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INSECTICIDE-RESISTANCE GENOTYPING OF ANOPHELES MOSQUITO

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Abstract

Anopheles Mosquito is a debilitating febrile and life threatening vector that causes malaria. The Mosquito is a parasite called Plasmodium. This route of malaria transmission still remains as bites from infected female anopheles mosquitoes. Environmental factors and behavioral patterns of vectors and human populations combine to provide favorable conditions for malaria transmission. Proven effective options to reduce morbidity and mortality include early diagnosis, combined with prompt effective therapy and malaria prevention through reduction of human-vector contact, especially with the use of insecticides.

Keywords: Anopheles Mosquito; Genotyping; Insecticide; Insecticide-Resistance

1. Introduction

Perceptions about malaria illness, particularly households' perceived susceptibility and beliefs about the seriousness of the disease, are important preceding factors for decision-making concerning preventive and curative actions (Rakhshani et al., 2003; Akinloye, 2024a). The understanding of the possible causes, modes of transmission, and individual preference and decision-making about the adoption of preventive and control measures vary from community to community and among individual households (Adongo, Kirkwood & Kendall, 2005). There have been a considerable number of reports about knowledge, attitudes, and practices relating to malaria and its control from different parts of Africa.

Malaria kills approximately 400,000 people in 2018 alone, 93% of these deaths in the sub-Saharan Africa. Nigeria alone account for the highest burden of malaria, at 25% of all global cases (WHO Malaria Report 2019). The major vectors of malaria in

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Nigeria are Anopheles gambiae s.s., Anopheles coluzzii, Anopheles arabiensis and the patchily distributed Anopheles funestus s.s. (Okorie, 2011). However, Anopheles coluzzii has become the dominant species reported in most parts of Nigeria in recent years, e.g. in the south western region (Samson, 2018), the south eastern region and the northern regions (Ibrahim, 2019).

It is expedient to note that mosquito bites are likened to Agnew's strain theory as explained by Omigbodun and Akinloye (2026) and briefly reflected in the study of Obakhume (2026). Achieving sustainable control of the disease depend on extensive public health promotional programs which focus on current and proven methods of malaria prevention and management. Thus, it is expedient to evaluate current knowledge of malaria prevention practices and management options as well as the uptake of the management options.

The malaria is caused by plasmodia of various types:

[1]. Plasmodium ovale,

[2]. Plasmodium malaria,

[3]. Plasmodium vivat, and

[4]. Plasmodium falciparum (FMOH, 2011).

Among these four species of plasmodia, plasomodium falciparum is said to be the cause of the most severe malaria illness and death throughout the world (WHO cited in FMOH, 2003). Plasmodium falciparum is also identified as being the most devastating to Nigeria (FMOH, 2004). According to Federal Ministry of Health (2011), the transmission of the parasite is facilitated through the bite of female anopheles mosquitoes.

One of the major ways to control malaria is through the use of insecticide that prevents these mosquitoes from feeding on people, as it is one of the main interventions recommended by the World Health Organization (WHO) for the control of malaria and the spread of the mosquito. The control of malaria vectors is reliant on the use of chemical insecticides through indoor residual spraying (IRS), long-lasting lasting insecticidal nets (LLINs) and Insecticide treated mosquito nets (ITNs) (Musiime, 2019). These interventions have reduced malaria transmission to half its level from year 2000 to 2015 (Bhatt, 2000). However, the major threat to these recorded successes is the issue of insecticide resistance in the major malaria vectors leading to retrogression and an increase in transmission.

