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Acetylcholinesterase and Knockdown Resistant Mutations in *Anopheles Gambiae S.L.* (Culicidae: Diptera) In Three Vegetations of the Niger Delta Region

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ABSTRACT: *Comprehending the genetic arrangement of resistant mosquito populations is essential for addressing biological and public health concerns as, spread of insecticide resistant alleles. Anopheles gambiae sensu lato, which spread malaria parasites comprises genetically different but morphologically indistinguishable species. This study: determine presence of resistant genes, in the lowland forest, mangrove and fresh water swamp in Rivers state, Niger delta. Sample collection was done from May to December. Laboratory-reared larvae collected from their breeding sites, were used for the molecular bioassay. Resistant genes were determined from DNAs extracted from the tissues of the samples and subjected to polymerase chain reaction (PCR), to identify knockdown and acetylcholinesterase resistance mechanisms. Acetylcholinesterase resistance mutation with accession number OQ802845 was found in all the three vegetations while knockdown resistance mutation was scantily found in the lowland forest vegetation only. The study makes available reference base-line data of various insecticide resistant genes in the Niger delta.*

KEYWORDS: acetylcholinesterase, knockdown, resistance, vegetation, insecticide.

INTRODUCTION

Malaria cases continued to rise as the global tally of malaria cases reached 247 million in 2021 compared to 245 million in 2020 and 232 million in 2019 (WHO, 2022). There were an estimated 619 000 malaria deaths globally in 2021 compared to 625 000 in the first year of the pandemic (WHO, 2022). In 2019, before the pandemic struck, the number of deaths stood at 568 000 (WHO, 2022). Several studies have demonstrated the efficacy of both indoor residual spraying (IRS) and long lasting insecticidal nets (LLIN) in curbing malaria incidence (Lengeler, 2004; Pluess *et al.*, 2010) and the efficacy in the use of microbial larvicides on mosquito breeding sites (Ekerette & Ebere, 2017; 2018) as a source reduction strategy. Three categories of mechanism confer resistance to neurotoxic insecticides in malaria vectors: alterations to metabolic genes or pathways, target site mutation, and cuticular thickening. Metabolic resistance resulting primarily from the amplification or up-regulation of detoxification enzymes (especially esterases, P450 monooxygenases, and glutathione *S*- transferases) occurs commonly and can confer high levels of resistance

(Ibrahim *et al.*, 2016). In metabolic detoxification, the insecticide is prevented from reaching its site of action by detoxification enzymes (Hemingway & Ranson, 2000). Some enzymes have been linked to resistance to a specific insecticide or class (Chiu *et al.*, 2008; David *et al.*, 2018) whereas others confer resistance across insecticide classes (Hancock *et al.*, 2018). Decreased target site sensitivity on the other hand, reduces the rate at which the insecticide binds to its target site (Ranson *et al.*, 2000) or changes to insecticide target sites are also prevalent and frequently associated with phenotypic resistance (Hancock *et al.*, 2018). Acetylcholinesterase target site alteration caused by a single mutation to the ace-1 gene in which amino acid substitution of glycine to serine at position 119 in the ace-1 gene typically confers cross-resistance to organophosphate and carbamate insecticides in *An. gambiae s.s.* (Weill *et al.*, 2004; Essandoh *et al.*, 2013). Cross-resistance to pyrethroids and the organochlorine DDT arise from knockdown resistance (KDR) mutations in the Vgsc gene which encodes the para voltage-gated sodium channel target site, in which two amino acid changes, in the sodium channel gene at codon 1014 and are involved in kdr in *An. gambiae s.s.*: a leucine to phenylalanine substitution (1014F) and a leucine to serine substitution (1014S) (Omer *et al.*, 1980; Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000). There is currently less evidence for cuticular thickening as a mechanism of resistance (Hancock *et al.*, 2018).

