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Insecticide Susceptibility and Resistance Detection in *Phlebotomus argentipes* Sandflies (Diptera: Psychodidae: Phlebotominae)

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INSECTICIDE SUSCEPTIBILITY AND RESISTANCE DETECTION IN *PHLEBOTOMUS ARGENTIPES* SANDFLIES (DIPTERA: PSYCHODIDAE: PHLEBOTOMINAE)

by

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ABSTRACT

Insecticide Susceptibility and Resistance Detection in

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by

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Utah State University, 2022

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Leishmaniasis is an important neglected disease in (sub)tropical rural regions worldwide. Approximately 12 million people are infected by a form of leishmaniasis annually, with the primary vector of the visceral form being transmitted by the vector *Phlebotomus argentipes* sandflies in India. Leishmaniasis currently has no known vaccine and treatment imposes a significant economic impact on already poor and marginalized populations, as well as severe, lingering side effects for afflicted individuals. The primary control method for sandfly populations for over half a century has been indoor residual spraying using insecticides. The selective pressure from utilizing insecticides over such a long period has resulted in selection for resistance which requires an assay to monitor and identify these populations.

A modified Center for Disease Control (CDC) bottle assay was used to assess *P. argentipes* susceptibility to permethrin, a synthetic insecticide in the pyrethroid family. A susceptibility curve was developed to determine the lethal concentration (LC) of permethrin capable of reducing populations by 50%, 75%, and 90%. Polymerase Chain Reaction (PCR) was optimized utilizing primers developed for the paralytic (*PARA*), a voltage-gated sodium channel, and acetylcholinesterase-1 (*Ace-1*), decoding region for the protein cholinesterase, genetic regions for *P. argentipes* which are specifically known to confer insecticide resistance.

This project utilized a Loop-mediated Isothermal Amplification (LAMP) assay, an alternative assay to PCR, which utilizes six specific primers and an isothermal temperature to amplify the genetic region of interest within the sandfly genome, to effectively and rapidly assess the degree of insecticide susceptibility in the vector *P. argentipes*. LAMP primers were designed specifically for the gene sequence associated with the *PARA* gene from a susceptible *P. argentipes*' colony. Two primer sets were developed: one to amplify *PARA* and a second to assess for codon mutation associated with insecticide resistance. Further testing with field-collected samples is required for optimization of this method.

An effective LAMP assay could allow integrated pest management professionals to rapidly determine the degree of susceptibility of sandfly populations collected from afflicted areas of the disease. Thus, allowing the disease vector to be controlled in a more timely and effective manner. (89 pages)

PUBLIC ABSTRACT

Insecticide Susceptibility and Resistance Detection in

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Shawna M. Hennings

Leishmaniasis is an understudied disease found predominantly in high heat and humidity areas. The disease is transmitted by sandflies which are blood-feeding, biting insects. There is currently no known vaccine for any form of leishmaniasis and treatment imposes a significant economic impact on already poor and marginalized populations, as well as severe, lingering side effects for afflicted individuals. Annually, there are millions of people around the world afflicted with a form of Leishmaniasis that is transmitted by species of sandflies. The primary sandfly population control method for over half a century has been indoor residual spraying using insecticides. The selective pressure from utilizing insecticides over such a long period has resulted in selection for resistance which requires an assay to monitor and identify these populations.

The goal of this thesis is to determine and assess insecticide susceptibility and resistance of the sandfly species, *Phelebotomus argentipes*. Research started by understanding the response of sandflies to particular insecticides of interest. This was completed by assessing levels of susceptibility of the P. argentipes colony to three insecticides which have been used extensively in India throughout the years.

Susceptibility curves were developed to determine lethal concentration (LC) of insecticides capable of reducing populations by 50%, 75%, and 90%.

An amplification assay (Polymerase Chain Reaction) was optimized to specifically amplify genetic regions associated with insecticide resistance for *P. argentipes* sandflies. Whereas, another amplification assay (Loop-mediated Isothermal Amplification) was developed as an effective and rapid procedure for integrated pest management (IPM) professionals to assess the degree of insecticide susceptibility in field collected populations of *P. argentipes* sandflies. This rapid detection of field collected samples could allow IPM professionals to determine the degree of susceptibility of sandfly populations collected from afflicted areas. Thus, allowing the disease vector to be controlled in a more timely and effective manner.

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CHAPTER 1

INTRODUCTION

Blood Feeding Insects. Insects that take blood meals from humans have been pests for thousands of years and would be no more than nuisances if not for the diseases that they vector and transmit.[1] There has been research done on a variety of blood feeding insects, including mosquitoes, ticks, and fleas, because of the bacterial, viral, and protozoan diseases they can transmit to humans. All of these vector-borne diseases impact the quality of life of human populations around the world.[2-4]

Mosquitoes, mostly from the *Anopheles* and *Aedes* genera, are known to vector the causative agents for the viral diseases such as Zika and dengue fever and the protozoan disease malaria. Mosquitoes have a wide geographical distribution but the species that transmit diseases are mostly found in tropical or subtropical regions.[2,3]

Zika is predominately in Central and South America and South East Asia.[5] Zika is a viral vector-borne disease caused by a member of the Flaviviridae family which is transmitted to humans by the infected bite of mosquitoes.[6] The Zika virus was discovered in 1947 and since that time multiple outbreaks of the disease have been documented around the world. The 2015 outbreak of Zika that occurred in Brazil spread and affected over 85 countries and territories with more than approximately 200,000 total cases.[7]

Dengue fever affects over 100 countries worldwide with approximately 2.5 billion people at risk.[8] This vector-borne disease has four serotypes that are transmitted from the infected bite of an *Aedes* mosquito. The serotypes have two disease presentations: dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF).[9] The average number of reported cases of DF/DHF have exponentially increased throughout the last 60 years starting at approximately 1000 cases per year in the 1950s, increasing by approximately 500,000 cases annually and upwards to 50 million cases by the 1990s, and continually increased to a current estimate of approximately 100 million cases per year.[8-11]

Malaria is a vector-borne disease caused by four known parasites from the *Plasmodium* genera and is transmitted by a bite from an infected *Anopheles* mosquito vector.[12] Malaria is the most important tropical disease with approximately 40% of the world's population at risk for infection.[13] In 2020, there were 241 million reported cases and over 500,000 deaths associated with malaria.[14]

Ticks, from the genus *Ixodes*, are mostly found in the United States, Europe, and parts of Asia.[15] Select species within the genera are of medical importance as vectors for the causative agent for bacterial Lyme disease. Lyme disease is caused by three species of a bacteria from the *Borrelia* genus.[16] There are an estimated 30,000 cases per year in the United States which makes it the most common vector-borne disease in the area.[17]

Fleas are known vectors for the causative agent for the bacterial form of plague.[4] Fleas are significant nuisance pests for domesticated animals and currently there are

no known fleas directly associated with humans.[18] The most common species of fleas associated with plague transmission is *Oropsylla montana* (Ground squirrel flea) in the United States and *Xenopsylla cheopi*s (Oriental rat flea) globally.[19] These species of fleas are known vectors for the causative agent for the bacterial form of plague, which is a re-emerging vector-borne disease. Plague is caused by the bacterium *Yersinia pestis* and is transmitted to humans by the infected bite of a flea.[18] The human flea, *Pulex irritans*, has poor vector competence for the transmission of *Y. pestis* compared to other species of fleas.[20] The disease was first characterized in 1894 and has been isolated from all continents. Approximately, 2000 cases are reported annually world-wide, with the United States averaging only about 3 human cases. The majority of plague cases are reported from African regions.[21]

The sandfly is an understudied blood feeding insect. They are the sole confirmed vectors for the transmission of the causative agents for the variety of forms of the neglected tropical disease Leishmaniasis.[22] There are over 30 species of phlebotomine sandflies but only two genera and their subspecies: *Phlebotomus* and *Lutzomyia*, are of medical importance.[23]

Leishmaniasis. Leishmaniasis is a neglected tropical vector-borne disease caused by the parasite *Leishmania*. The protozoan is vectored by the female phlebotomine sandfly and is transmitted when the infected sandfly takes a blood meal. The genus *Phlebotomus* transmit *Leishmania* species. The *P. argentipes* sandfly is the sole vector of visceral leishmaniasis in India. *P. papatasi* is a vector for cutaneous leishmaniasis.[24]

The estimated global impact of leishmaniasis is 350 million people in 88 countries at risk for a form of leishmaniasis. As of 2010, at least 12 million people annually are afflicted with a form of leishmaniasis with new cases ranging from 0.9-1.6 million each year.[25] The disease is most commonly found in rural areas of countries in the tropics, subtropics, and southern Europe. There are no vaccines or drugs available to prevent a Leishmaniasis infection.[26,27] There are three types of leishmaniasis disease presentations: cutaneous, mucocutaneous, and visceral.

The most common and widespread form is cutaneous leishmaniasis (CL), which causes skin lesions. These lesions can vary in size, appear at the site of the bite usually on exposed areas of the body, and last for up to a year after infection. The lesions can either be self-healing, or lead to widespread destructive ulceration that can cause permanent scarring.[28,29] As of 2004, through passive case detection, there were 1.5 million reported cases of CL globally.[30] The World Health Organization (WHO) regional specificities shows that the WHO Eastern Mediterranean region makes up 80% of all reported CL cases. The most recent epidemiological trend shows that new reported cases of CL range from 700,000 to 1.2 million.[26]

The less common mucocutaneous leishmaniasis (MCL) form is an infection in the mucosal routes of the body, such as near the mouth, ear, nose, and eyes. Mucocutaneous leishmaniasis can cause deformations by deteriorating soft tissue within the afflicted areas of the body.[29] Over 90% of worldwide cases occur in Bolivia, Brazil, Ethiopia, and Peru.[31]

The final and most deadly form is visceral leishmaniasis (VL). This disease presentation form occurs when the protozoan migrates beyond the skin into the lymph nodes and other soft tissue internal organs.[32] If left untreated, this disease state can be fatal, as it can lead to enlargement of the spleen and liver.[31] As of 2004, through passive case detection, there were 500,00 reported cases of VL globally.[30] The most recent epidemiological trend shows that new reported cases of VL are estimated at 400,000 or more cases.[26] The WHO regional specificities shows that the WHO African, Eastern Mediterranean, European, and South-East Asian regions all have countries that are endemic or highly endemic for VL.[31]

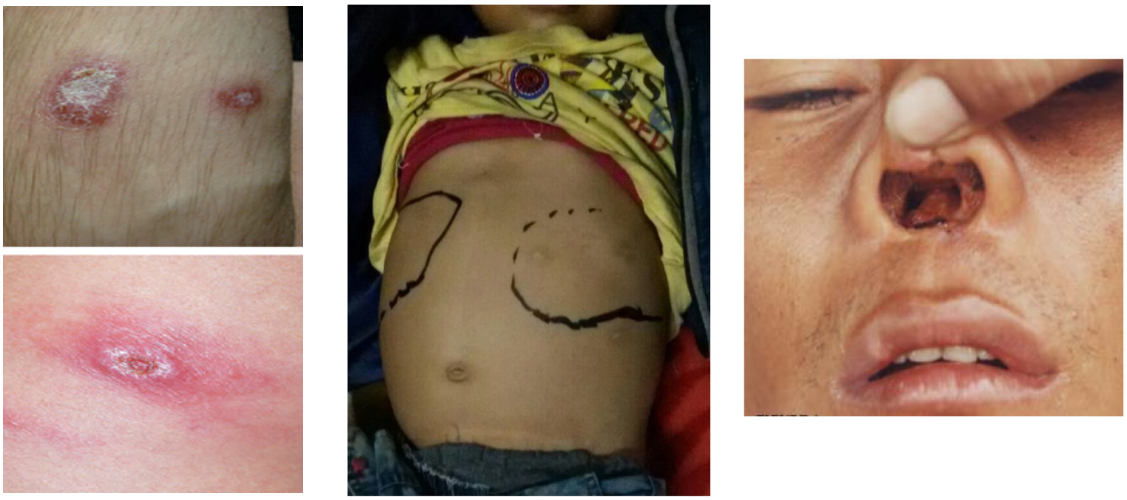

Fig 1.1 Left: cutaneous leishmaniasis depicting wet (top) and dry (bottom) skin lesions.[29] Middle: visceral leishmaniasis with outlined enlargements of spleen and liver demonstrating hepatosplenomegaly.[33] Right: mucocutaneous leishmaniasis with destruction of the nasal cavity.[34]

The country of India was widely prevalent for VL until 1947 when an insecticide spraying regimen was started to reduce mosquito populations and cases of malaria in humans. However, the spraying regimen also had an impact on the prevalence of the sandfly vector and resulted in a substantial decrease of VL cases.[35] Once the regimen

was stopped in 1977, an endemic outbreak of VL occurred with 100,000 reported cases.[36] Since 1977, VL has been considered endemic in India. As of 2007, the estimated number of cases of VL was 270,900.[37] The actual number of cases reported each year is a gross underestimation of the actual number of VL cases in India every year as official data is obtained only from passive case detection.[38] Figure 1.2 shows the overall trend of reported VL cases over a range of 40 years from three endemic countries.[39] As of 2020, VL in India is on a downward trend because of an elimination program that is being implemented.

