material in it is degraded. Another disadvantage is that the primers can not be used any longer due to a change or mutation in the original genome. These primers were designed for a specific piece of genome which is not longer the same. In addition, maybe the probe is not specific enough for the sequence you are trying to detect because that part has changed too. Molecular detection of nucleic acids is rapid, sensitive, and applicable to infectious agents that cannot be detected by cell culture. Because of its simplicity, PCR is a popular technique with a wide range of applications which depend on essentially three major advantages of the

method. First, PCR is rapid, and can be performed in a few hours, using relatively unsophisticated equipment; second, its extreme sensitivity allows amplifying sequences from minute amounts of target DNA; lastly, its robustness permits amplification of specific sequences from material in which the DNA is badly degraded or embedded in a medium from which conventional DNA isolation is problematic.

In an effort to compare assay accuracy[5], sensitivity[6] and specificity[7], a blinded laboratory evaluation compared an in situ enzyme immunoassay (EIA), VecTest assay (antigen screening test for the detection of West Nile and St. Louis Encephalitis (SLE) viruses in mosquitoes), and reverse transcription-polymerase chain reaction (RT-PCR) in pools of 50 mosquitoes (adult female *Culex tarsalis* Coquillet) [45]. Because most *Cx. tarsalis* tested for virus infection in California are collected host-seeking (attracted particularly to this host) by dry ice-baited traps, positive pools are expected to contain parous females that have incubated the virus for greater than 3 days. VecTest and RT-PCR were comparably sensitive (0.97- both VecTest and RT-PCR), and accurate (0.98- both VecTest and RT-PCR), detecting virus in pools containing females held for 3 days post inoculation with the virus; only RT-PCR detected SLE virus in pools on days 0-1 post inoculation [45], thus giving an advantage to RT-PCR against the VecTest method for earlier detection. The ability of detecting SLE virus by in situ EIA in WN-SLE mixed pools plates was compromised due to the rapid growth of WN, which make this method (EIA) the least practical of all. Detector antibodies used in the in situ EIA cross-reacted between SLE and WN viruses, reducing accuracy (0.90).

Table 2 shows a comparison of different criteria of all the methods discussed in this research. Molecular techniques are the most costly techniques in the table, but provide a smaller turnaround time for the most of the cases. This is possible if the piece of genetic material to clone or amplify the target sequence is available. A great degree of sensitivity, or a low limit of detection, is required to detect genes expressed at low levels in smaller quantities of total of genetic material. For reliable results, molecular assays must be very specific, detecting only single gene specific amplicons without secondary products. Even though PCR is a simple test for the detection of DNA from a single microorganism, in

[5] Accuracy means the correct positive plus negative assays/total.
[6] Sensitivity is the correct positive assays/true positives.
[7] Specificity refers to the correct negative assays/true negatives.

practice there is a problem with specificity in the method. In other words, the specificity alone does not tell us how well the test or experiment recognizes positive cases. We also need to know the sensitivity of the test. Specificity s sometimes confused with the precision or the positive predictive value. A test with very high specificity can have very low precision if there are far more true negatives than true positives, and vice versa. The generation of false positive reactions because of the amplification of contaminating DNA has been encountered, which include the products from previous PCRs, that it may also contain components which interfere with the amplification of the DNA [46]. Finally, reproducibility is a very important criterion when performing these techniques to insure the same results from the same samples.

Unlike the Bioassay technique, Molecular and Biochemical techniques do not need a large amount of insects of specimens. This is an advantage when a little amount of sample is provided. The sensitivity in the Molecular techniques is extremely high. The sensitivity of biochemical techniques is high, but it could be compromised if the sample is cross-contaminated. Bioassay method's sensitivity is also high, but the sensitivity will decrease to low when the test filter papers are deteriorated. This seldom occurs when samples are considered irretrievable or cannot be collected again.

Bioassays and Biochemical tests are more time consuming than Molecular techniques, but they are less expensive and easier to perform. There are some tests that have been marketed, and are available through WHO –Supplies for Monitoring Insecticide Resistance in Disease vectors ([31]). For example, the Bioassay Kit cost $42.00, which includes enough materials for 24 tests (that is $1.75 per sample). Table 3 shows a cost comparison between those methods. Equipment cost is an important factor at the time to develop and implement new techniques for the detection of infectious agents. Table 4 shows a comparison between the basic set up for serological and molecular biological techniques regarding the needed equipment in the laboratory. A total of $37,272.50 is needed to start a laboratory with serological methods capacity, as suppose to $53,734.47 for the molecular biological setting. A larger amount of money is required to start a molecular biology laboratory than a clinical laboratory.