Successful malaria control programmes depend on a good knowledge of the species (Ekerette *et al.*, 2023), abundance, distribution levels and dynamics of insecticide resistance in the local mosquito population, followed by continuous resistance monitoring in order to detect and monitor resistance to these insecticides. However, in most cases the implementation of vector control measures often precedes careful evaluation of the target mosquito population, putting the success of the control programme at risk. Constant monitoring of the susceptibility status of mosquito vectors is essential to forewarn existence of resistance (Ranson *et al.*, 2009). The three vegetation types in Rivers state create conducive environment for malaria vectors. No extensive spraying or major vector control programmes have been carried out previously in this area until 2013 when LLINs were distributed by the National Malaria and Vector Control Programme and IRS was carried out in some communities in the study area (Rivers State Government, 2021). Comprehensive knowledge of the factors underlying resistance is needed for the implementation of efficient vector control programmes including resistance management strategies. This raises the need for statewide and regular surveys for monitoring the insecticide susceptibility status of major vectors, detecting resistance genes and assessing their impact on vector control activities (Kelly-Hope *et al.*, 2008; Ekerette and Ebere, 2022a; 2022b).

MATERIALS AND METHODS

Study Area

Rivers state lies amid latitude 4‘45"N and longitude 6‘50"E and is for most part, low-lying pluvial state in southern Nigeria, located in the eastern part of the Niger Delta on the ocean ward extension of the Benue Channel. The inland part of the state consists of tropical rainforest, and towards the coast, the typical Niger Delta environment features, many mangrove swamps. Rivers state has a total land cover of 4,277 square miles. There was a recorded population of about 5,198,716 as of the 2006 census. The population increased to 7,303,900 with a density of 755.4/km^2 as of 2016 estimate. Population estimate in the three vegetation zones of Rivers state in 2016 were: lowland forest – 3,131,600, mangrove - 2,504,900, and fresh water swamp – 1,378,700 (RVSG, 2021). Neighbouring states include Imo, Abia and Anambra to the north, Akwa Ibom to the east and Bayelsa, Delta to the west. In the south, it is bounded by the Atlantic Ocean. Its landscape ranges from flat plains, with a network of rivers and tributaries. Mean temperatures are usually between 25 °C and 28 °C. Some parts of the state still obtain up to 150 mm (6 in) of rainfall during the dry season. Relative humidity hardly ever got below 60% and varies between 90% and 100% for most of the year (RVSG, 2021). The land surface of Rivers state is generally less than 20 m above sea level. As a lower Niger floodplain, it contains a greater silt and clay foundation and is more susceptible to

constant inundation by river floods. The floodplain's total thickness rises to about 45 m in the northeast and over 9 m in the beach ridge barrier zones to the southwest. On coastal sand ridges (lowland forest), the soils are mostly sandy or sandy loams. Various crops that grow well in the region include cocoyam, coconut, raffia palm and oil palm. The drier upland region of Rivers state covers 61% of landmass while the riverine areas, with a relief range of 2 m to 5 m, cover 39% (RVSG, 2021).

Two distinct climatic periods in the study area include a dry period from November to April and a rainy period which extends from May to October with a short break in August. Rivers state consist of metropolitan and rural dwellings as well as residential and industrialized areas spread across the three ecological regions. The major occupation in the metropolitan area is manufacturing and commercial industries while in the rural area, it is fishing and subsistence farming. The study area is also rich in crude oil mineral deposit, giving rise to extensive extraction and development activities cutting across the entire region. The major consideration in the division of the study area into three: lowland forest, freshwater swamp and mangrove vegetations, is that they represent different climatic/ecological regions, with different environmental conditions that may have varying effect on the distribution and the population genetics of the resistance mechanisms of *An. gambiae s.l.* There was ease of accessibility to the sample collection sites since it lies within areas of human settlement (Hamza *et al.,* 2014).