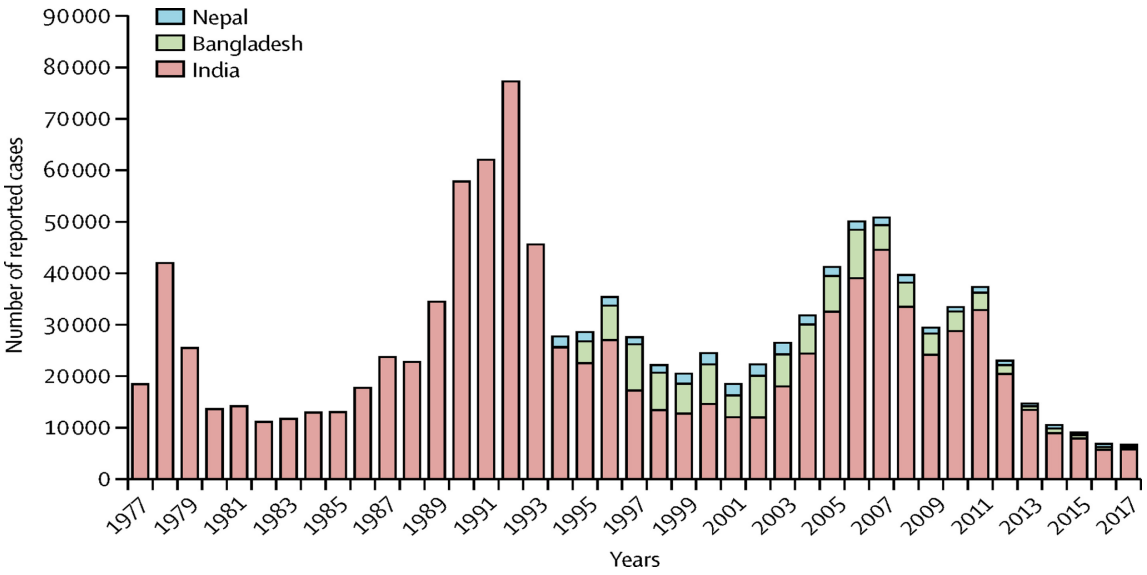

Fig 1.2 Visceral leishmaniasis (VL) trend from 1977 to 2018 showing number of reported cases for three endemic countries.[39]

Since India is endemic for the leishmaniasis disease, a plan has been proposed for the strategic elimination of the disease. The goal is to eliminate the causative agent which causes VL. In 2005, India implemented an elimination program which had four phases: preparatory, action, consolidation, and maintenance.[35,40] The action phase utilized vector control programs, disease surveillance, and community outreach. The

consolidation and maintenance phases goal is to maintain and control the burden of the disease to the goal level of annually less than 1:10,000 people affected by the disease.[35,40]

This elimination program has been effective with a steady decline of VL cases. However, as of 2020, India had a slight resurgence with greater than 1000 new reported cases of VL (Figure 1.3).[41] This resurgence was noted because of the inability of integrated pest management professionals to implement rounds of indoor residual spraying because of restrictions due to the Covid-19 pandemic. A steady decline of VL cases has been noted as restrictions are lifted and normal elimination programs have returned in 2021 and 2022.

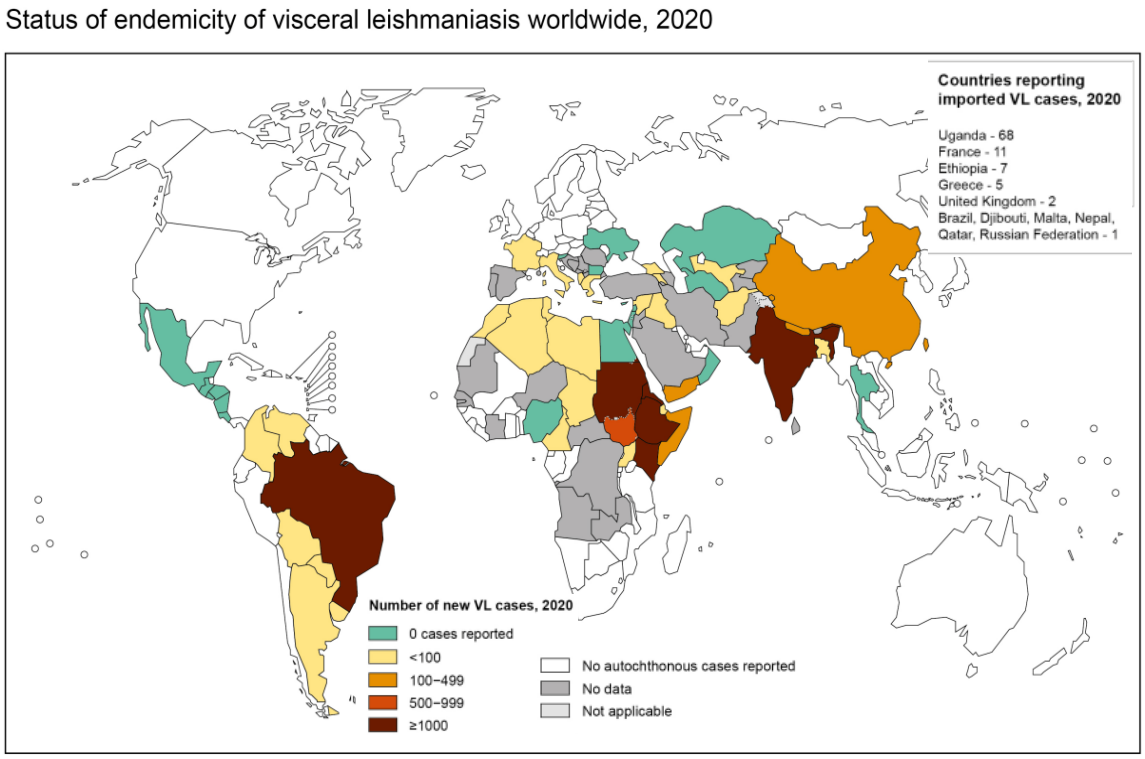

Fig 1.3 World map depicting the status of endemicity of visceral leishmaniasis as of 2020 for the report of new cases in different countries. Focus: India VL cases dark brown = +1000.[41]

Insecticides and Modes of Action. There are four main classes of insecticides implemented in vector control programs: carbamates, organophosphates, organochlorines, and pyrethroids. Carbamates and organophosphate insecticides have a similar mode of action; whereas, organochlorines and pyrethroid insecticides have a similar mode of action.

Carbamate and organophosphate insecticides are considered inhibitors of the acetylcholinesterase (AChE). Acetylcholinesterase are enzymes that help with the breakdown of acetylcholine (ACh), which is used as a neurotransmitter within neurons of the nervous system of biological systems. A proper balance of ACh is important for normal function of signaling of nerve impulses through the synaptic junction. For insects, AChE is only found primarily in the central nervous system. Both classes of insecticides are highly toxic to mammals.[42]

Organophosphate insecticides interact by phosphorylating the area of the junction that is normally acetylated and prevents the function of the AchE at the synaptic junction. This causes high levels of ACh to accumulate at the synaptic junction, which prevents the synaptic junction from signaling. This leads to the loss of nerve impulse resulting in death. The toxicity of organophosphates results when the molecule is activated by mixed function oxidases (MFO) through metabolic oxidation.[43]

Carbamate insecticides are derivatives of carbamate acid. They interact within the nervous system by creating a carbamylated enzyme-inhibitor complex that prevents the release of AchE. This results in constant high levels of ACh at the synaptic junction

and loss of nerve impulse because of the inability to signal. The carbamate molecule directly inhibits the AChE and does not require activation.[43]

Organochlorine and pyrethroid insecticides are considered sodium channel agonists. Sodium channels are present in almost all types of neuronal membranes and agonists for these channels cause them to activate or stay open. Organochlorine class of insecticides are resistant to degradation thus are highly persistent in the environment and can lead to major environmental health issues. Pyrethroids have low toxicity to mammals but are highly toxic to aquatic organisms, such as fish. There is also noted toxicity to beneficial insects such as bees which causes adverse effects or death.[44,45]

The organochlorine class of insecticides is divided into two subclasses: chlorinated alicyclic and dichloro-diphenyl-trichloroethane (DDT) type. The chlorinated alicyclic subclass of insecticide is a broad group which causes delayed neurological effects. This subclass binds to a site within the γ-aminobutyric acid (GABA) chloride ionophore complex. This leads to the inhibition of chlorine influx into the neuron and results in hyperexcitation. The DDT type subclass of insecticides primarily affect the peripheral nervous system by preventing deactivation or membrane depolarization of the synapsis which causes sodium leakage. This leads to a negative potential and hyperexcitability of the neuron. Thus, the neuron repetitively discharges disrupting the functionality of the nervous system which results in paralysis or death.[44,45]

Pyrethroids, which means “pyrethrum-like”, are a family of synthetic insecticides, consisting of cis and trans isomer configurations. Pyrethrins are naturally extracted from Chrysanthemums, whereas pyrethroids are chemically-modified pyrethrins, designed to

degrade slower in direct sunlight. This means that pyrethroids are less likely to be degraded by photolysis into inactive compounds.[46,47]

The pyrethroid family of insecticides is divided into two types based on the presence of a moiety in their chemical structure. Type 1 pyrethroids, such as permethrin, lack a cyano moiety at the alpha-position; whereas, type 2 pyrethroids, such as deltamethrin, contain the alpha-position cyano moiety.[44,48] As a synthetic insecticide, pyrethroids target the sodium channels of the nervous system that insects rely on for nervous impulses. The sodium channels are left open leading to multiple action potentials because of imbalanced sodium ions. This causes delayed inactivation and prevents normal nervous system function leading to paralysis (knockdown) and death.[49]

Type I pyrethroids produce a toxic response through the central and peripheral nervous system effect similar to a neurological syndrome which primarily produce very quick bursts of repetitive discharges due to the increased afterpotential. Type II pyrethroids cause lower amplitude of the action potentials and, eventually, total blocking of neural activity because of a marked depolarization of the membrane lasting several seconds.[44-46]

Insecticide History in India. Dichlorodiphenyltrichloroethane (DDT) was discovered in 1940 as an effective synthetic insecticide.[50] By 1947, DDT was used indiscriminately for many uses as a pesticide in agricultural fields and as an outdoor adulticide spray against adult mosquitoes around villages in India.[51] Then, from 1955-1969 DDT was used by the Global Malaria Eradication Programme for

indoor-residual spraying (IRS) as part of the chemical control of mosquitoes and to reduce the global impact of malaria.[52,53] During this time, it was noted that the population of sandflies was reduced in the areas exposed with DDT IRS, which resulted in decreased cases of VL.[54] Thus, the use of DDT IRS was implemented into integrated vector control and Indian VL elimination programmes from 1977-1995 which recorded reduced cases of VL during this time.[52]

The use of DDT IRS for vector control is administered twice annually in endemic areas. DDT is considered a persistent organic pollutant and it was recommended at the 2000 Stockholm Convention that it should be phased out of use. However, in 2006, India was allowed to use DDT in their VL elimination program because of the limited availability of alternative insecticides. Since the continued use of DDT, there has been a downward trend of vector susceptibility mainly because of suboptimal dosage used in IRS.[55,56]

As of 2015, India switched away from using DDT for IRS programs and shifted to using insecticides from the pyrethroid family, mainly permethrin and alpha-cypermethrin, to control sandfly populations in endemic areas.[57,58] However, DDT and the pyrethroid family have a shared target site, thus vector resistance to DDT could provide cross-resistance and reduce the effectiveness of the pyrethroid family of insecticides.[57]

Permethrin is a contact insect adulticide used for bed netting, IRS, and as an insect repellent. Permethrin is the only EPA approved factory treatment of clothing insect repellent. The amount of permethrin impregnated into clothing is a very low

concentration, thus exposure from treated clothing is extremely low.[59] The main use for permethrin is insect population control because of its fast-acting, low-dose insecticide properties. Permethrin is an effective insecticide against lice, ticks, mites, mosquitoes, sandflies, and other arthropods. Permethrin is one of the main insecticides used by India to reduce the population of medically important vectors, such as sandflies and mosquitoes, that carry diseases like leishmaniasis and malaria. Permethrin is usually applied using an ultra-low volume spray approach over a large area at low concentrations.[60,61]

Detecting Insecticide Resistance. It is important for integrated pest management (IPM) programs to manage insecticide resistance in vector populations by monitoring and evaluating insecticide susceptibility in vector species. The main way to assess insecticide susceptibility or resistance in vector species is using insecticide susceptibility bioassays. The two commonly used bioassay approaches are: the World Health Organization (WHO) exposure kit bioassay and the Centers for Disease (CDC) bottle bioassay.[62]