Adequate data gathering on the resistance profiles and mechanisms in the local malaria vector populations is a key to guide deployment of control tools using evidence-based strategies (WHO, Malaria Report 2016). Insecticide resistance in Anopheles gambiae s.l. has been reported in Nigeria as far back as the early 1960s. In the southern part of the country for example, resistance to dieldrin, lindane and dichlorodiphenyltrichloroethane (DDT), but susceptibility to malathion parathion, fenthion, and bromophos was reported (Self LS et al., 1966). Moreover, in Sokoto, in the northwest of the country resistance to dieldrin, DDT and benzene

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hexachloride (BHC) was reported early. Decades after these earlier efforts, data documenting the evolution of the malaria vectors, their insecticide resistance profiles and the underlying molecular mechanisms driving the resistance are still lacking, particularly in the Niger-Delta (south-south) region of the country.(Ramakrishna et al., 2005)

Malaria transmission is practically perennial in the coastal regions of Nigeria (including the Niger-Delta), due to the prolonged rainy season which provides Anopheles breeding sites year round. This is in contrast to the northern part of the country, with shorter rainy season (3–5 months) and thus relatively lower transmission rates (Okwa et al., 2009; Akinloye, 2024b). Unfortunately, where numerous strides have been made in determining the transmission profiles of malaria mosquitoes, insecticide resistance and the molecular mechanisms driving it in other parts of the country, e.g. in southwestern Nigeria and in the north, e.g. little information is available from the south-south/Niger-Delta region. Baseline information on the abundance of Anopheles and its distribution (Okwa et al., 2009).

Insecticide resistance profiles are available in this region, without actually going deep into studying the molecular mechanisms involved. Equally, also there is a dearth of information on the potential impact of the resistance on the malaria control tools such, as the efficacy of bed nets, as recently conducted in other parts of the country. As well, information on the role of target-site resistance mechanisms like the 1014F/S knockdown resistance (kdr) mutations (Ranson et al., 2000) is also lacking in south-south of Nigeria, compared to other parts of the country where frequencies of these mutations have been described in several studies (Okorie et al., 2015).

2. Literature Review

Mosquito insecticide resistant in Nigeria

In this study we will be diving into the insecticide resistance genotyping of anopheles mosquito. The review of literature on this case study will be conducted. Anopheles gambiae Giles is one of the most important malaria vectors in Africa, killing more people than HIV/AIDS or any other killer disease. And an endemic throughout Nigeria accounting for 25% of infant mortality (Federal Ministry of Health, 2005). Malaria is a life-threatening disease caused and spread by Plasmodium parasite and female mosquito vectors, respectively (WHO, 2018).

It is the world's most important parasitic disease of public health interest. (Aribodor et al., 2016) According to the latest world malaria report, African region continues to have an alarmingly high incidence rate of malaria – 92% of malaria cases and 93% of malaria deaths. Which according to WHO (2019), Nigeria accounts for 25% of the cases in Africa. In Northwest Nigeria, a prevalence range of 60% to 65% has been reported in different studies in Kano State despite the high frequency of use of insecticide-treated nets (ITNs) and indoor residual spraying in the area.(Dawaki et al., 2016, Oladele et al., 2018; Akinloye, 2026).

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In human, malaria is caused by Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, and Plasmodium malariae– and the infection by P-falciparum has been reported to pose the greatest threat (Geleta et al., 2016).Anopheles gambiae complex constitutes the main vector that transmits one of the most threatening Plasmodium species – P falciparum in sub-Saharan Africa (Kabula et al., 2011). The A gambiae complex consists of 7 species (A gambiae sensu strict (s.s.), Anopheles arabiensis, Anopheles quadriannulatus species A and B, Anopheles melas, Anopheles merus, Anopheles bwambae) which are morphologically indistinguishable.

Recently, it has been reported that A gambiae sensu stricto exists in 2 molecular forms, denoted M (now A coluzzii) and S-form (now A gambiaes.s.), which can be distinguished by differences in a 4-Mbregion located in the X chromosome (Ranford-Cartwright et al., 2016). This increases the number of species to eight (8). A gambiae sensu stricto (s.s.), A coluzzii, andA arabiensis constitute 3 out of the 6 species considered to exhibit the most vectorial capacity (Coetzee et al., 2013). Ecologically, A melas, Amerus, and A bwambae are salt-tolerant and are described assalt water or mineral water species, while the others are obligate freshwater species (Kengne, 2019).