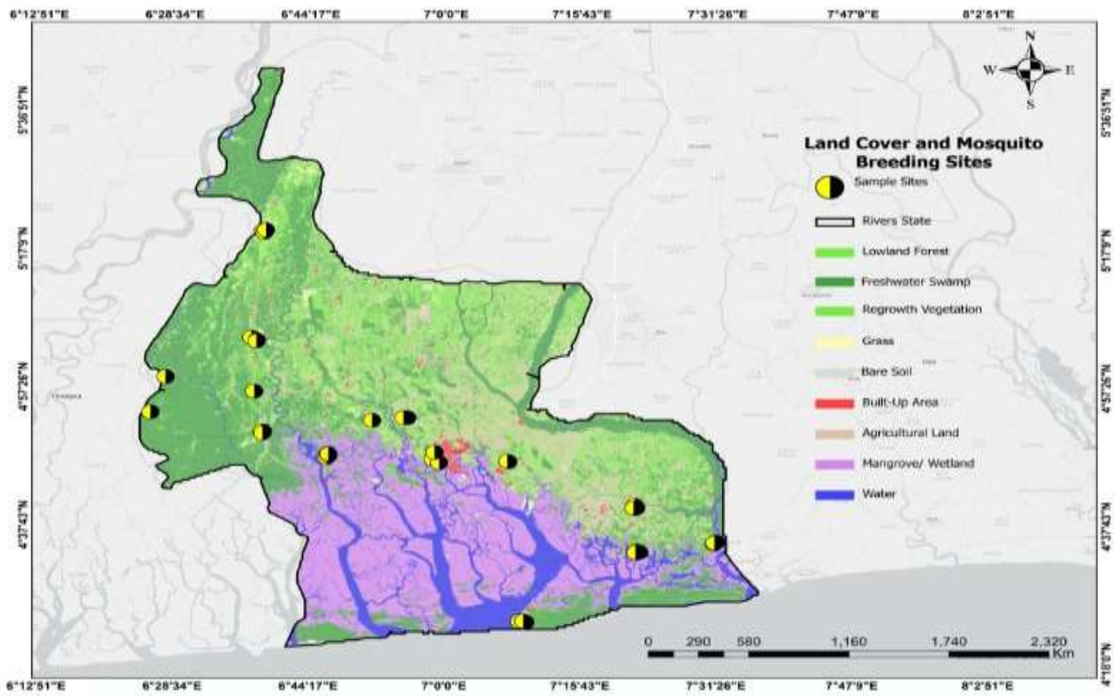

Figure 1. Map of Rivers State in the Niger Delta Region showing the Ecological Zones and Sampled Breeding Sites of *An. gambiae s.l.*

Mosquito Sampling

Many prospective sampled breeding sites of Anopheles mosquitoes included borrow pits, vehicle tyre imprint, footprints, temporary sunlit pools, ditches in construction sites, puddles formed alongside lakes, gutters, rain puddles, ponds and stagnant waters, among others, in the fresh water swamp, mangrove and lowland forest vegetations. The sampling was carried out from May 2019 to December 2019 and also from May 2020 to October 2020. This was to ensure that sample collection was done in the period of consistent rainfall, when there was abundance of larvae, Standard dipper (400 ml) with one (1 m) handle was used to collect larvae from different breeding sites (WHO, 2015). Figure 1 shows a number of breeding sites from which samples were collected.

Rearing of Mosquito Larvae

Larvae that were not from the same breeding sites in a particular ecological zone were assembled together, kept alive and held in loosely covered plastic cans after which they were conveyed to the insectary of Malaria Vector Surveillance and Insecticide Resistance Monitoring Laboratory of the Department of Animal and Environmental Biology, Rivers State University, Port Harcourt, Nigeria. The development of the mosquitoes for this study was carried out at the laboratory where the larvae were reared to adulthood following the procedures of Gerberg *et al.* (1994). In the laboratory, the *Anopheles* larvae and the breeding water from the sampling sites, which were conveyed in a plastic cans were gradually poured into a white tray. A 3 ml-rubber pipette was used to pick the larvae from the white tray into another plastic bowl. This technique was used to sort the *Anopheles* larvae into plastic bowls holding de-chlorinated water. The bowls were enclosed with nets fastened with elastic bands and kept on platforms which held water below as a trap, to prevent crawling insects such as ants, from climbing onto the platform to invade and eat up the larvae. The larvae were nourished with ground biscuits every two days and observed through to adult development and appearance. Recently developed adults were separated into females and males, by means of aspirator (length-60cm: half of it made of glass and the other half made of rubber, diameter-1cm), to pick them individually from the adult cage. Females possess non-plumose antennae with palp almost as long as proboscis while the males possess plumose antennae with palp almost as long as proboscis and distended at ends/tips. Adults were placed in screen cages and fed constantly on 10% glucose solution. Cages were held at 26°C - 29°C and 74% - 82% relative humidity.