The WHO exposure kit bioassay is the accepted standard when it comes to collecting susceptibility data or assessing resistance in a vector population. The kit is a prepared tube lined with papers impregnated with a discriminating concentration of an insecticide, a determined concentration in a set time used to differentiate between susceptible and resistant phenotypes within a population. The WHO bioassay kit exposes over 100 insects inside a tube that has a discriminating concentration of insecticide for at least an hour. The WHO bioassay has been used for sandfly research, but the discriminating concentrations are determined from mosquito susceptibility work. There

is also the issue that only WHO approved insecticides for vector control are available and only in select concentrations.The WHO bioassay range of susceptibility as determined from mosquitoes and the discriminating dose of insecticides is: greater than 98% mortality is susceptible, between 98-90% mortality is possible resistance (tolerance), and less than 90% mortality is resistance.[56,63]

The CDC bottle bioassay is another approach to collecting susceptibility data and assessing resistance in a vector population. The bioassay is performed in bottles that have been coated with an insecticide concentration. The CDC bottle bioassay uses less insects compared to the WHO kit. The CDC bottle bioassay procedure is simple and can utilize any concentration and any insecticide. To determine susceptibility, the CDC bottle assay uses a similar range to the WHO bioassay and is determined from mosquitoes. The discriminating dose range of an insecticide is defined as: greater than 98% mortality is susceptible, 97-80% is the possibility of resistance and needs to be further tested, and less than 80% is resistant.[62,64]

To adequately assess resistance for both of these bioassays, it is necessary to have accurate diagnostic doses and times. A diagnostic dose is the lowest concentration of insecticide that causes 100% mortality in a vector population in the shortest time span. This leads to the issue of the difference in defining resistance between the two bioassays. The WHO bioassay considers resistance less than 90% mortality in a population; whereas, the CDC bioassay considers resistance if there is less than 80% mortality in the population. This definition of resistance is based on mosquito research, thus sandflies

need their own diagnostic doses and definition of resistance because of the potential physiological and size differences between the two vector species.[63-65]

Mechanisms of Resistance. The indiscriminate and overuse of insecticides has put immense selective pressures on populations of vectors. Insecticide resistance has been documented in mosquitoes, ticks, fleas, beetles, sandflies and many other insect vectors. Resistance in vector populations can be broken down into four categories: reduced penetration, behavior avoidance, target-site insensitivity (TSI), and metabolic detoxification (MD). There is evidence across major vector species of TSI and MD to each of the four main classes of insecticide.[66]

The resistant mechanisms for reduced penetration and behavior avoidance are less studied than the other forms. The reduced penetration resistance mechanism means that the insecticide is less effective on entering the insect's system because of thicker or altered cuticles. For example, a study has revealed a documented case of lowered penetration of the organophosphate class insecticide in the vector *Culex tarsalis*.[67] The behavior avoidance resistance mechanism means that the vector changes its habits to actively interact with an insecticide less often. Another study using three mosquito vectors showed altered behavior to avoid the presence of pyrethroid treated nets which caused a shift in biting times.[68]

Metabolic detoxification (MD) occurs from the overproduction of specific enzymes to break down the insecticide into detoxified products. The most common enzymes used for MD resistances are: carboxylesterases, glutathione-S-transferases (GST), and cytochrome P450-dependent monoxygenases.[66] MD via carboxylesterases

is the main mechanism of organophosphate and carbamate insecticide resistance in mosquitoes.[69] MD by GST is effective against organochlorine and pyrethroid insecticides.[66] MD by cytochrome P450-dependent monoxygenases can detoxify the active substance in an insecticide before its impact can affect the insect making it an effective resistance mechanism against all classes of insecticides.[70]

Metabolic detoxification has been documented in mosquitoes and has been attributed to the overexpression of detoxification enzymes, such as GST.[71] This resistant phenotype has been noted across different regions and species of mosquitoes. In two DDT selected resistant strains of mosquitoes from different regions the GST gene has been directly linked to DDT resistance.[72]

GST associated insecticide resistance has been documented in a wide range of agricultural pests as well as medically important vectors such as sandflies.[73] A study by Ashraf et al, found approximately 20 GST in the *Phlebotomus papatasi* and 40 GST in the *Lutzomyia longipalpis* genomes respectively. The highest percentage of GST belonged to GST delta, which is an insect specific class, and GST xi class. They found no GST epsilon class within the sandfly genome. These delta and epsilon classes of GST, have been associated with insecticide resistance in mosquitoes. The xi class was thought to be mosquito specific.[74]

Target-site insensitivity (TSI) happens when a point mutation occurs causing the site that insecticides interact with to be less sensitive. Insecticides affect the nervous system of the insect in three main parts: AChE, γ-aminobutyric acid (GABA) receptors, and sodium channels. Mutations in each of these parts have been documented across

major insect vectors which confers insecticide resistance. The AChE resistance is associated with a mutation in the gene for the protein acetylcholinesterase which is called *Ace-1*. Sodium channel resistance is associated with a mutation in the α-subunit of the paralytic (*PARA*) voltage gated sodium channel (*Vgsc*).[47,63,66,75,76]

The polymorphism in the *PARA* region causes what is known as a knockdown resistant (kdr) mutation in the pyrethroid and DDT target site. sandfly populations from multiple districts of India have demonstrated high resistance to the WHO discriminating dosage of 4% DDT.[54] This mutation is the primary mechanism underlying DDT resistance and pyrethroid tolerance in *P. argentipes* because the two classes of insecticide share a similar target site. This results in cross-resistance and reduces the pyrethroid sensitivity of the sodium channel.[57,77,78]

Mutations in the *Ace-1* and *PARA* genes vary in prevalence depending on location of the vector but similar mutations are seen across various vector species. A point mutation at the 119 codon in the *Ace-1* gene has been identified in very low frequency in different mosquito populations.[79] There are three documented point mutations that occur across insect vectors associated with the 1014th codon of the *Vgsc* gene.[57] This codon is the target site for DDT and pyrethroid family insecticides. These mutations have been associated with insecticide resistance in mosquitoes, cockroaches, and sandflies.[57]

Loop Mediated Isothermal Amplification Assay. Loop Mediated Isothermal Amplification (LAMP) assay is a novel technique that utilizes a set of four primers designed to recognize 6 specific regions of a target DNA sequence that amplifies

specifically, efficiently, and rapidly under isothermic conditions. Additionally, a loop primer can be used with the other primer sets to facilitate amplification and reduce time needed to accumulate a large number of copies of target DNA. With the loop primer set, the LAMP technique is able to identify 8 distinct areas of the target DNA for higher specificity.[80]

LAMP can be visualized using a turbidity technique, gel electrophoresis or DNA dye. The LAMP technique has a natural by-product of pyrophosphate ion which is a white precipitate. Thus, the amount of DNA amplified can be determined by the amount or absence of the precipitate. Another technique is to perform a gel electrophoresis to determine if an appropriate size band for the target DNA sequence has appeared. The last visualization technique is to use dyes that change in the presence of a large amount of DNA (high copy number). This allows for real time confirmation that amplification of the target DNA has occurred.[81]

LAMP is considered an alternative method to PCR that does not require a thermocycler because the entire reaction process occurs at a constant temperature of 65ºC. Since LAMP occurs at isothermal conditions, such as utilizing a heating block, this technique would be ideal for field settings and rural areas that do not have access to PCR equipment. The LAMP process creates a stem-loop final product of DNA that is highly specific because it utilizes inner and outer primer pairs that recognize six distinct areas on your target DNA, which overall accumulates approximately 10^9 copies of the target DNA in just about an hour. The LAMP technique is as sensitive as qPCR with a detection limit

of 1-10 copies/μL based on reaction conditions and has no cross reactive sensitivity with samples containing similar DNA to the target DNA.[80,82,83]

LAMP is unique in that the assay can occur in minimal buffer and a range of pH and still quantify DNA. A DNA dye, specifically phenol red, reacts to the addition of a deoxyribonucleotide triphosphate (dNTP) by the DNA polymerase that gives off a hydrogen ion and results in a pH change in the reaction. Phenol red goes from a reddish pink in neutral to alkaline conditions to yellow in the presence of acidic conditions, which can easily be viewed by the naked eye. An acidic environment in LAMP means that extension of the DNA has occurred. A study by Tanner et al, showed that LAMP is sensitive enough with a dye to change color in only 15 minutes. Another study by Poole et al (2017), showed that LAMP with a DNA dye can detect positive results with as little as 0.01ng of target DNA present in the sample.[84,85]

LAMP can be utilized as an effective screening tool with little specialized equipment. Thus is ideal for detecting insecticide susceptibility in field populations of sand fields. This assay would allow IPM professionals to screen and visually detect insecticide susceptibility of sandfly populations right at the field site because of the single temperature used to run the assay.

Overview of Chapters. The focus of this thesis is to further understand insecticide susceptibility and detection of possible resistance in sandflies. The objective is to develop baseline susceptibility data for *P. argentipes* to add to a growing repository of sandfly data that can be used by IPM professionals in vector control programs. The

secondary objective is to utilize sandfly genetic data to develop an assay for detection of insecticide susceptibility in the vector *P. argentipes*.

Chapter 2 describes the development of insecticide susceptibility curves using permethrin, α-cypermethrin, and DDT for a laboratory population of *P. argentipes*. Dose response curves (DRC) of each insecticide were created using a range of concentrations and utilizing the CDC Bottle assay method for assessing insecticide susceptibility. The DRC determined baseline susceptibility data for the laboratory *P. argentipes* colony to each respective insecticide. Then an optimized polymerase chain reaction (PCR) was utilized to visualize and sequence the *PARA* and *Ace-1* genes associated with insecticide resistance within the *P. argentipes* genome.

The findings from this research give IPM professionals baseline data for *P. argentipes* which can be used when attempting to rapidly distinguish susceptible and resistant colonies of sandflies in field settings. The genetic data obtained from the optimized PCR will allow for further development of a screening tool, such as LAMP, to determine susceptibility of sandflies populations.

Chapter 3 will utilize LAMP to develop an assay to determine insecticide susceptibility versus resistance in a lab population of *Phlebotomus argentipes* from India. Primers were developed for the *PARA* region of the *P. argentipes* genome potentially associated with insecticide resistance. Two sets of primers were developed: 1) the general region of the gene of interest and 2) to sit on the 1014th codon to screen for mutations in the gene of interest.

The hypothesis of this research is that LAMP will effectively amplify the gene of interest associated with insecticide resistance. Specific aims include understanding insecticide susceptibility and resistance of the vector *P. argentipes* by developing dose response curves to insecticides of interest and optimizing a LAMP technique to effectively detect susceptibility at the *PARA* gene in populations of sandflies that can be utilized in field settings.

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CHAPTER 2

Assessing Insecticide Susceptibility of Laboratory *Phlebotomus argentipes* Sandflies (Diptera: Psychodidae: Phlebotominae)

Abstract

Background: *Phlebotomus argentipes* (Diptera: Psychodidae: Phlebotominae) is the primary vector for the *Leishmania* parasite that causes the neglected tropical disease visceral leishmaniasis. In endemic localities, chemical control via synthetic insecticides has been the primary means of sandfly population control for the past half century. This intense selective pressure has resulted in the potential evolution of insecticide resistance in specific field populations, limiting vector control options for integrated pest management (IPM) professionals. Improved baseline estimates of susceptibility to currently and historically used insecticides, along with the amplification and sequencing of the genes associated with resistance, could provide for more targeted application of insecticides, and in turn slow the evolution of resistance in sandflies.

Methods: The objective of this study was to determine the susceptibility of a *P. argentipes* laboratory colony to three insecticides and their lethal concentrations (LC). This was completed by using a modified version of the Center for Disease Control and Prevention (CDC) bottle assay. Glass bottles were internally coated in a range of concentrations. *Phlebotomus argentipes* sandflies were inserted into these bottles and mortality was recorded following a specific exposure period and a 24 hour waiting

period. Dose-response survival curves (DRC) were generated for each insecticide. Based on the DRC, LC values causing 50, 75, and 90% mortality were determined for each insecticide. A polymerase chain reaction (PCR) protocol was optimized to amplify specific genes of interest associated with insecticide resistance in the *P. argentipes*' genome.

Results: *Phlebotomus argentipes* baseline susceptibility data was collected for α-cypermethrin, permethrin, and dichloro-diphenyl-trichloroethane (DDT), along with LC's values corresponding to 50, 75, and 90% mortality of a population. An optimized PCR protocol was developed to effectively amplify and sequence the genetic regions in *P. argentipes* associated with target-site insensitivity insecticide resistance.

Conclusion: This study will provide IPM professionals baseline insecticide susceptibility data for *P. argentipes* to two synthetic pyrethroids and the historically used compound DDT. A repository of sandfly susceptibility data, including ranges of LC to different insecticides is an important tool for effective vector control in field populations. The PCR protocol with primers specific to the genes of interest in the *P. argentipes* genome can be utilized for future research associated with insecticide resistance or susceptibility detection in sandflies.