Besides the equipment, the personnel factor is also different and important to take into consideration when performing these methods. These techniques require personnel

trained with specialized skills and special training to work with this particular equipment. On average, a person skilled to work in a clinical laboratory holds at least an associate degree (medical laboratory technician), but most of the time a four year degree (medical technologist) is earned. While a person working at a molecular biology laboratory holds at least a bachelors' degree up to a doctorate degree. The job titles are various for these kind of scientists. They are some biologists, microbiologists, chemists, molecular biologists, etc., working on the molecular biology laboratory. The labor cost for both laboratories' workers are different as well. They can differ from earning $10-$15 an hour in a regular laboratory, up to $20-$35 an hour for the most specialized laboratory.

Most scientists prefer screen samples with the easiest, accessible and least expensive methods of detection, in this case bioassays and biochemical's techniques. Once the samples had been screened by these methods, the few positives samples (if present), are processed through more sensitive and costly methods such as molecular techniques. In the case of working with a conserved sample, a screening process could be done using a bioassay technique. Bioassays are also used for routine monitoring due to its lower cost. If few presumptive positive samples are detected, confirmation (meaning repeat a sample to verify the result with a different methodology) of those positive samples using a molecular technique such as Conventional PCR can be performed. Molecular techniques are also useful if you need to verify if any mutations have taken place. In the case of suspicion of a contaminated sample is best to process it with a less specific test such as the bioassay method. It is important to consider the number of samples to be processed; supplies or materials, and technology available to process a big workload of samples, use the molecular technique. It will help managing a big number of samples in the least amount of time.

Table 2. Detection Methods Comparison

Method	Cost/ test in $US	Speed	Sensitivity Level	Accuracy Level	Reproducibility	Specificity Degree	No. of amount/specimens needed	Use/Comments
Bioassay (uses cell culture for viral growth0	1.75	< 48 HRS or up to two wks when viral growth is needed	High, but low when test papers are deteriorated	High	Difficult	High	Large number of insects	Measures time intervals for resistance levels. Determines if resistance is present or not, but not the type of mechanism.
Biochemical -filter paper or microtiter plates	3.71	3 HRS, or within minutes for microtiter plates.	High, but medium when sample is cross-contaminated	High, but low or none when sample is cross-contaminated.	Easy	High	Individual insects or specimens	Establishes resistance's mechanisms. Extra equipment might be needed.
Molecular (Conventional PCR)	12-25	~4 HRS	High	High	Easy	Extremely High	Individual insects or minute amount of DNA (20-200 copies).	
Molecular (Real-Time PCR)	25-62	~1 HR	Extremely High	High	Easy	Extremely High	Individual insects or minute amount of DNA (20-200 copies).	

Table 3. Adapted from WHO, 2001- Cost of Supplies to Monitor Insecticide Resistance.

ITEM	COST PER UNIT (US$)	NOTES	Cost per sample (US$)
Biochemical- Insecticide impregnated paper- DDT 4%	12.00	Each box with 8 papers.	1.50
Biochemical- Insecticide impregnated paper- Malathion 5%	18.00	Each box with 8 papers.	2.25
Biochemical- Insecticide impregnated paper- Permethrin 0.75%	18.00	Each box with 8 papers.	2.25
Bioassay Kit (for a specific insecticide)	42.00	24 tests	1.75
Bioassay kit- Mosquitoes (Larvae resistance to development inhibitors)	59.00 per kit	24 tests	2.46

Table 4. Equipment cost for Serological and Molecular methods[8].

Method	Item	Price in Dollars
Serological	96 well Microplate reader [4]	$20,577.50
	Centrifuge-Refrigerated [1]	$8,865.00
	Heating Blocks [3]	$109.00
	Incubator [2]	$2,513.00
	Magnetic Stir Bar [2]	$27.55
	Microcentrifuge [2]	$2,327.80
	Minicentrifuge [3]	$227.00
	Vortex [2]	$269.12
	Water Bath [2]	$2,356.53
Total	$37,272.50	
Molecular	Centrifuge-Refrigerated [1]	$8,865.00
	Magnetic Stir Bar [2]	$27.55
	Microcentrifuge [2]	$2,327.80
	Gel System [3]	$325.00
	Gel System-Minigel [3]	$478.00
	Imaging System [3]	$12,000.00
	Minicentrifuge [3]	$227.00
	Real-Time PCR LightCycler [5]	$27,500.00
	Transilluminator [3]	$1,715.00
	Vortex [2]	$269.12
Total	$53,734.47	

[8] Companies are as follows: 1-Eppendorf, 2-Fisher Scientific, 3-ISC BioExpress, 4-Molecular Devices, 5-Roche Diagnostics.