Morphological Identification of *An. gambiae s.l.*

Individuals belonging to the *An. gambiae s.l.* group were set aside through their morphological distinct features from other anopheline mosquitoes using the morphological identification skills of Gillies and De-Mellion (1968) and Gillies and Coetzee (1987).

DNA Extraction from *An. gambiae s.l.* Mosquito Tissue

DNA was extracted from the tissues of *An. gambiae s.l.* samples aided by the use of Quick-DNA™ Tissue/Insect Miniprep Kit produced by Zymo Research, U.S.A., in line with the producer's guidelines. Resistant or susceptible *An. gambiae s.l.* mosquitoes, experimented with a well-known insecticide from a known ecological zone, was put into a 1.5 ml Eppendoff tube. 50 µl BashingBead™ Buffer was further added to the Eppendoff tube and crushed slowly with a crushing stick until it was totally pounded and crushed. 350 µl of BashingBead™ Buffer was additionally put into the tube to make-up the volume to 400 µl and the tube was vortexed, to allow for overall integration of the solution. The ZR BashingBead™ Lysis Tube (2.0 mm) was centrifuged in a micro centrifuge at 10,000 rpm for 60 seconds. A Zymo-Spin™ III-F Filter was positioned in a Collection Tube and the supernatant was placed onto it and centrifuged at 8,000 rpm for 60 seconds. The Collection Tube with its deposit was collected and the Zymo-Spin™ III-F Filter was thrown away. 1,200 µl of Genomic Lysis Buffer was put into the tube containing the deposit in the Collection Tube and vortexed for 10 seconds. The Zymo-Spin™ IICR Column 1 was put inside a Collection Tube and 800 µl of the mixture from the step above was moved into the Collection Tube and centrifuged at 10,000 rpm for 60 seconds. The remainder of the mixture in the Collection Tube was not thrown away but was put into use at a later time. The content which passed through the filtrate from the Collection Tube was thrown away and the same Zymo-Spin™ IICR Column1 was kept back in the Collection Tube. The remaining of the mixture up to that time in the step above was moved into the Collection Tube and centrifuged at 10,000 rpm for 60 seconds. The content which passed through the deposit from the Collection Tube was thrown away and the same Zymo-Spin™ IICR Column1 was positioned in a new Collection Tube. 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin™ IICR Column in the new Collection Tube and centrifuged at 10,000 rpm for 60 seconds. 500 µl g-DNA Wash Buffer was put into the Zymo-Spin IICR Column in the Collection Tube and centrifuged at 10,000 rpm for 60 seconds. The Zymo-Spin™ IICR Column was moved into a clean 1.5 ml Eppendoff tube and 100 µl DNA Elution Buffer was added. The Zymo-Spin™ IICR Column in the Eppendoff tube was centrifuged at 10,000 rpm for 30 seconds, so that the DNA could be deposited at the bottom

of the Eppendoff tube. The deposited solution of the Eppendoff tube containing the *An. gambiae s.l.* DNA was preserved at -20°C for further PCR analysis (Quick-DNA™, n.d.).