Background

Phlebotomine sandflies are important vectors for human disease throughout the world. Among these species, females obtain a blood meal from mammals, including humans. As they feed, they can vector medically important parasites such as *Leishmania*. This is

commonly observed within the sandfly genera: *Phlebotomus* and *Lutzomyia*[1], with *Phlebotomus argentipes* being the main vector for *Leishmania donovani*, the causative agent of the neglected tropical disease visceral leishmaniasis throughout India.[2]

The *Leishmania* parasite causes the vector borne disease Leishmaniasis, which can occur in three presentations: cutaneous, mucocutaneous, and visceral.[3] The visceral form, also known as kala-azar (KA), is endemic in 10% of India's total at risk population.[4] This at risk population is characterized as living in developing or rural areas with poor sanitation, high humidity, and temperature. In India and other afflicted areas, this totals approximately 350 million people.[5]

Historically, four types of insecticides have been used in vector control programs to reduce populations of vectors such as sandflies: 1) organochlorines, 2) organophosphates, 3) carbamates, and 4) pyrethroids. These insecticides interact with the nervous system of insects and either inhibit acetylcholinesterase (AChE), as seen with organophosphates and carbamates, or act as sodium channel agonists, as observed with organochlorine and pyrethroids.[6,7] This study focuses on organochlorines and pyrethroid classes of insecticides which have been used as chemical control on sandfly populations in India.

Organochlorine insecticides were first introduced as broad-spectrum insecticides in the late 1930s. The most common and overused organochlorine insecticide is dichloro-diphenyl-trichloroethane (DDT). This insecticide had been used for decades indiscriminately to reduce mosquito populations and the spread of malaria and dengue virus.[8] However, since the 2000 Stockholm Convention, organochlorine insecticides are

considered persistent organic pollutants and have been phased out of use because of their environmental impact. As of 2015, India was the last country to still be using organochlorine insecticides, specifically DDT.[8]

Pyrethroid insecticides are synthetic derivatives of pyrethrins and are highly effective against agricultural pests.[9] The pyrethroid family of insecticides are mostly used in vector control programs, but historically have been used as agricultural pesticides. Within a 10 year period between the 1960s and 1970s, many different pyrethroids were developed for agriculture use because of their limited environmental impact and photolytic stability.[10] The most common uses for this class of insecticides are: impregnated bed nets for mosquito or sandfly population control and ultra low volume spraying of buildings.[11]

The over and misguided use of insecticides as means for vector control has led to elevated insecticide resistance.[12] It is important for integrated pest management professionals to monitor sandfly populations that are actively targeted with insecticides. The continued usage and increasing amounts of insecticides exacerbates the problem of resistance.

The resistant phenotypes most often present in populations of exposed pests are target-site insensitivity (TSI) or metabolic detoxification.[12] TSI occurs when an amino acid substitution takes place in the voltage-gated sodium channel (*Vgsc*), which causes reduced sensitivity of the target site to organochlorine and pyrethroid family classes of insecticides.[11] The TSI polymorphisms that cause two knockdown resistant (kdr) mutations in the pyrethroid and DDT target site genes have been identified. The genes of

interest that have been extensively documented in other vectors and connected to insecticide resistance are a paralytic (*PARA*) voltage-gated sodium channel gene and acetylcholinesterase-1 (*Ace-1*), encoding the protein for synaptic transmission. The *PARA* gene is associated with organochlorine and pyrethroid class of insecticides because their mode of action is sodium channel agonists.[7] The *Ace-1* gene is associated with the organophosphate and carbamate classes of insecticides because their mode of action is as inhibitors of AChE.[6] A voltage-gated sodium channel mutation is the primary mechanism underlying DDT resistance in *P. argentipes*.[13] Thus, the TSI channel mutation associated with DDT resistance could provide cross-resistance to the pyrethroid family of insecticides because of their shared target site in insect genomes.

There have been many documented populations of vectors that demonstrate resistance to a variety of insecticides. A study by Dinesh et al. (2010) documented that field populations of *P. argentipes* throughout India demonstrate DDT resistance when exposed to a discriminative dosage of DDT (4% for 1 hour).[2] A discriminative dosage is determined to be double the concentration required to kill a susceptible population of the species.[14] A population of *Phlebotomus papatas*i from Sudan has also demonstrated resistance to discriminative dosages of 5% malathion (organophosphate) and 0.1% propoxur (carbamate).[15] Throughout regions of Africa, field populations of the mosquito species *Anopheles gambiae* have demonstrated pyrethroid resistance.[16] A TSI point mutation in the target site for DDT and the pyrethroid family of insecticide has been documented in a population of mosquitoes, *Anopheles subpictus*, from Sri Lanka resulting in increased insecticide resistance.[12] All of these insecticides are important for their uses in vector control programs and documented cases of resistance hinder

control methods. Thus, it is important that these populations are monitored using susceptibility bioassays to make changes to insecticide programs to effectively combat and slow the development of resistance.

The two methods used to evaluate a vector's susceptibility to insecticides are: 1) the World Health Organization (WHO) exposure kit bioassay and 2) the Center for Disease Control (CDC) bottle bioassay. These assays allow integrated pest management (IPM) professionals to effectively assess a vector population's susceptibility to insecticides. The WHO exposure kit bioassay uses paper impregnated with predetermined concentrations of insecticides and is widely accepted because of its ability to be used on numerous insect vectors. Drawbacks include its expense, as well as limited concentrations to evaluate, and the availability of other insecticides.[17,18] In contrast, the CDC bottle bioassay is both portable and less expensive than the WHO bioassay kit. This protocol allows for a bottle to be coated by any concentration of insecticide of interest. The bioassay can be used to measure mortality at the end of the exposure test to determine susceptibility.[17,19]

The continued use of insecticides as part of vector control means the continual need for baseline susceptibility data for the vector populations. A study by Denlinger et al. (2015), provided baseline susceptibility data to ten insecticides for two different sandfly species, *Lutzomyia longipalpis* and *P. papatasi*.[20] However, more direct baseline data is needed for *P. argentipes* to provide IPM professionals information necessary for appropriate vector control methods in India.

The objective of this study was to quantify, using a modified CDC bottle bioassay, the susceptibility of laboratory *P. argentipes* to permethrin, α-cypermethrin, and DDT. A dose response survival curve was produced for each insecticide. From the survival curves, lethal concentrations (LC) for 50, 75, 90% mortality values were determined. A PCR protocol was optimized using specific primers to effectively amplify the *PARA* and *Ace-1* genes in the *P. argentipes* genome. The primers and protocol can be adopted by integrated pest management professionals to assess susceptibility in field populations of sandflies and make informed decisions in regards to vector control program usage. The *PARA* and *Ace-1* gene regions are substantially documented to be associated with insecticide resistance from studies of other vectors.

Methods

Susceptibility Bioassay

Sandfly Colony

Insecticide-susceptible *Phlebotomus argentipes* sandfly colony at Utah State University (USU) was derived from long-established colonies maintained at the Walter Reed Army Institute of Research (Silver Spring, MD). All life stages were reared at USU at 25˚C, 85% relative humidity, and a photoperiod of 12:12 (L:D) h.[21] Larvae were fed a composted 1:1 mixture of rabbit feces and rabbit food; whereas, adults were provided 30% sucrose-water solution daily on saturated cotton balls. Adult female *P. argentipes* were blood-fed on anesthetized mice placed inside holding cages twice weekly.[17, 22]

The protocol and use of SKH1 hairless mice was approved by the USU's Institutional Care and Use Committee.

Insecticides

Three technical-grade insecticides were used in this study from two different classes of insecticide. Two insecticides from the pyrethroid family of insecticides were used: Type I, permethrin (Chem Service, Inc., West Chester, PA), and Type II, α-cypermethrin (Sigma-Aldrich, St. Louis, MO). One organochlorine was used, dichloro-diphenyl-trichloroethane (DDT) (Sigma-Aldrich, St. Louis, MO). *Phelbotomus argentipes* was exposed to concentrations of each insecticide as provided in Table 2.1. All insecticide dilutions were prepared in acetone and kept in glass bottles following the CDC bottle bioassay procedure.[23]

Table 2.1 Concentrations of insecticides used in the CDC bottle bioassay to expose laboratory *P. argentipes* sandflies

Insecticide	Species	Concentration (µg insecticide per bottle)
DDT	***P. argentipes***	1, 3, 5, 10, 15, 20, 30, 40, 55, 75, 85, 100, 120, 135, 145, 175, 200
Permethrin	***P. argentipes***	1, 3, 5, 6.5, 8, 10, 12. 15, 20, 25, 30, 35, 40, 45, 50, 65, 80, 100
α-Cypermethrin	***P. argentipes***	0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4

Insecticide Exposure Tests

Preparation of the exposure bottles was performed as outlined in Denlinger et al. (2015). This study exclusively used 1,000ml glass bottles and 4 ml of insecticide to coat the

inside of each bottle. Utilizing the CDC bottle assay procedure as outlined in Denlinger et al. (2015), *P. argentipes* was exposed to all insecticides for 60 minutes.[20] Mortality was scored after a 24 hour holding period. If mortality in the unexposed control group ranged from 5 to 20%, the mortality of the experimental bottles was corrected using Abbott's formula.[24] This formula accounts for the error of natural death in the control group of insecticide susceptibility assays to obtain accurate mortality results. If the unexposed control mortality was less than 5%, the error is considered small and the replicate is not corrected. If the unexposed control mortality was greater than 20%, the entire replicate was excluded from further analysis.[23,24]

Survival Curves

A dose-response survival curve was created for each insecticide using QCal software.[25] A logistic regression model was used to estimate the LC 50, 75, and 90 for the susceptible *P. argentipes* sandfly colony to each insecticide. Mortalities corrected with Abbott's formula were rounded to the nearest whole insect.[20]

Molecular Assay

DNA Extraction

Whole sandflies were collected from the susceptible laboratory *P. argentipes* colony. Total DNA was extracted from a single sandfly using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Each sandfly was mechanically homogenized in 180µL molecular grade water and 3 glass beads at 30 Hz for at least 2 minutes using a

Retsch MM400 (Retsch, Haan, Germany). To ensure all buffer has passed through and that the sample was dry, each centrifuge step was repeated, except for the final elution step.

Sequencing of PARA and Ace-1 Gene Fragments

The *PARA* and *Ace-1* gene fragments for the susceptible *P. argentipes* colony were amplified following the protocol as outlined in Denlinger et al. (2017). Briefly, amplification utilized a thermal cycler protocol with a gradient temperature increase during the annealing step of 1℃ for each cycle up to 35 cycles.[26] The primer sets and sequences are provided in Tables 2.2. The primers are positioned on the *PARA* gene to surround three codons of interest at the 1011th, 1014th, and 1016th positions because of known association with TSI insecticide resistance in other vectors. The primers are positioned on the *Ace-1* gene to surround the 119th codon position because of known association with TSI insecticide mutation in other vectors.[26]

Table 2.2: *PARA* and *Ace-1* gene primer pair and sequences

Species	Gene	Primer Pairs	Primer Sequence (5' → 3')
P. argentipes	*PARA*	Para Nested-2 F	GTRTTCCGTGTGYTGTGC
		Para Nested-2 R	ATCCGAAATTGCTCAAAA
	Ace-1	F12	CAACGGATAAGGGGAAGG
		R8	AAACCTGTGATCGTACAC

All polymerase chain reaction (PCR) products were visualized using gel electrophoresis with a 1% TAE agarose gel at 100V for 45 minutes. Select PCR products for the *PARA* and *Ace-1* gene fragments from the susceptible *P. argentipes* colony were

purified using Qiagen's QIAquick PCR Purification kit. Samples were sent to the USU CIB Genomics Core lab (USU, Logan, UT) for Sanger sequencing. All sequences were verified by comparison to the Nucleotide database in Genbank.[27]

Results

Susceptibility Bioassay

Survival Curves

A dose-response survival logistic regression analysis was performed for the susceptible *P. argentipes* sandfly colony to estimate the LC 50, 75, and 90% mortality values to all three insecticides as seen in Table 2.3.