Their public health importance stems from their high anthrophilicity and potential to exploit and adapt to diverse environmental conditions occasioned by human activities – directly or indirectly (Kabula et al., 2011). This is an indication that they are able to evolve different mechanisms for survival under different environmental conditions defined by various physicochemical parameters. The major vectors of malaria in Nigeria are Anopheles gambiae s.s., Anopheles coluzzii, Anopheles arabiensis and the patchily distributed Anopheles funestus s.s. (Coetzee et al., 2013, Federal Ministry of Health (FMoH) 2005).

However, Anopheles coluzzii has become the dominant species reported in most parts of Nigeria inrecent years, e.g. in the south western region (Fagbohun et al., 2019), the south eastern region (Chukwuekezie et al., 2020) and the northern regions (Ibrahim et al., 2019, Ibrahim et al., 2014). Insecticide resistance in Anopheles gambiae s.l. has been reported in Nigeria as far back as the early 1960s. In the southern part of the country for example, resistance to dieldrin, lindane and dichlorodiphenyltrichloroethane (DDT), but susceptibility tomalathion parathion, fenthion, and bromophos was reported (Self, 1966). Moreover, in Sokoto, in the northwest of the country resistance to dieldrin, DDT and benzene hexachloride (BHC) was reported early (Ramakrishna, 2005).

Decades after these earlier efforts, data documenting the evolution of the malaria vectors, their insecticide resistance profiles and the underlying molecular mechanisms driving the resistance are still lacking, particularly in the Niger-Delta (south-south) region of the country. Malaria transmission is practically perennial in the coastal regions of Nigeria (including the south-south), due to the prolonged rainy season which provides Anopheles breeding sites year round (19-21). This is in contrast to the northern part of the country, with shorter rainy season (3-5 months)

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and thus relatively lower transmission rates (Federal Ministry of Health (FMoH) 2013).

Unfortunately, where numerous strides have been made in determining the transmission profiles of malaria mosquitoes, insecticide resistance and the molecular mechanisms driving it in other parts of the country, e.g. in southwestern Nigeria (Fagbohun, 2019; Awolola, 2009) and in the north, e.g. (Ibrahim, 2014, Olatunbosun-oduola et al., 2019; Akinloye, 2025), little information is available from the south-south/Niger-Delta region. Baseline information on the abundance of Anopheles and its distribution (Oringanje et al., 2013), insecticide resistance profiles (Atting et al., 2019) are available in this region, without actually going deep into studying the molecular mechanisms involved.

Equally, also there is a dearth of information on the potential impact of the resistance on the malaria control tools such, as the efficacy of bed nets, as recently conducted in other parts of the country (Ibrahim et al., 2019, Awolola et al., 2014). As well, information on the role of target-site resistance mechanisms likethe 1014F/S knockdown resistance (kdr) mutations (Martinez-Torres et al., 1998, Ranson et al., 2000) is also lacking in south-south of Nigeria, compared to other parts of the country where frequencies of these mutations have been described in several studies (Chukwuekezie et al., 2020, Okorie et al., 2015).

From literature, the resistance profiles of An. coluzzii from Port Harcourt, Niger-Delta, Nigeria was investigated, establishing high resistance to pyrethroids and DDT, with evidences from qPCR (over expression of CYP6Z2 and CYP6M3 especially) and synergist bioassay with piperonylbutoxide implicating cytochrome P450s in driving permethrin resistance. Analysis of the voltage gated sodium channel also revealed the presence of 1014F kdr mutation at high frequency, whereas 1014S was not detected. The 1575Ymutation was detected but only at a very low frequency (Okorie et al., 2015).