CHAPTER 4

Conclusions

Appropriate monitoring of vector resistance to insecticides is an integral component of planning and evaluation of insecticides uses in vector-borne control programs. The initial step in identifying a potential problem with the effectiveness of vector control programs is to detect changes in the susceptibility of a population of vectors. Identification of resistance mechanisms helps determine the cross-resistance spectrum, facilitates the choice of alternative insecticides, and allows detailed mapping of areas with resistant populations.

Molecular biology techniques offer a way to detect resistance that is based on mechanisms that involve changes at the level of nucleic acids. It is sensitive, and applicable to infectious agents that cannot be detected by cell culture. Molecular techniques are rapid, therefore, can be performed in a few hours. The detection of a DNA sequence could effectively help us to detect insecticide resistance at an early stage because it can detect fewer resistant insects, therefore earlier stage of emergent resistance. Despite all the advantages that molecular techniques offer, it has a disadvantage that needs to be considered, the requirement for an existing characterized DNA sequence. It will sometimes have to be used in conjunction with another detection method. In addition, another disadvantage of PCR is the potential for generation of false positive reactions due to the amplification of contaminating DNA.

Conducting studies to detect the susceptibility status of mosquito vectors populations will assist to plan an effective mosquito control program. Molecular techniques have made it possible to determine the extent to which resistance can develop and to detect resistant genotypes long before their frequency increases sufficiently to cause failures in insect control programs.

It is important to consider the impact of pesticides on beneficial insects, and use products at recommended rates and spray intervals to minimize undesired effects on parasitoids and predators. Pest control measures used will determine the way in which

resistance will evolve in the future. Our increasing knowledge of the underlying mechanisms, and the availability of sensitive and rapid diagnostic methods for their identification, opens the way to make rational choices of insecticides. Integrated vector control strategy should be considered and resistance surveillance in mosquito needs to be conducted regularly.

Early detection of resistance is essential to prevent both unnecessary insecticide application and disease transmission. Early detection is of major importance in the control of diseases with high mortality levels or in this research case insecticide resistance in mosquitoes. At this point of time a combination of well managed and developed bioassays and molecular methods is the most beneficial strategy to detect insecticide resistance. Detecting resistance early is one part of the management strategy; the other is to have alternative treatments available when the previous are no longer effective. Using sound Integrated Pest Management (IPM), insecticide rotations and thoroughness in treating will ensure positive results in insecticide resistance management programs. Therefore it is imperative to keep studying pesticides and their modes of action, thus, we can create and use the less toxic chemical that would cause less damage to our environment.

GLOSSARY

Anthropophilic - human-seeking or human-preferring, especially with reference to: 1) bloodsucking arthropods, denoting the preference of a parasite for the human host as a source of blood or tissues over an animal host; and 2) dermatophytic fungi which grow preferentially on humans rather than other animals.

Cuticulin - hard covering of invertebrates: a hardened noncellular layer secreted by and covering the epidermis in many invertebrates.

Diapausing - a period of hormonally controlled quiescence, esp. in immature insects, characterized by cessation of growth and reduction of metabolic activity, often occurring seasonally or when environmental conditions are unfavorable.

Deoxiribonucleic acid (DNA) - Double-stranded molecule, consisting of paired nucleotide units grouped into genes and associated regulatory sequences. These genes served as blueprints for protein construction from amino-acid building blocks.

Dichlorodiphenyltrichloroethane (DDT) - organochlorine contact insecticide that kills by acting as a nerve poison.

Endophagic - An endophagic mosquito is a mosquito that feeds indoors.

Endophilic - An endophilic mosquito is a mosquito that tends to inhabit/rest indoors. Endophilism facilitates the blocking of malaria transmission through application of residual insecticides to walls.

Hazard - the risk or danger of poisoning when a chemical is used or apply.

Heterocyclic derivatives - the ring structures are composed of different or unlike atoms. One or more of the carbon atoms is displaced by oxygen, nitrogen or sulfur and the ring may have three, five or six atoms.