Table 2.3: QCal logistic regression parameters and lethal concentration (LC) values causing 50, 75, and 90% mortality in laboratory *P. argentipes* sandflies exposure to three insecticides with the CDC bottle bioassay

Insecticide	Species	LC 50 (µg insecticide per bottle) [LL, UL]*	LC 75 (µg insecticide per bottle) [LL, UL]*	LC 90 (µg insecticide per bottle) [LL, UL]*
DDT	*P. argentipes*	36.91 [33.07, 41.19]	78.27 [69.99, 87.52]	165.97 [140.13, 196.59]
Permethrin	*P. argentipes*	11.72 [10.74, 12.78]	20.79 [18.94, 22.80]	36.86 [32.19, 42.22]
α-Cypermethrin	*P. argentipes*	1.24 [1.13, 1.36]	2.04 [1.81, 2.31]	3.37 [2.81, 4.05]

*LL = Lower 95% confidence limit, UL = Upper 95% confidence limit

For each insecticide, the LC are almost double the previous value which is represented by the sigmoidal shape of the logistic curve as the values approach the 100% mortality asymptote. Figure 2.1, shows the survival curves of the susceptible laboratory *P. argentipes* sandfly colony to each insecticide.[28]

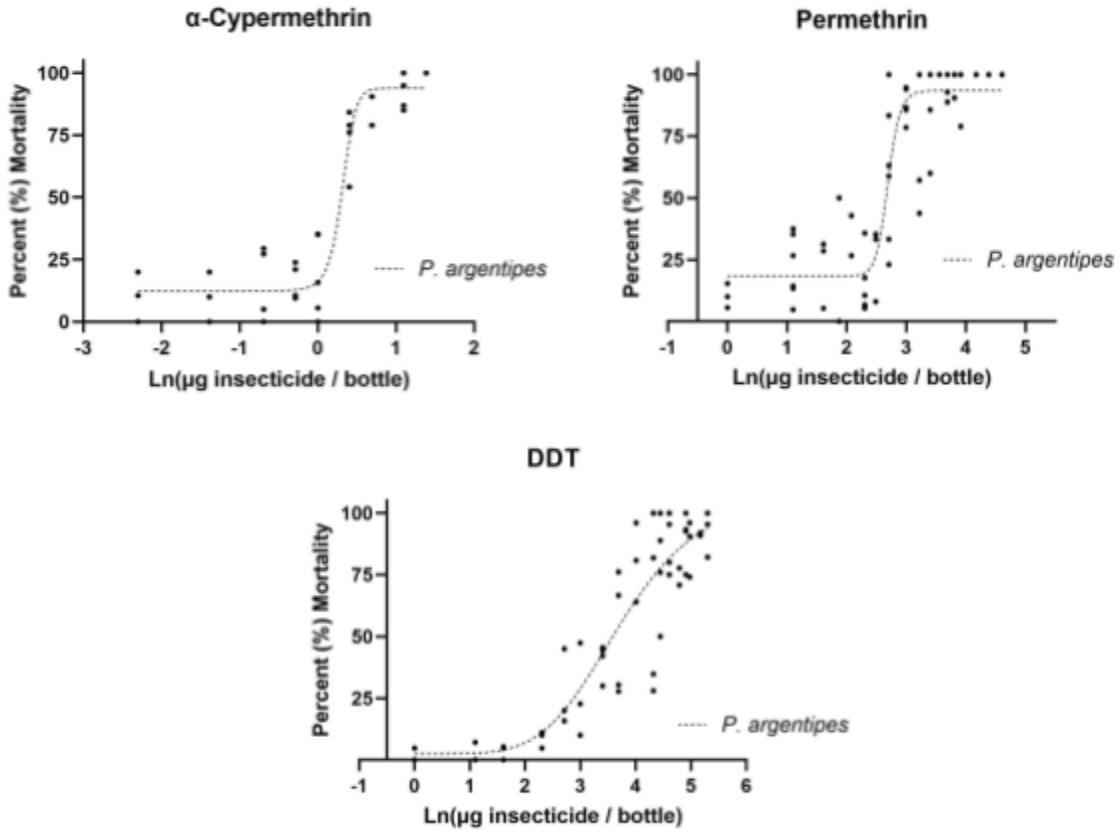

Figure 2.1 *Phlebotomus argentipes* sandfly dose response survival curves to α-cypermethrin, permethrin, and DDT

Table 2.1, showcases the difference in concentrations used between the two insecticides from the pyrethroid family. For *P. argentipes*, it takes ten times the concentration of a Type I pyrethroid to have the same effect as the Type II at all LC values (Table 2.3). *P. argentipes* is highly susceptible to α-cypermethrin, as all the LC values are less than 5µg α-cypermethrin per bottle, which is the lowest values for any of the insecticides evaluated.

Sandflies needed to be exposed to almost double the DDT concentration of insecticide compared to either of the pyrethroids used to obtain similar mortality rates. *P. argentipes* LC 90 for DDT (165.97 µg DDT per bottle) was four times greater than

permethrin and 50 times greater than α-cypermethrin's values. DDT had the largest LC values out of the insecticides used and demonstrates that a larger concentration is required to elicit a knockdown effect on *P. argentipes* compared to pyrethroid insecticides.

Molecular Assay

Sequencing of PARA and Ace-1 Gene Fragments

PCR primers were optimized for the susceptible *P. argentipes* colony. The primer sets for both gene fragments specific to *P. argentipes* can be found in Table 2.2. Both PCR amplified gene segments were able to be visualized as shown in Figure 2.2.

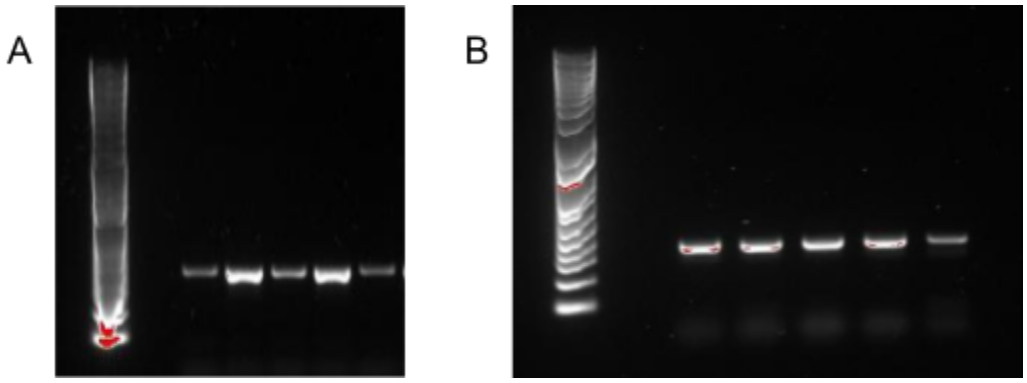

Fig 2.2 Visualization of the respective gene fragment from the susceptible *P. argentipes* colony using gel electrophoresis on a 1% TAE agarose gel A. *PARA* DNA gene fragment each band is ~250 base pairs in length. B. *Ace-1* DNA gene each band is ~1000 base pairs in length

The purified PCR products were sequenced for both genes and codons of interest have been marked for their known part in TSI insecticide susceptibility. (Figures 2.3 and 2.4) The primers target a specific region of the *PARA* gene to capture three codons, 1011th, 1014th, and 1016th, of interest associated with TSI insecticide resistance. There

is an intron that is inserted within the 1016th codon of the *PARA* gene. The primers target a specific region of the *Ace-1* gene to capture the 119th codon of interest that is associated with TSI insecticide resistance. Thus, the *Ace-1* gene is only sequenced out to ~300 base pairs; whereas, the entire gene is approximately 1000 base pairs in length.

```
Alignment: P. argentipes Ace-1 fragment

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
SB7-2-F12-      TCAATCTCCA GCAGGCAGAG GTGGACGCGT GGATGGGGTC CCGTACGCAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
SB7-2-F12-      AGCCACCTGT TGGGCCATTG CGCTTCCGAC ACCCACGGCC GGCCGAACGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
SB7-2-F12-      TGGTCCGGGA TCCTCAATGC CACCACTCCG CCAAACACGT GTGTTCAGAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
SB7-2-F12-      TGTCGATACG CTCTTCGGCG ACTTTCCGGG CGCCACTATG TGGAATCCTA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
SB7-2-F12-      ACACGAATCT CACAGAGGAT TGTCTCTACA TCAACGTTGC GGTGCCACAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
SB7-2-F12-      CCACGCCCAA AGAACTCACC CGTCATGCTG TGGATCTTCG GTGGTGGATT

                ....|....| ....|....| ....|....| ....|....| ...
                       310        320        330        340
SB7-2-F12-      CTACTCAGGA ACCTCTACGC TGGATGTGTA CATCACAGGT TTA
```

Fig 2.3 Sequence for the *Ace-1* DNA gene fragment for the susceptible *P. argentipes* laboratory colony. The region highlighted is the 119th codon

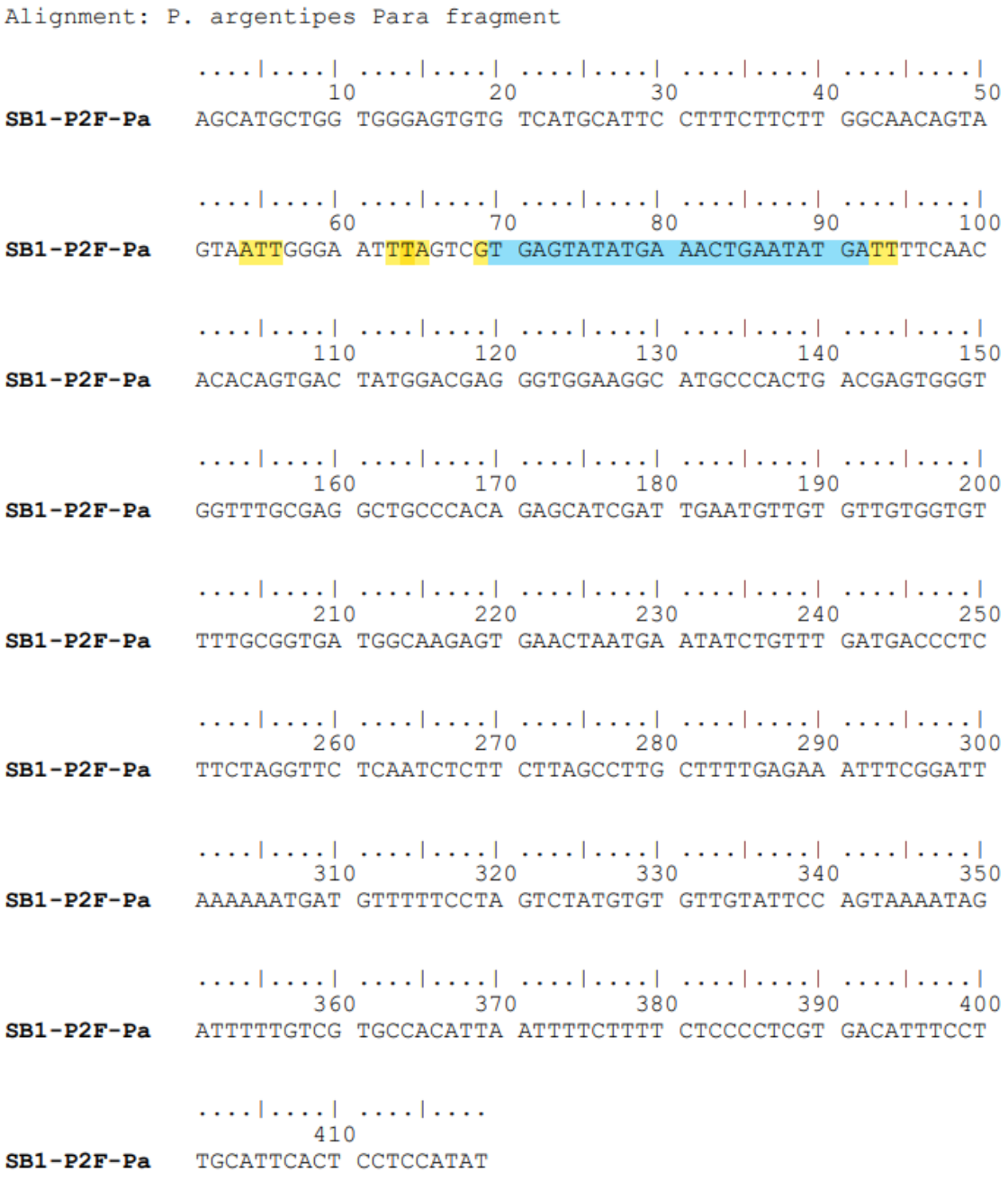

Fig 2.4 Sequence of the *PARA* DNA gene fragment for the susceptible *P. argentipes* laboratory colony. The yellow highlighted regions are the 1011, 1014, 1016th codons, respectively. The blue highlighted region is an intron segment in the 1016th codon

Each sequence was run through the NCBI BLAST for alignment and analysis of similar sequences. All PCR sequences for the *PARA* gene from the *P. argentipes* sandfly colony had high alignment with a *P. argentipes* isolate voltage-gated sodium channel gene. There was partial alignment with a *Lutzomyia longipalpis* (sand fly species) isolate paralytic gene. All PCR sequences for the *Ace-1* gene from the *P. argentipes* sandfly colony came back with only two results. There was a high alignment to a *P. papatasi* (AChE) mRNA sequence and partial alignment with *Anopheles darlingi* isolate AD6 acetylcholinesterase-1 (*Ace-1*) gene.[29]

Discussion

The first objective of this study was to quantify the susceptibility of laboratory *P. argentipes* to permethrin, α-cypermethrin, and DDT. These findings are significant in that they provide IPM professionals baseline susceptibility data for *P. argentipes,* a medically important vector. Moreover, my work revealed the modified CDC bottle bioassay is an effective tool for recording *P. argentipes* susceptibility to important insecticides used in vector control programs.