Mosquitoes identification to species level

Anopheles mosquito is a mosquito of a genus which is particularly common in warmer countries and includes the mosquitoes that transmit the malaria parasite to humans. it is one of the best known, because of its predominant role in the transmission of the most dangerous malaria parasite species (to humans) – Plasmodium falciparum. Genomic DNA (gDNA) can be extracted from female anopheles mosquitoes randomly selected fromthe cages. The gDNA was utilised in the identification to the species level using theSINE200 PCR. All mosquitoes were confirmed as An. coluzzii with a band of ~479 bp onagarose gel. Also, legs individually pulled out from randomly selected 60 adult femalesused for qPCR were extracted for species identification, as above, with all the females confirmedas An. coluzzii (Martinez-Torres et al., 1998)

Genotypic DNA was extracted from the legs of individualF0and F1female mosquitoes using Qiagen QIAxtractorrobot. Species Identification to the molecular level was carried out as previously done (Santolamazza et al., 2008). All phenotypedF1and F0 An. gambiae s.l. mosquitoes were analysed for species

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identification. Primers included in the reaction were those that detect sibling species of An. Gambiae complex, including An.arabiensis, An. coluzzii. An. Gambiaes.s and Anopheles melas; which are the relevant vectors of malaria in Ghana (Bass, 2007).

3. Methods

Morphological identification of vector species

All adult mosquitoes subjected to the test were identified at the NAVRC laboratory using the keys of Gillies and DeMeillon (1968), Gillet (1972) and Gillies and Coetzee (1987). Morphological identification was conducted using the Gilles and Coetzee 25 characteristic under a Zeiss ×10 light microscope. The upper margins of the fore wings contained some dark spot (wing spots), a feature common to all Anopheles mosquitoes. The palpis are elongated and segmented into 3 parts with a pale spot on the second dark area.

Around 30 An. gambiae (s.l.) were selected randomly by site from live and dead mosquitoes (15 from pirimiphos-methyl tests and 15 from deltamethrin tests) and subjected to polymerase chain reaction (PCR) amplification for species identification and resistance mechanism detection. Genomic DNA from whole female mosquitoes was extracted according to the standard procedures of Collins et al.,. 1987. Extracted DNA was re-suspended in 50µl PCR grade water.

PCR for molecular species identification

PCR tests were performed with universal and species specific primers for the An. gambiae complex. The SINE200 PCR protocol (19) was used to simultaneously identify members of the An. gambiae complex and their former molecular forms.

Case study Species identification by PCR method by Tagman

The specimens were identified to species and molecular forms by the method of Santolamazza et al. (2017) using species-specific primers for A gambiae complexes - the SINE200 forward and reverse primers (forward 5′-CGCTTCAAGAATTCGAGATAC-3′ and reversed 5′-CGCTTCAAGAATTCGAGATAC-3′). Fifty-six A gambiae mosquitoes (30 from Sharada and 26 from Wailari) were used. The extracted DNA pellets were subjected to polymerase chain reaction (PCR) (Taqman).

The reactions were carried out in a 25 µL reaction tube which contained 1 pmol of each primer, 0.2 mM of each dNTP,1.5 mM MgCl2, 2.5 U Taq polymerase, and 0.5 µL of templateDNA extracted from a single mosquito. Thermocycler conditions were 94°C for 10 minutes followed by 35 cycles of 94°Cfor 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute, with a final elongation at 72°C for 10 minutes, and a 4°C hold. The resulting products were analysed on 1.5% agarose gelsstained with ethidium bromide at 85 V, with low- and high molecular-weight bands corresponding to fragments containing or lacking the targeted SINE200, respectively.

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4. Result and Discussions

Insecticide resistance

The resistant phenotype is an insect that survives a dose of insecticide that would normally have killed it is relatively easy to monitor with direct insecticide bioassays. However, in many cases the actual molecular mechanisms responsible for the resistant phenotypes are still unknown. The availability of the A. gambiae genome will allow us to determine the exact molecular changes that have resulted in resistant phenotypes. For example, up-regulation of one or more members of the cytochrome P450gene families can produce broad-spectrum insecticide resistance. Currently, the mechanisms that regulate insecticide resistance are poorly understood. Drosophila may not be a good model in this respect because it is not an insectpest, and so is not subjected directly to large scale insecticide control programs. In contrast, A. gambiae has multiple resistance mechanisms that have been field-selected in both East and West Africa through exposure to DDT and pyrethroids (Hemingway & Ranson, 2000).