Mode of action - How a pesticide block some metabolic process. The sum of anatomical, physiological and biochemical interactions and responses that result in toxic action of a chemical, as well as the physical (location) and molecular (degradation) fate of the chemical in the organism.

Mechanism of action or resistance - Biochemical and biophysical responses of the organism that are associated with the pesticidal action.

Orthologs - genes in different species that have evolved from a common ancestral gene by speciation and generally retain a similar function in the course of evolution. Normally, orthologs retain the same function in the course of evolution. Identification of orthologs is critical for reliable prediction of gene function in newly sequenced genomes.

Pesticides - agents employed by humans to destroy or control pests.

Polytene - of multi-stranded chromosome: describes a giant chromosome with distinct chromosome bands in polyploid cells of some two-winged flies, comprising multiple copies of a chromosome aligned side by side.

Ribonucleic acid (RNA) - Any of a class of single-stranded molecules transcribed from DNA in the cell nucleus or in the mitochondrion or chloroplast, containing along the strand a linear sequence of nucleotide bases that is complementary to the DNA strand from which it is transcribed.

Sclerite - layer of arthropod's skeleton: a hard plate or layer of chitin or calcium on the outer skeleton of an arthropod.

Synergism - Increased activity resulting from the effect of one chemical on another.

Synergists - Materials used with insecticides to synergize or enhance the activity of the insecticides, thus maximizing the effect of the insecticide. The mode of action is to bind to oxidative enzymes (oxidases) that would otherwise degrade the insecticide.

Tolerant - Capable of withstanding effects.

Toxicity - is the inherent poisonous potency of a compound under experimental conditions.

APPENDICES

APPENDIX A- Insecticide resistance Action Committee (IRAC) Mode of Action Classification for United States Products: Showing the Acetylcholine esterase inhibitors and gated chloride channel antagonists (GABA) groups.

IRAC Mode of Action Classification v5.1, September 2005 - Ag Uses		
Main Group - Primary Site of Action		
Chemical Subgroup or exemplifying Active Ingredient		
Active Ingredient	**Product Name**	**Registrant**
Group 1 - Acetylcholine esterase inhibitors		
1A - Carbamates		
Aldicarb	Temik®, Bolster™	Bayer CropScience, Amvac
Carbaryl	Sevin®	Bayer CropScience, Drexel, Gowan, UAP-Loveland, Wilbur-Ellis
Carbofuran	Furadan®	FMC
Formetanate	Carzol® SP	Gowan
Methiocarb	Mesurol®	Gowan
Methomyl	Lannate®	DuPont
Oxamyl	Vydate®	DuPont
Pirimicarb	Pirimor®	Syngenta
Thiodicarb	Larvin®	Bayer CropScience
1B - Organophosphates		
Acephate	Orthene®	Cheminova, Micro Flo, TENKOZ, United Phosphorus, Valent
Azinphos-methyl	Guthion®	Bayer CropScience, Micro Flo
Chlorpyrifos	Govern™, Lock-On®, Lorsban®, Nufos®, Warhawk™, Whirlwind™, Yuma™	Dow AgroSciences, Makhteshim Agan NA, Agriliance, Drexel, Gowan, Helena, TENKOZ, UAP-Loveland
Diazinon	Diazinon	Drexel, Gowan, Helena, Makhteshim Agan NA, Micro Flo, UAP-Loveland, Wilber-Ellis
Dimethoate	Dimethoate	Agriliance, Britz, Drexel, Gowan, Helena, Micro Flo, UAP-Loveland
Disulfoton	Di-syston	Bayer CropScience
Ethoprophos	Mocap®	Bayer CropScience
Fenamiphos	Nemacur®	Bayer CropScience
Fosthiazate	Nemathorin®	ISK
Malathion	Fyfanon®, Malathion	Agriliance, Cheminova, Gowan, Helena, Micro Flo, UAP-Loveland
Methamidophos	Monitor®	Bayer CropScience
Methidathion	Supracide®	Gowan
Methyl parathion	Penncap-M®	Cerexagri
Naled	Dibrom®	Amvac
Oxydemeton-methyl	MSR® Spray Concentrate	Gowan
Phorate	Phorate, Thimet®	Agriliance, Micro Flo, UAP-Loveland, Amvac
Phosmet	Imidan®	Gowan
Pirimiphos-methyl	Actellic®	Agriliance
Profenofos	Curacron®	Syngenta
Tebupirimfos	Aztec®, Defcon™	Bayer CropScience, Amvac
Terbufos	Counter®	BASF
Group 2 - GABA-gated chloride channel antagonists		
2A - Cyclodiene organochlorines		
Endosulfan	Thionex®	Drexel, Makhteshim Agan NA
2B - Phenylpyrazoles (Fiproles)		
Fipronil	Regent®	BASF