The dose response survival curve and the LC 50, 75, 90% mortality values for each insecticide are important tools for vector control programs in assessing susceptibility in field populations. An optimized PCR technique is an effective tool to generate DNA fragment sequences of *PARA* and *Ace-1* specific to *P. argentipes* and allow for evaluation of potential mutations associated with TSI insecticide resistance. The WHO differentiates vector populations based on their susceptibility to insecticides where

greater than 98% mortality is susceptible while less than 90% mortality is resistant. The range between 90-98% is considered possible resistance and a population within that range is subject to further susceptibility testing to determine the degree of resistance.[30]

A study by Denlinger et al. (2015) utilized two different species of sandflies: *L. longipalpis* and *P. papatasi*, to generate susceptibility data. Comparatively, at higher LC values, *P. argentipes* is much more susceptible to permethrin than both of the other species of sandflies. The *P. argentipes* permethrin LC 90 value (36.86 µg permethrin per bottle) is half as much as the *L. longipalpis* value (82.40 µg permethrin per bottle) and fifth as much as *P. papatasi* (188.58 µg permethrin per bottle).[20]

The opposite trend is seen in the DDT exposure data; however, differences in exposure time must be addressed. Both *L. longipalpis* and *P. papatasi* required a 2 hour exposure time to record LC in reasonable dosage range whereas *P. argentipes* only required an hour of exposure to have similar results. In preparatory tests with DDT, 2 hours of exposure for the *P. argentipes* susceptible colony was utilized following the procedure as outlined in Denlinger et al. (2015); however, high mortality was seen in all concentrations even after the 24 hour recovery period. Thus, the 2 hour exposure time was too significant for accurate mortality data collection for *P. argentipes* species of the sandflies as compared to the data collected from *P. papatasi* and *L. longipalpis*. Therefore, the exposure time for *P. argentipes* was limited to one hour for DDT to collect LC values.[20]

The LC 50 value for *L. longipalpis* for DDT is 28.36 µg DDT per bottle, which is slightly lower than *P. argentipes* (36.91 µg DDT per bottle), while *P. papatasi* is half the

value at only 15.08 µg DDT per bottle. However, the LC 90 values for both *L. longipalpis* and *P. papatasi* are double, 218.58 and 295.98 µg DDT per bottle respectively, to elicit a similar response as seen in *P. argentipes* (165.97 µg DDT per bottle).[20]

These comparisons show that DDT requires a significantly higher concentration across different species of sandfly vectors to have a similar effect as permethrin from the pyrethroid family. These data, in addition to evidence of organochlorine insecticides acting as persistent compounds in the environment, support that insecticides such as DDT should be completely phased out of vector control programs. This aligns with the 2000 Stockholm Convention decision to phase out the use of persistent organic pollutants, such as DDT. As of 2015, India was the only country producing and using DDT as a control method for vector populations. As of 2022, most vector control programs in India have shifted to using the pyrethroid family of insecticides in lieu of DDT.[8,31] A study by Raghavendra et al. (2010) demonstrated the effects of DDT resistance in vector populations decades after their last exposure to the insecticide. This shows a lasting DDT resistance that could confer cross-resistance to the pyrethroid family of insecticides because of the similar mode of action and target site and the importance of rotating insecticides to delay resistance within a vector population.[32]

The second objective was to generate the DNA sequence gene fragments of *PARA* and *Ace-1,* genes that are known to be associated with TSI insecticide resistance in vectors. The primers specific to *P. argentipes* utilizes an optimized PCR protocol to reliably detect two genes of interest associated with TSI insecticide resistance within the *P. argentipes* genome. The susceptible phenotype sequence from the laboratory colony

allows for comparison and detection of point mutations at the codons of interest which could confer insecticide resistance in a field population.

The importance of sequencing the *PARA* and *Ace-1* genes within the *P. argentipes* connects research across many vector species to identify the susceptible phenotype and be able to identify codons associated with insecticide resistance. Sandflies, like many other vectors, have the potential to develop insecticide resistance through TSI from a point mutation within the genome. Research on mosquitoes has demonstrated similar amino acid substitutions across different taxa. The most common is a leucine to phenylalanine mutation (L→F) within the *PARA* gene at the 1014th codon and a glycine to serine mutation (G→S) within the *Ace-1* gene at the 119th codon.[33,34] These codons of interest have been associated with conferring TSI insecticide resistance are also present in different sandfly species at the same specified locations as mosquito vectors.[26]

The BLAST results for the *PARA* gene fragment aligning with a sequence that is associated with the L1014_TTA codon demonstrates that the 1014th codon in the *P. argentipes PARA* gene shows no TSI mutation. This supports that the laboratory *P. argentipes* colony exhibits the susceptible phenotype of the *PARA* gene.[13]. The sequence results for both gene fragments support the specificity of the primers to the appropriate location in the *P. argentipes*' genome.

Future research will determine a diagnostic time and dose for *P. argentipes* to α-cypermethrin, permethrin, and DDT. The diagnostic dose is the lowest concentration of an insecticide that will cause 100% mortality in a population within a given time, the

diagnostic time.[23] These values are important reference points for IPM professionals to understand insecticide susceptibility of a vector and to be able to implement an effective vector control plan on the population.

For the continued use of chemical insecticides on vector populations, IPM professionals need to continually monitor insecticide resistance. The incorporation of baseline susceptibility data into vector control programs will ensure appropriate insecticide dosages are applied at appropriate times and for effective detection of *PARA* and *Ace-1* genes within field populations of sandflies to screen for insecticide resistant mutations.

Conclusion

It is important for IPM professionals to have baseline susceptibility data for *P. argentipes* to insecticides commonly used in vector control programs. A repository of sandfly susceptibility data including ranges of lethal concentrations to different insecticides is an important tool for effective vector control in field populations. The PCR protocol with primers specific to the *PARA* and *Ace-1* genes associated with insecticide resistance in the *P. argentipes*' genome can be utilized for future research associated with detection and evaluation of insecticide resistance in field populations of sandflies.

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CHAPTER 3

Loop-Mediated Isothermal Amplification Assay to Differentiate Pyrethroid Susceptibility in *Phlebotomus argentipes* Sandflies (Diptera: Psychodidae: Phlebotominae)

Abstract

Background: Leishmaniasis is a neglected tropical disease that is caused by the *Leishmania* protozoan and vectored by the *Phlebotomus argentipes* sandfly. This disease causes significant socioeconomic impact on already marginalized populations. Integrated pest management (IPM) professionals use insecticides in the form of indoor residual spraying to reduce sandfly populations, which in turn reduces the caseload of leishmaniasis.

Many IPM professionals work in areas with limited access to proper scientific equipment. Therefore, having a rapid and effective assay to assess susceptibility of sandfly populations is necessary. A Loop-mediated isothermal amplification (LAMP) assay gives IPM professionals a diagnostic tool for the paralytic voltage-gated sodium channel (*PARA)* gene associated with target site insensitivity (TSI) insecticide resistance. TSI mutations limit the effectiveness of insecticides' ability to interact with the target binding site within the insect's nervous system. The LAMP assay allows for quicker responses from IPM professionals to make changes in pest management programs to effectively use insecticides on sandfly populations.

Methods: A permethrin-resistant *P. argentipes* laboratory sandfly colony was generated using lethal doses of permethrin on subsequent generations to artificially influence the evolution of resistant mutations. A susceptible *PARA* gene fragment sequence was obtained using optimized polymerase chain reaction (PCR) to develop LAMP primer sets specific for the *PARA* genetic region of the *P. argentipes* genome. A colorimetric assay was designed to visually identify amplification of the gene by a noticeable pH color change.

Results: A LAMP assay was developed to amplify a portion of the *PARA* genetic region of the *P. argentipes* genome. Two primer sets were developed: 1) to amplify the *PARA* genetic region and 2) to sit on the 1014th codon to identify if a mutation is present in the *PARA* genetic region. The LAMP assay allows for amplification results within 1 hour and at a constant temperature of 65°C. The LAMP assay did not produce a white precipitate and the colorimetric assay did not show positive color change for amplification of either primer set.

Conclusion: The LAMP assay will be beneficial to IPM professionals that work at field sites as it does not require specialized equipment. The developed primer sets did produce bands for the LAMP assay, but did not produce a color change for the colorimetric assay. The LAMP assay needs further optimization to distinguish between susceptible and resistant DNA profiles. Future research could focus on optimization of the colorimetric LAMP assay for rapid detection of field collected samples at field sites.

Background

Visceral Leishmaniasis (VL) is a neglected tropical disease with 350 million people at risk of infection worldwide. There is an annual incidence of approximately 0.9-1.6 million new cases and over 20,000 deaths worldwide.[1] Visceral Leishmaniasis endemic transmission is found in 10 countries, including India, Sudan, and tropical regions of Africa.[1] This disease predominantly affects rural areas and can cause severe socioeconomic impacts on afflicted individuals.[2]

The leishmaniasis disease is caused by the *Leishmania* parasite, which is transmitted from the bite of an infected female phlebotomine sandfly. In India, the causative agent of VL is *Leishmania donovani* and is transmitted by the *Phlebotomus argentipes* sandfly.[3,4] Visceral leishmaniasis is lethal in 95% of cases if left untreated. There are effective treatments, but they are expensive for afflicted individuals . There is currently no known vaccine for VL.[5,6] Therefore, prevention and control of sandfly populations using synthetic insecticides is the main strategy in regards to elimination of VL in India. Thus, in 2006, India enacted an elimination strategy combining societal education and outreach with vector control programs to reduce the impact of VL to approximately 1 in 100,000 people.[7,8]

Integrated vector management programs rely on insecticide resistant information for effective indoor residual spraying regimens. However, improper application and extreme conditions, high heat and humidity, require continued reapplication of insecticides over many years. This process has put a selective pressure for resistance on sandfly populations which has resulted in multiple documented cases of resistant sandfly

colonies in endemic areas such as India, Sudan, and across the Middle East and south-east Asia.[9]

Insecticide resistance has been documented in many insect vectors especially to synthetic insecticides such as pyrethroids. The pyrethroid family of insecticides is used by India in its VL elimination strategy. These insecticides interact with the insect's nervous system at the target site, the paralytic (*PARA*) voltage-gated sodium channel.[9,10] The *PARA* gene has been extensively documented in other vectors and connected to target-site insensitivity (TSI) insecticide resistance. TSI occurs when an amino acid substitution takes place in the voltage-gated sodium channel (*Vgsc*), which causes reduced sensitivity of the target site to pyrethroid family classes of insecticides.[11] The most common substitution occurs at the 1014th codon of the *PARA* gene which is seen across different insect vectors.[12,13] Thus it is important for the continued effectiveness of chemical control via insecticides within the Indian elimination strategy to have molecular assays to differentiate pyrethroid susceptibility in sandfly populations.

Therefore, it is important that integrated pest management (IPM) professionals have an effective and rapid method to assess susceptibility in sandfly populations. Most sandfly populations are in rural areas of endemic countries. Field sites are used to monitor the endemic areas and have limited access to specialized equipment. Thus, an assay is needed for field sites to be able to effectively and rapidly differentiate susceptibility in sandfly populations. This will allow IPM professionals to make informed decisions on the application and use of insecticides against the population of sandflies.

The standard for amplification of DNA is polymerase chain reaction (PCR), which requires the use of a thermocycler to fluctuate temperatures from 96 to 4℃. PCR assay uses a thermostable polymerase to separate and amplify DNA with the help of primers.[14] Thus for ideal amplification to occur, the PCR protocol must be conducted in a lab setting with access to the specialized equipment. A field site in a rural area does not always have access to such specialized equipment, which leads to delayed diagnostics of a field collected sample. Thus, IPM professionals have to collect and transport field samples to labs with the necessary equipment, or potentially outsource samples to third party labs and wait for results to be returned to make informed decisions about the insecticide susceptibility of the populations. This outsourcing potentially leads to sample degradation in transit; whereas, a live sample taken directly from a field collected sample can result in a more accurate diagnostic.

Loop-mediated isothermal amplification (LAMP) assay allows researchers in a remote field site to run their own diagnostics on field collected samples. The LAMP assay is run at a constant 65 °C and utilizes a warmstart polymerase which can be worked on at the bench without ice. The polymerase does not separate the DNA strands but displaces them enough to allow the primers to anneal and create dumbbell or loop shaped structures to allow for amplification without the need for specialized equipment.[15] Thus, with specialized primers for the *PARA* gene of the *P. argentipes* sandfly genome, field sites could utilize a LAMP assay on a heating block to rapidly differentiate pyrethroid susceptibility in field collected samples of sandfly populations.

The objective of this study was to develop a LAMP assay to differentiate pyrethroid susceptibility in a laboratory colony of *P. argentipes* sandfly. A LAMP assay

was designed and two primer sets were developed: one to recognize the PARA gene and another to sit on the 1014th codon. Then a colorimetric LAMP assay was developed using pH dye to visualize the amplification of the *PARA* gene fragment.

Methods

Colony Set-up

Sandfly Colony

An insecticide-susceptible *Phlebotomus argentipes* sandfly colony at Utah State University (USU) was derived from a long-established colony maintained at the Walter Reed Army Institute of Research (Silver Spring, MD). All life stages were reared at USU at 25˚C, 85% relative humidity, and a photoperiod of 12:12 (L:D) h.[16]. Larvae were fed a composted 1:1 mixture of rabbit feces and rabbit food; whereas, adults were provided 30% sucrose-water solution daily on saturated cotton balls. Adult female *P. argentipes* were blood-fed on anesthetized mice placed inside holding cages twice weekly.[17,18] The protocol and use of SKH1 hairless mice was approved by the USU's Institutional Care and Use Committee.