Research into insecticide resistance is obviously ripe for the move from the static genome map to the functional genomics approach, which will allow us to understand the evolution of resistance in these complex organisms through modulation of gene expression. Material from East Africa has already been subjected to standard genetic quantitative trait loci (QTL) mapping, which has defined a polytene chromosome region within which the regulator ofP450 gene expression must be encoded (Aribodor, 2016).

The availability of the A. gambiae genome sequence now allows us to use new molecular microsatellite markers from the sequence to narrow down this control region to a few kilobases of DNA. Open reading frames can then be identified and candidate genes analysed for function with recently developed anopheline transformation technology. Regulatory genes controlling the expression of glutathione transfer (enzyme families that are important for protecting insect cells from insecticides)will be similarly defined from QTLs that are already mapped to the A. gambiaepolytene chromosomes.

In Culex mosquitoes and aphids, elevate desterases confer organophosphate resistance through gene amplification, with multiple copies of DNA amplicons of about 30 kb being integrated stably into the insect genome, either contiguously or, in the case of aphids, sometimes disparately. The resistant phenotype results from a complex tissue-specific interplay of differential up regulation of these amplified genes and in the case of aphids involves changes in DNA methylation. The sequenced A. gambiae genome is from an insecticide-susceptible strain, and there are obvious orthologs for the amplified Culex esterases (Djouaka, 2016).

To date, there is no evidence of esterase gene amplification–based resistance in any Anopheles species. However, as new resistant strains of A. gambiae are

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investigated, amplifications may well be found, and analysis of the genome sequence surrounding the amplicons will allow us to elucidate the size ofthe units and may shed light on the amplification mechanisms. The availability of the complete A. gambiae genome sequence should stimulate arapid shift in research aimed at improving the management of insecticide resistance. If the problem of resistance is subdivided into three stages:

[1]. Detection,

[2]. Monitoring, and

[3]. Management

Then the benefits of this genome sequence become obvious. The genome sequence will enable access to the major regulatory genes involved in resistance, particularly if orthologous regulators control metabolically based resistance in insects generally. For example, management of resistance in practice currently involves basic rotations and mixtures or mosaics of different insecticides. Access to insect-specific metabolic enzyme regulators will provide a target for “add-ons” to current insecticides, which should expand their natural life-span by blocking common resistance pathways while leaving mammalian toxicity unaffected.

Insecticide resistance can result from direct changes to the proteins that normally bind to the insecticides. For example, mutations in sodium channels (the target of DDT and pyrethroids) and in acetylcholinesterase (AChE; the target of organophosphates and carbamates) have been well documented in many insect species including, in the case of sodium channels, mosquitoes. The Anopheles genome will provide information on the target site genes, facilitating cloning and mutagenesis studies to determine the precise nature of the mutations and to aid in predicting interactions between insect proteins and insecticides. In the longer term, this could lead to new insecticidal molecular targets. This approach may be especially important for AChE as there is increasing evidence for multiple AChE genes from the Anopheles and Drosophilagenome databases (Hemingway and Ranson (2000).

Two AChEgenes are apparent in the A. gambiae genome as detailed by Ranson et al.,. in this issue, and to date no resistance-linked mutations have been identified in mosquitoes predominantly in studies on the sex-linked AChE gene. The Anopheles genome in conjunction with that of Drosophila also provides sequences of nicotinic acetylcholine receptor subunits, which will facilitate their cloning from other insect species. This receptor is the target of an important new group of agrochemicals, but until now studies of insect receptors have relied on co-expression of insect genes with a vertebrate subunit (Djouaka, 2016).Many instances of resistance, resulting from a change in a single regulator gene, may trigger complex cascades of expression of unrelated genes.

The presence of the full genome sequence will prompt a move away from the reductionist approach that has dominated the last two decades of resistance

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research. Such an approach has tended to result in a lack of appreciation for how the large physiological changes that often accompany resistance can influence other characteristics of the insect vector.