APPENDIX B- Insecticide resistance Action Committee (IRAC) Mode of Action Classification for United States Products Continued: Showing the Sodium channel modulator, Nicotinic Acetylcholine receptor agonists/antagonists, Nicotinic Acetylcholine receptor agonists (allosteric) (not group 4), and chloride channels activators groups.

IRAC Mode of Action Classification v5.1, September 2005 - Ag Uses		
Main Group - Primary Site of Action		
Chemical Subgroup or exemplifying Active Ingredient		
Active Ingredient	**Product Name**	**Registrant**
Group 3 - Sodium channel modulators		
Pyrethroids		
beta-cyfluthrin	Baythroid® XL	Bayer CropScience
Bifenthrin	Annex™, Bifenture, Brigade®, Capture®, Discipline™, Double Threat™	Amvac, FMC, Helena, TENKOZ, United Phosphorus
Cyfluthrin	Aztec®, Baythroid®, Renounce®, Leverage®	Bayer CropScience
lambda-cyhalothrin	Karate® Zeon, Mystic™ Z, Silencer™, Taiga™ Z, Warrior® Zeon	Agriliance, Helena, Makhteshim, Syngenta
gamma-cyhalothrin	Proaxis™, Prolex™	TENKOZ, UAP-Loveland
Cypermethrin	Ammo®, Battery™, Up-cyde™	Agriliance, Helena, TENKOZ, UAP-Loveland, United Phosphorus
zeta-Cypermethrin	Mustang®, Mustang® Max	FMC
Deltamethrin	Battalion™, Decis®	Arysta, Bayer CropScience
Esfenvalerate	Asana® XL	DuPont
Fenpropathrin	Danitol®	Valent
tau-Fluvalinate	Mavrik®	Wellmark
Permethrin	Ambush®, Arctic™, Perm-up, Pounce®	Agriliance, Amvac, FMC, Gowan, Helena, Micro Flo, TENKOZ, UAP-Loveland, United Phosphorus
Tefluthrin	Force®	Syngenta
Pyrethrins (pyrethrum)		
Pyrethrins (pyrethrum)	Pyganic®	MGK
Group 4 - Nicotinic Acetylcholine receptor agonists / antagonists		
4A - Neonicotinoids		
Acetamiprid	Assail®, Intruder™	Cerexagri, DuPont, Nisso
Clothianidin	Belay™, Clutch™, Poncho®	Arysta, Bayer CropScience
Dinotefuran	Venom™	Valent
Imidacloprid	Admire®, Admire® Pro, Alias™, Couraze™, Gaucho®, Imidacloprid 4F, Macho™, Pasada™, Provado®, Trimax™, Leverage®	Albaugh, Bayer CropScience, Cheminova, Makhteshim Agan NA
Thiacloprid	Calypso™	Bayer CropScience
Thiamethoxam	Actara®, Centric®, Cruiser®, Platinum®, T-Moxx™	Syngenta
4B - Nicotine		
4C - Bensultap, Cartap hydrochloride, Nereistoxin analogues		
Group 5 -Nicotinic Acetylcholine receptor agonists (allosteric) (not group 4)		
Spinosyns		
Spinosad	Double Threat™, Entrust®, SpinTor®, Success®, Tracer®	Dow AgroSciences, FMC
Group 6 - Chloride channel activators		
Avermectins, Milbemycins		
Abamectin	ABBA™, Agri-Mek®, Zephyr®, Avicta®	Makhteshim Agan NA, Syngenta
Emamectin benzoate	Denim™, Proclaim®	Syngenta
Milbemectin		

APPENDIX C- Insecticide resistance Action Committee (IRAC) Mode of Action Classification for United States Products Continued: Showing the Juvenile hormone mimics, microbial disruptors, and unknown compounds groups.