Development of Resistant-Selected Colony

Approximately 1000 adult *P. argentipes* from the insecticide-susceptible laboratory colony (generation P) were exposed to a predetermined lethal concentration of permethrin to develop a permethrin resistant laboratory colony. This was completed following the protocol from Denlinger et al. (2015), using LC75 and LC50 of permethrin

that caused 75% and 50% mortality in *P. argentipes* sandfly populations.[19] 20.79 µg/ml permethrin and 11.72 µg/ml permethrin served as LC75 and LC50 respectively. After a 24 hour waiting period post exposure, the surviving females were blood-fed consecutively over a three day period and allowed to oviposit. This process was repeated with successive generations. The resistant-selected colonies were kept under the same conditions as the insecticide-susceptible colony.[18]

Approximately 100 sand files were collected from each generation post exposure and blood-feds of each permethrin-selected colony. The collected samples were suspended in 100% ethanol and stored in -80℃. A control population of equal number of sandflies was collected from the insecticide-susceptible colony at the start of each resistant colony attempt.

Molecular Assay

DNA Extraction

Whole sandflies were collected from the susceptible and permethrin-exposed *P. argentipes* colonies. Total DNA to represent the population was extracted from 15 sandflies using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Each sample was mechanically homogenized in 180µL molecular grade water and 3 glass beads at 30Hz for at least 4 minutes using a Retsch MM400 (Retsch, Haan, Germany). To ensure all buffer has passed through and that the sample was dry, each centrifuge step was repeated, except for the final elution step. All DNA samples were kept in -20℃ for storage.

Loop-mediated Isothermal Amplification Assay

A paralytic voltage-gated sodium channel (*PARA*) gene fragment PCR product from the insecticid-susceptible *P. argentipes* colony was purified using Qiagen's QIAquick PCR Purification kit. Samples were sent to the USU CIB Genomics Core lab (USU, Logan, UT) for Sanger sequencing. The *PARA* fragment sequence was verified by comparison to the Nucleotide database in Genbank.[20] The sequence was cleaned and used as a guide in Primer Explorer V4 software to create Loop-mediated isothermal amplification (LAMP) specific primer sets as seen in Table 3.1.[21]

The LAMP assay was prepared in 25µL reactions containing: 2µL dNTP mix (2.5mM) (Invitrogen); 3µL 10x Isothermal amplification buffer; 1µL $MgSO_4$; 1µL *Bst* 2.0 WarmStart DNA Polymerase (8,000 U/ml) (NEB); 1µL of each primer. The forward inner primer (FIP) and backward inner primer (BIP) have a concentration 40µM, whereas the forward/backward outer primers have a concentration of 10µM; and 2µL of target DNA. The FIP and BIP primer pairs consist of F2+F1c and B2+B1c respectively, while the outer primers are F3 and B3. The LAMP reactions were run at a constant 65℃ for 1 hour. All LAMP products were visualized using gel electrophoresis with a 2% TBE agarose gel at 50V for two hours.

Table 3.1 *PARA* Loop-mediated isothermal amplification primer sets and sequences. Primer set 38 is the normal *PARA* gene fragment. Primer set M is the *PARA* gene fragment mutation with the F3 primer placed on the 1014th codon.

Species	Gene	Primer Set	Primer Sequence (5' → 3')
P. argentipes	*PARA*	38 - F3	TGGGAATTTAGTCGAGT
		38 - F2	ACTGAATATGATTTTCAACACAC
		38 - F1c	CAAACCACCCACTCGTCAGTG
		38 - B3	AAGAGGGTCATCAAACAGAT
		38 - B2	TTCACTCTTGCCATGACC
		38 - B1c	CTGCCCACAGAGCATCGATT
P. argentipes	*PARA*	M - B3	ATTGCTCAAAAGCAAGGC
		M - B2	TAAGAAGAGATTGAGAACCT
		M - B1c	GTGTTTTGCGGTGATGGCAA
		M - F3	TTAGTCGTGAGTATATGAAACTG
		M - F2	TTCAACACACAGTGACTATGG
		M - F1c	CAAACCACCCACTCGTCAGTG

Colorimetric LAMP Assay

The colorimetric LAMP assay utilized a Warm Start Colorimetric LAMP 2x Master Mix (NEB) and was set up in 25µL reactions. The colorimetric LAMP reactions contained: 12.5µL WarmStart Colorimetric LAMP 2x Master Mix; 1µL of each primer in working concentrations (FIP/BIP = 40µM and outer primers = 10µM); 0.5µL of $MgSO_4$; and 2µL of target DNA. The colorimetric LAMP reactions were run at a constant 65℃ for 1 hour. Detection of positive amplification was determined visually using the pH indicator color change from pink to yellow.

Results

Colony Survival

The percent survival of the permethrin-selected *P. argentipes* colonies are provided in Table 3.2. None of the colony attempts made it further than the F_2 generation as the offspring (F_3) were not viable past the egg stage. The permethrin-selected colonies only exposed to a lethal concentration (LC) of 75% (20.79 µg insecticide per bottle) demonstrated a decline from the initial percent survival of the insecticide-susceptible generation (P) for the respective colonies to the final offspring generation.

Table 3.2 Percent survival and the number of *P. argentipes* exposed for each generation of the permethrin-selected colonies.

	Percent (%) Survival (Number of Flies Exposed) *Phlebotomus argentipes*		
Generation	Permethrin-Selected Colony 1	Permethrin-Selected Colony 2	Permethrin-Selected Colony 3
P	25.8 (1200)[a]	35.8 (2267)[a]	9.1 (2420)[a]
F_1	9.6 (1515)[a]	50.7 (2194)[a]	27.7 (971)[b]
F_2		10.8 (2170)[a]	22.3 (807)[b]

[a]LC75 exposure [b]LC50 exposure

Permethrin-selected colony 1 only survived one generation and declined from 25.8% survival to 9.6% survival. Permethrin-selected colony 2 survived two generations and showed an increase in survival from the initial percent survival at 35.8% to 50.7% survival in the first offspring generation (F_1). However, the permethrin-selected colony 2 then declined from the 50.7% survival to 10.8% survival in the second offspring generation (F_2). The permethrin-selected colony 3 utilized an initial exposure at the LC75 which resulted in 9.1% survival. Then each subsequent exposure was at the LC50 (11.72

µg insecticide per bottle) which showed an increase in percent survival to 22.3% by the second offspring generation (F_2).

Molecular Assay

Loop-mediated Isothermal Amplification Assay

The LAMP primer sets (Table 3.1) were developed for the *PARA* region of the *P. argentipes* sandfly genome. The 38 primer set is a normal LAMP primer set for the detection of the gene and amplification. The M primer set is a mutation specific LAMP primer set that the outer primer F3 5' sits on the 1014th codon of the *PARA* gene.

No white precipitate formed or was visually noticeable after the 1 hour incubation of the LAMP assay with any primer set as demonstrated in Fig 3.1. A slight difference in DNA profiles can be seen between the primer sets as demonstrated in Fig 3.2. There was interaction between the primers and carryover was noticed in the negative reagent controls for all LAMP assay reactions as demonstrated in Fig 3.2.

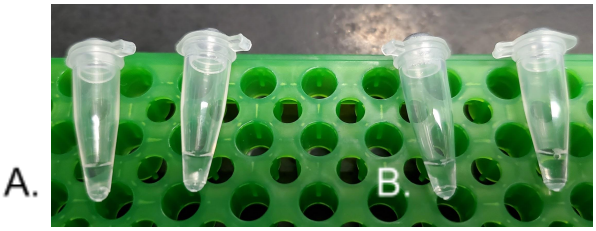

Fig 3.1 LAMP assay reactions using DNA from the insecticide-susceptible sandfly colony. A. 25µL LAMP assay containing 38 LAMP primer set showing before and after views of the reaction. B. 25µL LAMP assay containing M LAMP primer set showing before and after views of the reaction

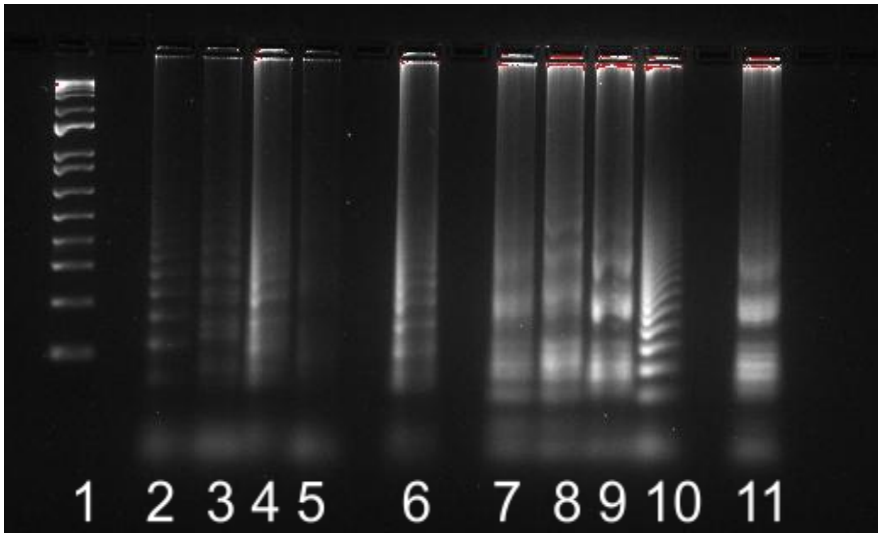

Fig 3.2 Visualization of LAMP M primer set wells 2-5 (left) and LAMP 38 primer set wells 7-10 (right) showing banding patterns and differences in DNA profiles. Wells 6 & 11 are negative reagent controls for the respective primer sets. Well 1 is a 1kb DNA ladder in 2% TBE agarose gel electrophoresis

LAMP assays were run on DNA extracted from insecticide-susceptible and permethrin-selected sandflies. Amplification was noticeable in all LAMP assay reactions utilizing the M LAMP primer set with all DNA samples (Fig 3.3). Thus, no mutation or visual presence of permethrin resistance was noted in the permethrin-selected *P. argentipes* colony attempts.

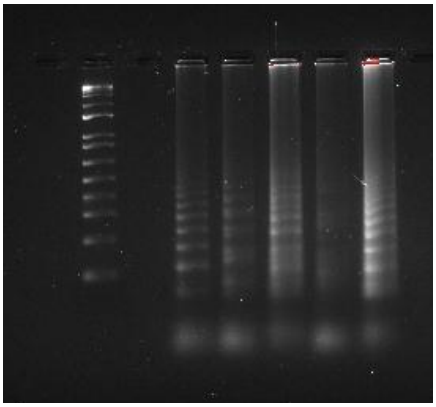

Fig 3.3 Visualization of LAMP M primer set utilizing DNA from a permethrin-selected *P. argentipes* sandfly colony using 2% TBE agarose gel electrophoresis

Colorimetric LAMP Assay

There was no visual color change detected in any of the colorimetric LAMP assay reactions with either primer set. It was noted that the negative reagent control for the colorimetric LAMP assay would shift to an orange color as demonstrated in Fig 3.4c but after being stored in -20℃ for 5 hours would revert back to the original pink color.

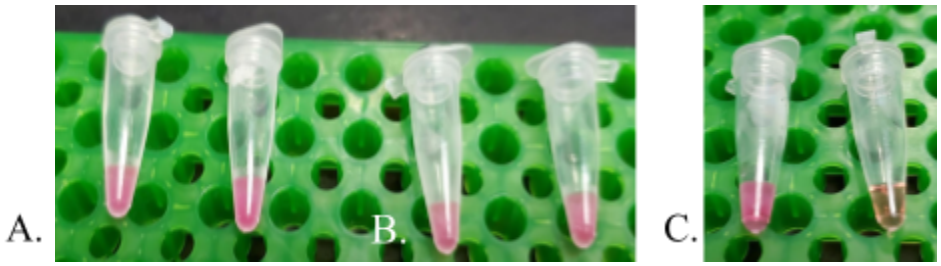

Fig 3.4 LAMP colorimetric assays for F1 generation from a permethrin-selected *P. argentipes* sandfly showing before (left) and after (right) for each primer set. A. Colorimetric LAMP assay reaction using 38 normal LAMP primer set. B. Colorimetric LAMP assay reaction using M LAMP primer set. C. Colorimetric LAMP assay reaction negative reagent control of M primer set

The colorimetric LAMP assay was run on gel electrophoresis to ensure amplification. Figure 3.5 shows that the colorimetric LAMP assay is positive for amplification.