For example, many of the large scale-up regulations of enzyme families that accompany insecticide resistance result in profound changes in oxidative stress levels in the cells where these enzymes are expressed. These are often the identical tissues in which parasites or viruses reside during transmission by insect vectors from one human to another. Microarray and cell biology approaches are being used to define mechanisms in A. gambiae that enable this insect vector to be refractory to infection by malaria and other parasites. Available data already strongly indicate that an enhanced ability of the refractory insects to tolerate oxidative stress is integral to their ability to resist parasite infection (Hemingway, 2000).

The convergence of these two lines of resistance research will be greatly facilitated by our ability to look at regulation patterns in different phenotype combinations across the whole genome. There are already indications that these are not mutually exclusive systems; for example, filarial parasites fail to develop in highly insecticide-resistant Culex mosquitoes (Hemingway, 2000). Overall, the A. gambiae genome provides us with exciting opportunities to move from knowledge and understanding of how resistance genes work to the practical application of that knowledge in the field. This will fundamentally improve the control of malaria and other important vector-borne diseases and will contribute to the wider studies of resistance in agricultural pests.

Effectiveness of insecticide resistance

Effective control of malarial vector amid spreading insecticide resistance presents enormous logistics challenges. Monitoring and understanding the dynamics in relation to some environmental elements such as climate, physicochemical properties are key to addressing the challenges. The impact of environmental elements on mosquito oviposition, larval development/distribution, larval density, and development has been reported in some endemic areas of Africa. However, studies that have investigated mosquito vector species, resistance/susceptibility status, and physicochemical properties of the breeding environment are still lacking in Nigeria. While Kabula et al. (2017) studied insecticide resistance in A gambiae s.i. relative to physic chemical properties within Accra metropolis, Awolola et al.(2014) investigated the kdr mutations in M and S forms of Agambiae s.s. in Southwest Nigeria and more recently Ibrahim et al. (2002) characterized the transmission and resistance profiles of A coluzzii population in Nigeria. Both studies from Nigeria did not consider the possible association of abiotic factors of breeding sites to resistance profiles of the sampled mosquitoes.

Second, despite these studies, it is important to note that the mosquito insecticide resistance phenomenon is dynamic and thus necessitates continuous study to monitor the trend. It is upon this premise that this study was carried out to characterize the mosquito species, resistance profile, and physicochemical

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properties of 2 breeding sites – Wailari (residential) and Sharada (industrial) – in Kano state, Northwest Nigeria.

WHO Case studies

WHO Insecticide susceptibility/resistance bioassay tests case-study

Insecticide susceptibility/resistance bioassay tests were carried out using World Health Organization (WHO) susceptibility test kits and standard procedures with 4 replicates of 20 to 25non-blood-fed adult female mosquitoes. The assays were carried out in batches by exposing the mosquitoes to papers impregnated with a recommended concentration of a given insecticide (purchased from Universiti Sains, Penang, Malaysia) in the assay tubes. The following insecticides (classes) were used:

[1]. 0.75% permethrin (a pyrethroid: inhibitor of closure of voltage-gated sodium channels);

[2]. 4% DDT (organochlorine: of closure of voltage-gated sodium channels);

[3]. 4%bendiocarb (carbamate: reversible inhibitor of acetylcholinesterase),and

[4]. 0.1% malathion (organophosphate: cholinesteraseinhibitor) and 0.75% permethrin + 5% piperonyl butoxide (PBO)(a synergist that inhibits cytochrome P450 oxidase system andcarboxyesterase).

Each replicate was exposed to the insecticide impregnated filter paper for 60 minutes. During the exposure period, the number of mosquitoes knocked down was recorded after 15, 30, 45, and 60 minutes. The mosquitoes were then transferred into the holding tube and fed on glucose solution via a pad of a cotton wool soaked in 10% glucose solution and placed on the mesh-screen end of the holding tubes. The time taken to achieve 50% of population knockdown (KDT50) was assessed using log-probit analysis. Mortality was determined 24-hourpost-exposure by counting the number of dead and alive mosquitoes.