IRAC Mode of Action Classification v5.1, September 2005 - Ag Uses		
Main Group - Primary Site of Action		
Chemical Subgroup or exemplifying Active Ingredient		
Active Ingredient	**Product Name**	**Registrant**
Group 7 - Juvenile hormone mimics		
7A - Juvenile hormone analogues		
Kinoprene	Enstar®	Wellmark
Methoprene	Apex®, Diacon®, Extinguish®	Wellmark
7B - Fenoxycarb		
Fenoxycarb	Award®	Syngenta
7C - Pyriproxyfen		
Pyriproxyfen	Esteem®, Knack®, Seize®	Valent
Group 8 - Compounds of unknown or non-specific mode of action (fumigants)		
8A - Alkyl halides		
Methyl bromide	Brom-o-Gas, Haltox, Terr-o-Gas	
8B - Chloropicrin		
Chloropicrin	Pic-clor, Telone®	Dow AgroSciences, TRICAL
Group 9 - Compounds of unknown or non-specific mode of action (selective feeding blockers)		
9A - Cryolite		
Cryolite	Kryocide®	Cerexagri, Gowan
9B- Pymetrozine		
Pymetrozine	Fulfill®	Syngenta
9C - Flonicamid		
Flonicamid	Beleaf™, Carbine™	ISK, FMC
Group 10 - Compounds of unknown or non-specific mode of action (mite growth inhibitors)		
10A - Clofentezine		
Clofentezine	Apollo®	Makhteshim Agan NA
Hexythiazox	Hexygon®, Onager®, Savey®	Gowan
10B - Etoxazole		
Etoxazole	Zeal™	Valent
Group 11 -Microbial disruptors of insect midgut membranes (includes transgenic crops expressing Bacillus		
11A1 - B.t. subsp. Israelensis		
Bacillus thuringiensis subsp. *israelensis*	Gnatrol®	Valent
11A2 - B. sphaericus		
Bacillus sphaericus		
11B1 -*B.t. subsp. Aizawai*		
Bacillus thuringiensis subsp. *aizawai*	Agree®, XenTari®	Certis, Valent
11B2 - B.t. subsp. Kurstaki		
Bacillus thuringiensis subsp. *kurstaki*	Crymax®, Biobit®, Deliver®, Dipel®, Javelin®, Lepinox®	Certis, Valent, Wilbur-Ellis
11C - B.t. subsp. tenebrionis		
Bacillus thuringiensis subsp. *tenebrionis*	Novodor®	Valent

APPENDIX D- Insecticide resistance Action Committee (IRAC) Mode of Action Classification for United States Products Continued: Showing the Inhibitors of oxidative phosphorylation, Inhibitors of chitin biosynthesis, mitochondrial complex electron transport inhibitors, and Disruptors of molting chemicals groups.

IRAC Mode of Action Classification v5.1, September 2005 - Ag Uses		
Main Group - Primary Site of Action		
Chemical Subgroup or exemplifying Active Ingredient		
Active Ingredient	**Product Name**	**Registrant**
Group 12 - Inhibitors of oxidative phosphorylation, disruptors of ATP formation (inhibitors of ATP synthase)		
12A - Diafenthiuron		
12B - Organotin miticides		
Azocyclotin		
Cyhexatin		
Fenbutatin-oxide	Vendex®	DuPont, Griffin
12C - Propargite		
Propargite	Comite®, Omite®	Chemtura
Group 13 - Uncouplers of oxidative phosphorylation via disruption of proton gradient		
Chlorfenapyr		
DNOC		
Group 14 - vacant		
Group 15 - Inhibitors of chitin biosynthesis, type 0, Lepidopteran		
Benzoylureas		
Diflubenzuron	Dimilin®, Micromite®	Chemtura
Novaluron	Rimon®	Chemtura, Makhteshim Agan NA
Group 16 - Inhibitors of chitin biosynthesis, type 1, Homopteran		
Buprofezin		
Buprofezin	Applaud®, Courier®	Nichino
Group 17 - Molting disruptor, Dipteran		
Cyromazine		
Cyromazine	Trigard®	Syngenta
Group 18 - Ecdysone agonists / molting disruptors		
18A - Diacylhydrazines		
Methoxyfenozide	Intrepid®	Dow AgroSciences
Tebufenozide	Confirm®, Mimic®	Dow AgroSciences
18B - Azadirachtin		
Azadirachtin	Aza-direct™, Ecozin®, Neemix®	Amvac, Certis, Gowan, PBI Gordon
Group 19 - Octopaminergic agonists		
Amitraz		
Group 20 - Mitochondrial complex III electron transport inhibitors (Coupling site II)		
20A - Hydramethylnon		
Hydramethylnon	Amdro® Pro	BASF, Wilbur-Ellis
20B - Acequinocyl		
Acequinocyl	Kanemite™	Arysta
20C - Fluacrypyrim		
Fluacrypyrim		
Group 21 - Mitochondrial complex I electron transport inhibitors		
METI acaricides		
Fenpyroximate	Fujimite®	Nichino
Pyridaben	Nexter®, Pyramite™	BASF, Wilbur-Ellis
Rotenone		