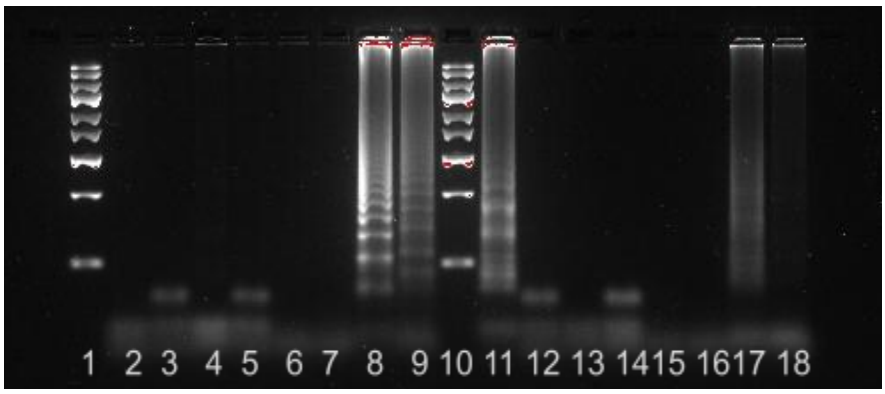

Fig 3.5 Visualization of colorimetric LAMP assay using M LAMP primer set and DNA from the F1 generation of a permethrin-selected *P. argentipes*. Wells 8 and 9 show positive amplification for the colorimetric LAMP assay. Wells 17 and 18 show faint streaking in the negative reagent controls for the colorimetric LAMP assay. Wells 1 and 10 have a 1kb DNA ladder using 2% TBE agarose gel electrophoresis

Discussion

Developing an insecticide-resistant laboratory colony of *P. argentipes* sandflies is very challenging. The ideal method to evaluate insecticide susceptibility is by developing a resistant colony from field collected samples, but the challenge and ability to collect enough sandflies from a particular region is difficult and often prohibited. Thus, researchers resort to using laboratory colonies as a baseline for comparison. A sandfly laboratory colony is good for insecticide susceptibility research, but an insecticide-resistant colony could allow for additional studies into vector fitness, and further understanding of the development of resistant mechanisms. However, a limitation of developing an insecticide-resistant laboratory colony is the volume of sandflies needed for exposure to the insecticide at different life stages. When sandflies are exposed, there often is still a significant death rate. There needs to be enough flies that will survive to blood-feed and oviposit to allow for enough offspring to make it through the flies' long life cycle.

To develop the *P. argentipes* insecticide-resistant colony, a LC75 was used as the selective pressure to drive the population towards resistance. A study by Denlinger et al. (2017) showed that propagating an insecticide-resistant colony is possible by using sub-diagnostic doses of insecticides. This method though still saw large fluctuations in percent survival of the sandflies. Intervals of no exposure between generations were also performed in an attempt to increase the number of available sandflies in the colony for future insecticide exposure.[13] Therefore, a build-up approach could potentially be attempted in future research to develop an insecticide-resistant colony. This can be

completed by increasing the insecticide exposure concentration X amount with subsequent generations.

Developing an insecticide resistant colony from a long established laboratory colony presented issues. These sandflies have been propagated under lab conditions for hundreds of generations, thus potentially allowing for bottleneck genetic effects. Bottleneck effects can occur when only a select number of a species survive or exist in an area and then repopulate.[22] The new population only has a limited variation of possible alleles available. This can therefore make it difficult to mirror mutations and variation that would normally be seen in field populations.[22] Also, the laboratory colony has significantly smaller numbers than field populations, thus making the probability of a beneficial mutation occurring at a particular loci in alleles very unlikely.

These laboratory conditions present the possibility that, during the resistant colony attempts with so few female sandflies surviving to blood feed and oviposit, other genetic influences come into play within the population. Another possibility is because of the small population size present within the resistant colony that natural selection does not occur but rather genetic drift. Genetic drift is a random event and causes fixation of alleles, regardless of benefit or cost to the organisms. Thus, genetic drift of limited alleles within the permethrin-selected resistant colony could have drifted a non-beneficial allele to fixation which could have lead the resistant colony towards extinction.[13,23,24]

One success of this research was the development of two primer sets that can be used in a Loop-mediated isothermal amplification (LAMP) assay to assess the presence and differentiate the susceptibility of the *PARA* gene fragment in *P. argentipes* sandfly

populations. The *PARA* gene fragment is found within the paralytic voltage-gated sodium channel associated with part of the insect's nervous system. This region has also been described as being associated with the target site of synthetic insecticides from the organochlorine and pyrethroid families of insecticides. The *PARA* gene fragment contains three codons of interest that are highly conserved across insect vector species and are associated with potential resistance through target-site insensitivity (TSI) mutations.[10] These TSI mutations reduce the effectiveness of organochlorine and pyrethroid classes of insecticides because the insecticide molecules are no longer able to effectively interact with the target site within the nervous system.[11] Therefore, these primer sets can be used by researchers at field sites to rapidly run diagnostics on field collected samples to assess the susceptibility of the *PARA* gene fragment within the population to make informed decisions to integrated vector control programs as needed in endemic areas.

Initially, the LAMP products were run on 1% agarose gels using TAE buffer, which is typically used for most PCR products. However, unlike traditional PCR that only utilizes a primer pair and results in a single band, the LAMP assay creates dumbbells/ loops in the DNA, allowing for strand displacement and making regions accessible for primer insertion and DNA sequence amplification. This results in creating a banding pattern on an electrophoresis gel of different molecular weights, depending on how many loops stem off the original DNA strand. Thus, the 1% agarose gel in TAE caused the LAMP fragment to disappear or streak considerably even at low voltage. The streaking made the bands from the amplification very hard to visualize.

Hence, a different electrophoresis buffer was used in an attempt to clean up some of the streaking and allow for cleaner DNA bands to be visible. TBE was substituted in

the electrophoresis procedure, along with a 2% agarose gel and low voltage, in order to optimize and improve the visualization of the LAMP products.[25] It should be noted that the LAMP assay does cause streaking in most gel electrophoresis regardless of voltage, as there is such a variety of molecular weights created from the loop structures in the assay.

The M LAMP primer set was developed using a *PARA* gene fragment from an insecticide-susceptible *P. argentipes* sandfly colony. The primer set was designed to allow the forward outer primer, F3, to sit on the 1014th codon of the *PARA* gene fragment, which is associated with target-site insensitivity in multiple insect vectors. Thus, if a mutation were to occur at this codon, it is hypothesized that the F3 primer will not attach and amplification will not occur. This is because the F3 primer is necessary for the non-cyclic amplification of the target DNA that occurs after the loop has been formed.[26] Therefore, it would allow integrated pest management (IPM) professionals a rapid diagnostic tool to screen for susceptibility in populations.

There is an issue with the M LAMP primer set as currently designed. Amplification of the *PARA* region may occur regardless of the forward outer primer's lack of attaching. This is because of the design of the LAMP assay to create loops the forward inner primer (FIP), specifically the F2 primer attaches first and creates space for the F3 primer to attach. Thus, some amplification may occur because the FIP forms the loop structure, allowing the backward primers to amplify the DNA strand.[26] Therefore, future research should focus on redesigning the M LAMP primer set so that the F2 primer from the FIP and forward outer primer, F3, both sit on the 1014th codon.

This would result in no amplification occurring at that site if a mutation did occur and the strand would not be displaced.

A LAMP assay can utilize the use of additional primers called loop primers that are designed to accelerate amplification by binding to additional sites not accessed by the other primers.[25] However, neither of these LAMP primer sets were developed to include loop primers. The loop primers help to facilitate the loop forming and amplification but are not required for the LAMP assay to function. It is noted that the addition of the loop primers may help to speed up the amplification process and help the production of the white precipitate which acts as a visual cue that the LAMP assay underwent successful amplification of the gene fragment.

A limitation of this research was the access of DNA from other vector species to ensure specificity of the primer sets. LAMP assay is inertly sensitive because of the primers recognizing six unique regions of the DNA sequence. But, it is documented across different insect vectors that mutations and codons within the *PARA* gene are conserved.[27,28] Therefore, a future direction could show specificity across different sandfly species and that amplification will not occur in other vector species such as mosquitoes.

The colorimetric LAMP assay was not successful in visually showing a color change in response to the amplification process. There was no positive color change from the pink to yellow for either LAMP primer set. The only noted color change occurred within the reagent controls which randomly turned orange, but no sample containing target DNA changed color. Upon review of technical protocols from New England

Biolabs, procedures suggests using a 10x primer solution and adding only 2.5µL to the 25µL reaction.[29] This technique variation could potentially result in using the same concentration of primers, but less volume in the final reaction, and this limiting primer-primer dimers.

Another limitation noted during the colorimetric LAMP assay is that only one DNA dye was attempted. There are other DNA dye or fluorescence that could have been used that may be a better avenue for visualizing the LAMP assay amplification by eye. Future research could attempt using hydroxynaphthol blue, which ultimately may work better with the LAMP assay for a rapid visualization of the presence or lack of amplification.

Conclusion

The Loop-mediated isothermal amplification (LAMP) assay has many challenges but can be a great diagnostic tool as a rapid assay to differentiate the susceptibility of the *PARA* gene fragment in field collected samples at field sites. This will allow integrated pest management (IPM) professionals to make informed decisions faster on the susceptibility status of populations of *P. argentipes* sandflies. The developed primer sets can ensure the presence of the *PARA* gene fragment and distinguish populations that may have TSI mutations that can confer insecticide resistance. The colorimetric LAMP assay allows for rapid visual detection using a pH dye that changes color based on amplification using the primer sets. Therefore, giving IPM professionals that work at field sites another tool to

assist in insecticide programs and differentiate pyrethroid susceptibility in populations of sandflies.

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CHAPTER 4

CONCLUSION

Leishmaniasis is a neglected tropical disease that affects millions of people worldwide. This deadly disease is vectored by different species of Phlebotomine sandflies. There is no known vaccine and treatment is expensive for afflicted individuals as the disease is endemic to poor, rural regions. Thus, chemical control using synthetic insecticides is important to reduce the populations of sandflies, which in turn lowers the caseload of leishmaniasis in these afflicted areas. However, continuous use of insecticides over decades places an immense selective pressure on the development of resistance mechanisms in sandfly populations.

The development of resistance mechanisms limits the effectiveness of insecticides and prolongs the possibility of an elimination strategy to reduce the prevalence of leishmaniasis in endemic regions like India. Therefore, it is important for sandfly populations to be continually monitored and assays be developed to rapidly identify or visualize genes associated with insecticide resistance. This will allow integrated pest management (IPM) professionals to make informed decisions about the usage of insecticides in vector control programs in order to slow the progression of insecticide resistant mechanisms.

In this thesis, the focus is on the *Phlebotomus argentipes* sandfly, the sole vector of the *Leishmania* protozoan that causes visceral leishmaniasis in India. The goal of this dissertation was to assess insecticide susceptibility in the vector and develop a

loop-mediated isothermal amplification (LAMP) assay for the detection of the paralytic voltage-gated sodium channel (*PARA*) gene that is apart of the insect's nervous system. The *PARA* gene is the target site for synthetic insecticides from the pyrethroid and organochlorine classes. Knockdown mutations in this genetic region cause target-site insensitivity (TSI) to insecticides which confers insecticide resistance.

My goal in Chapter 2 was to determine baseline susceptibility of *P. argentipes* sandflies to three different synthetic insecticides. The three insecticides used were: permethrin, α-cypermethrin, and dichloro-diphenyl-trichloroethane (DDT). These insecticides were selected as they have been used in vector control programs in India. We confirmed baseline susceptibility for *P. argentipes* using a dose-response assay for assessing insecticide-susceptibility. This baseline data determined lethal concentrations of the three insecticides for the vector which allows IPM professionals a comparison to field collected samples.

Another goal of Chapter 2 was to optimize a polymerase chain reaction (PCR) assay for the amplification of two genes associated with insecticide resistance in different insect vector species. Primer pairs were developed for the amplification of the *PARA* gene fragment containing the 1011th, 1014th, and 1016th codons and the *Ace-1* gene fragment containing the 119th codon which confer insecticide resistance through target site insensitivity (TSI) mutations. Sequences of the gene fragments from the insecticide-susceptible *P. argentipes* sandfly colony showed the presence of each of these codons of interest and confirmed the positive amplification of the *PARA* and *Ace-1* gene fragments.

My goal in Chapter 3 was to develop a LAMP assay for the amplification of the *PARA* gene fragment containing the 1014th codon for the *P. argentipes* sandfly. Two primer sets were developed: 1. Allows for the detection of the *PARA* gene fragment and 2. Forward outer primer (F3) positioned to directly sit on the 1014th codon to assess for mutations in the *PARA* gene fragment. This assay allows for rapid diagnosis of field collected samples of *P. argentipes* sandflies to differentiate insecticide susceptibility in the *PARA* gene fragment so IPM professionals can make quicker decisions on the use of insecticides in a vector control program.

Another goal of Chapter 3 was the development of a colorimetric LAMP assay for the visual detection of amplification. The colorimetric assay utilizes a pH dye color change which can be determined by eye after 1 hour of amplification. Thus, depending on which primer set is used the absence of or positive amplification allows IPM professionals information about the susceptibility of the field collected samples. This allows for quicker response to the development of resistance in sandfly field populations.

A future direction is the optimization of the LAMP procedure, including use of colorimetric assay. The colorimetric assay did not give conclusive results and thus, requires additional revisions to the protocol. Another approach for the colorimetric LAMP assay is to look into the use of different DNA dyes or fluorescences. The LAMP primer set was developed to sit on the 1014th codon of the *PARA* gene fragment. Additional work needs to be performed to allow for both the forward outer primer (F3) and the forward inner primer (FIP), specifically the F2 primer, to sit on the codon to ensure no amplification occurs in the presence of a mutation at that site.

Another important future direction is the continued research into baseline susceptibility data for *P. argentipes* sandflies to different insecticides. A repository of susceptibility data for sandflies to different insecticides is important for IPM professionals to make informed decisions in regards to vector control programs. Until a vaccine is created for leishmaniasis, chemical control of the vector population continues to be one of the most important options for reducing the incidence and caseload of leishmaniasis in endemic regions.