An adult mosquito is considered to be alive if it was able to fly regardless of the number of legs remaining. Any knocked down mosquito, whether or not it has lost a leg or wing, was considered moribund and counted as dead. Mosquitoes were classified as dead or knocked down if they were immobile or unable to stand or take off. On completion of the susceptibility test, the mosquitoes were transferred individually to clearly labelled tubes with a lid for airtight locking (separating dead and alive mosquitoes into separate tube) for preservation until further analysis.

For the synergist bioassay to establish the potential involvement of metabolic enzyme system using PBO and permethrin, replicates of about 20 to 25 female mosquitoes were pre-exposed to 5% PBO for 30 minutes in a tube. They were subsequently transferred to a tube containing permethrin for 1 hour. The mosquitoes were treated as in conventional bioassay described above and mortalities scored after 24 hours.

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WHO insecticide susceptibility test

The result generally showed that DDT, permethrin, and permethrin + PBO recorded a significantly($P < .05$) poor knock down (low percentage knock down and longer median knockdown time). The pre-exposure to synergist, PBO, recorded a significant but low improvement in the knock down potential of permethrin. The 24-hour mortality assay result revealed a very high mortality (no resistance)for malathion and bendiocarb (with low level of resistance)and low mortality and high resistance to DDT, permethrin, and permethrin + PBO. The pre-exposure with PBO did not increase the mortality observed with permethrin beyond 60%.Comparatively, lower mortality was observed in Walari relativeto Sharada; however, a significant ($P < .05$) difference in mortality between the 2 sites was only observed with DDT.(Killeen, 2018).

Evolution of WHO insecticide susceptibility test

The WHO insecticide susceptibility bioassay that is the main subject of these revised and expanded procedures is a simple direct response-to-exposure test. Mosquitoes are exposed to known concentrations of an insecticide for a fixed period of time, and the number of fatalities is recorded at least 24 hours after exposure. In its present form, the test is designed to distinguish between baseline susceptibility and resistance to insecticides in adult mosquitoes. Thus, the test is intended to be used as a field and laboratory surveillance tool, with the limitation that it gives little information on the underlying mechanism(s) conferring resistance (where detected) or the strength of that resistance.

The test equipment and method has changed relatively little since WHO first recommended the use of a standard bioassay technique to detect insecticide resistance in the early 1960s. Any methodological changes that have been made over the years have been fairly minor, relating largely to the test conditions and controls, and to the insect sampling protocols (Moyes, et al., 2013). Changes incorporated into the 2013 update of the guidelines related primarily to data interpretation criteria, the addition of discriminating concentrations for certain new insecticide active ingredients, a brief description of tests for resistance mechanisms and a synopsis of the bottle bioassay developed by the US Centers for Disease Control and Prevention (CDC).

The original impetus to develop the 2013 version of the guidelines arose from recommendations of an informal WHO-GMP consultation convened in May 2010. The consultation reviewed the current status of insecticide resistance in malaria vectors in order to identify strategies for delaying the emergence of resistance. The impetus for the current revision stems from an informal consultation convened in December, 2015 to revise and adapt the current procedures. The aim was to provide a stronger focus on producing operationally meaningful data to enable policy-making decisions for vector control. Recognizing that insecticide resistance has a central role in monitoring the effective planning and implementation of insecticide-based vector control, and taking into account resistance management, a WHO consultation in April 2016 made several recommendations about the future

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direction of insecticide resistance detection and monitoring. In addition to highlighting the need for an immediate scale-up in susceptibility testing and the establishment of data reporting mechanisms, the consultation recommended an expanded three-step bioassay protocol:

Step 1: Detect the presence of insecticide resistance phenotypes in a population using discriminating concentration bioassays, according to the method outlined in the 2013 publication (WHO, 2016).

Step 2: Assess the strength of phenotypic resistance by performing bioassays using five and 10 times (5× and 10×) the discriminating concentrations of insecticides.

Step 3: Determine the involvement of metabolic resistance mechanisms by assessing the effect of a synergist such as piperonyl butoxide (PBO) on the resistance phenotypes detected in Steps 1 and 2.