APPENDIX E- Insecticide resistance Action Committee (IRAC) Mode of Action Classification for United States Products Continued: Showing the voltage-dependent sodium channel blockers; Lipid synthesis, Neuronal, and Aconitase inhibitors; Synergists; and miscellaneous inhibitors.

IRAC Mode of Action Classification v5.1, September 2005 - Ag Uses		
Main Group - Primary Site of Action		
Chemical Subgroup or exemplifying Active Ingredient		
Active Ingredient	Product Name	Registrant
Group 22 - Voltage-dependent sodium channel blockers		
Indoxacarb		
Indoxacarb	Avaunt®, Steward®	DuPont
Group 23 - Inhibitors of lipid synthesis		
Tetronic acid derivatives		
Spirodiclofen	Envidor®	Bayer CropScience
Spiromesifen	Oberon®	Bayer CropScience
Group 24 - Mitochondrial complex IV electron transport inhibitors		
24A - Aluminum phosphide		
24B - Cyanide		
24C - Phosphine		
Group 25 - Neuronal inhibitors (unknown mode of action)		
Bifenazate		
Bifenazate	Acramite®	Chemtura
Group 26 - Aconitase inhibitors		
Fluoroacetate		
Group 27 - Synergists		
27A - P450-dependent monooxygenase inhibitors		
Piperonyl butoxide	Exponent™	MGK
27B - Esterase inhibitors		
Group 28 - Ryanodine receptor modulators		
Flubendiamide		
un - Compounds with unknown mode of action		
una - Benzoximate		
unb - Chinomethionat		
unc - Dicofol		
Dicofol	Dicofol, Kelthane®	Dow AgroSciences, Gowan, Makhteshim Agan NA
und - Pyridalyl		
Pyridalyl		
ns - Miscellaneous non-specific (multi-site) inhibitors		
nsa - Borax		
nsb - Tartar emetic		
Tartar emetic		

APPENDIX F- Insecticide resistance Action Committee (IRAC) Mode of Action Classification used for Vector Control.

Insecticide Resistance Action Committee
www.irac-online.org

IRAC Mode of Action (MoA) Classification for active ingredients useful in vector control[1]

Primary Target Site of Action	Group	Subgroup	Chemical subgroup	Examples
Acetylcholinesterase inhibitors	1	A	carbamates[2]	bendiocarb, propoxur
		B	organophosphates[2]	fenitrothion, pirimiphos-methyl, malathion, temephos
GABA-gated chloride channel antagonists	2	B	fiproles	fipronil
Sodium channel modulators	3		DDT, pyrethroids and pyrethrins	allethrin, bifenthrin,, lambda-cyhalothrin, alpha-cypermethrin, deltamethrin, cyfluthrin, permethrin, etofenprox, phenothrin, transfluthrin
Nicotinic acetylcholine receptor agonists	5		spinosyns	spinosad
Juvenile hormone mimics	7	A	juvenile hormone analogues	methoprene, hydroprene
		C	pyriproxifen	pyriproxifen
Microbial disrupters of insect midgut membranes	11	A1	*Bacillus thuringiensis var. israelensis*	
		A2	*Bacillus sphaericus*	
Inhibitors of chitin biosynthesis	15		benzoylureas	diflubenzuron, triflumuron, novaluron

1. Including larvicidal and adulticidal insecticides. This mode-of-action classification is edited and updated yearly to include new products; please refer to www.irac-online.org for the complete mode of action list.
2. Not all compounds within the OPs are cross-resistant. Different resistance mechanisms that are not linked to target site of action, such as enhanced metabolism are common for the OPs (Figure 1). Some of these metabolic resistance mechanisms are sometimes specific to a particular subgroup or particular compounds within the OPs. As a result, there are proven examples of the successful management of resistance to a particular compound or subgroup of compounds within the OPs using OP compounds from a different subgroup.

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