This expanded version is intended to make it easier to generate comprehensive data that will be useful in making decisions on how to respond to the presence of insecticide resistance. The CDC bottle bioassay offers options to test 1×, 2×, 5× and 10× concentrations. Data from these bioassays increase confidence that multiples of the discriminating concentrations are informative and are applicable to WHO susceptibility bioassays. WHO continues to recommend that the standard WHO susceptibility tests should continue to be a primary method by which resistance is detected. However, it was considered necessary to update the existing resistance-monitoring procedures to also highlight the need for operationally meaningful data. Consequently, a technical consultation was convened to inform updates to the procedures. Specific objectives identified were to:

[1]. Expand current WHO test procedures to generate more operationally meaningful data for monitoring insecticide resistance in malaria vectors in order to align with new developments in insecticide resistance management;

[2]. Provide procedures for resistance intensity bioassays using 5× and 10× the discriminating concentrations;

[3]. Advise on test procedures for assessing the effect of synergists (e.g. PBO) on the expression of resistance phenotypes; and

[4]. Provide an updated list of “discriminating concentrations” for adult mosquitoes for the insecticides recommended for use in malaria vector control. Insecticide resistance is a quantitative trait that varies in its intensity.

Therefore, it is further recommended that routine susceptibility monitoring using the expanded WHO insecticide susceptibility test be supplemented by additional genetic testing (polymerase chain reaction, PCR) and biochemical testing (enzyme activity assays). Supplementary test methods for determining the underlying mechanisms of resistance, their intensities of expression and tools for tracking the spread of resistance are important for decision-making to manage insecticide resistance. The procedures for larvicide susceptibility testing are still considered valid; hence, they

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are not repeated in the present document. Those interested in the evaluation of larvicides are advised to refer to the original documents, which are available from WHO (1976, 1970).

5. Conclusion

The WHO susceptibility bioassay is a direct response-to-exposure test. It measures mosquito mortality to a known standard concentration of a given insecticide, either with a discriminating concentration or with intensity concentrations. The concept of discriminating (or diagnostic) concentration is now well established, and has been widely adopted for the purposes of testing and monitoring insecticide resistance in mosquitoes and other disease vectors (WHO, 2016). The use of discriminating concentrations in routine insecticide resistance monitoring is explained. Discriminating concentrations have been established under standardized laboratory conditions for all insecticides currently in use in malaria control programmes. They have been reported in previous versions of the guidelines WHO (1963) and are updated again in this revision.

The anopheline species used in that study were An. aconitus, An. albimanus, An. arabiensis, An. dirus, An. freeborni, An. gambiae s.s., An. maculatus, An. minimus and An. stephensi. Since then, discriminating concentrations have been established for a further four insecticide compounds, although as yet these are tentative, pending confirmation by WHOPES through a multicentre validation. Insecticide susceptibility test papers impregnated with insecticides at the appropriate discriminating concentration are supplied as part of the WHO test kits manufactured by Universiti Sains Malaysia and coordinated by (WHO Malaria Report, 2017) WHO has applied the use of discriminating concentrations in one of the following two ways:

[1]. Twice the lowest concentration that gave systematically 100% mortality (i.e. LC100) after 60 minutes exposure and a holding period of 24 hours of a susceptible laboratory strain or a susceptible field population of mosquitoes; or

[2]. Twice the LC99.9 values determined by baseline susceptibility testing against a susceptible laboratory strain or a susceptible field population of mosquitoes. A susceptible population is one that has not been subjected to insecticidal pressure and in which resistant individuals are either absent or rare.

The WHO recommended discriminating and intensity bioassay concentrations of insecticides for determining susceptibility of adult anopheline mosquitoes using WHO insecticide susceptibility tests. It also gives the concentrations for synergists for synergist-insecticide bioassays. Provides the methodology for carrying out the bioassays. In most countries, insecticide resistance monitoring for other vectors (e.g. Aedes mosquitoes) is also necessary.

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Conflicts of Interest

The authors declare no conflict of interest.

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Author Responsibility Statement

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