Table 1: Samples of DNA Extract of *An. gambiae s.l.* in each well of the Agarose Gel for PCR Analysis

Agarose gel well number	Samples of *An. gambiae s.l.* in the agarose gel wells		
	Lowland forest vegetation	**Fresh water swamp vegetation**	**Mangrove Vegetation**
1	Permethrin resistant	Lambda-cyhalothrin resistant	Lambda-cyhalothrin resistant
2	Deltamethrin resistant	DDT resistant	Permethrin resistant
3	Deltamethrin resistant	Primiphos-methyl resistant	DDT resistant
4	Lambda-cyhalothrin resistant	Deltamethrin resistant	Primiphos-methyl resistant
5	DDT resistant	Propoxur resistant	Deltamethrin resistant
6	DDT resistant	Permethrin resistant	Alpha-cypermethrin resistant
7	DDT resistant	Bendiocarb susceptible	Bendiocarb susceptible
8	Propoxur resistant		
9	Primiphos-methyl resistant		
10	Primiphos-methyl resistant		
11	Alpha-cypermethrin resistant		
12	Primiphos- methyl susceptible		

Knockdown Resistance (KDR) in *An. gambiae s.l.* by PCR

GoTaq flexi DNA polymerase kit (Promega) and Primers (Sigma) were used according to Martinez-Torres *et al.,* (1998). The two primer sequences used include: Agd1- 5' ATA GAT TCC CCG ACC ATG 3' (Forward Primer)
Agd2- 5' AGA CAA GGA TGA TGA ACC 3' (Reverse Primer)
Agd3- 5' AAT TTG CAT TAC TTA CGA CA 3' (Forward Primer)
Agd4- 5' CTG TAG TGA TAG GAA ATT TA 3' (Reverse Primer)

The lyophilized primers were rehydrated by nuclease-free water to 300 µM stock solution, from this stock aliquots 30 µM were used for the primers. Only DNase free reagents were used. DNA extracted samples, all PCR reagents and rehydrated primers were stored at -20°C. In the master mix cabinet, the PCR components mixed included buffers, primers, and enzymes according to the recommended procedure. The procedure was done on ice in a designated clean area. 5 X Green Go Taq Buffer (10 µl), 25 mM $MgCl_2$ (2.5 µl), 2.5 mM each dNTPs (2 µl), Agd1 – 30 µM (1 µl), Agd2 – 30 µM (1 µl), Agd3 – 30 µM (1.5 µl), Agd4 – 30 µM (1.5 µl), PCR water (25 µl), GoTaq flex DNA polymerase 5u / µl (0.5 µl), Volume / tube (45 µl), DNA template (5 µl), Total Reaction volume (50 µl).

Sufficient master mix was prepared according to the number of samples tested and two extra reactions to compensate for pipetting errors. The PCR master mix was prepared, thoroughly vortexed and briefly spun down (5-10 seconds) before a known fraction of appropriate amount was put per well of the PCR strip. The strip was tapped gently on the bench to settle the content. It was then placed in the standard Thermocycler that had been pre-programmed with the appropriate cycling parameters as follows: Hold (95°C for 5 minutes), 35 cycles (94°C for 30 seconds; 46°C for 30 seconds; 72°C for 30 seconds), Hold (72°C for 7minutes). The PCR products were verified by running them on a 2% agarose gel at 120 Volts for at least 1 hour 30 minutes. Agarose gel electrophoresis was used for size separation of the PCR product by comparison with a DNA ladder (a molecular weight marker). The DNA molecules had a net negative charge because of their phosphate backbone, so they migrated toward the positive electrode. The shorter the DNA molecules, the faster they can migrate through the gel, while the longer the DNA molecules, the slower they migrate because they are more impeded by the gel. Gel was stained by gel red for 10 minutes, then DNA bands were visualized by ultraviolet trans-illuminator. DNA bands in

positive sample indicated successful amplification of the target sequence. DNA samples of *An. gambiae s.l.* that were resistant and susceptible to various insecticides, were used to determine the presence or absence of KDR genes in the *An. gambiae s.l.* and its pattern across the different insecticides tested in the three vegetations. DNA extract from each of the set of mosquito samples tested with a particular insecticide, were put into each well of the agarose gel. From Table 1, in the lowland forest vegetation, only sample 5 was used, samples 6 and 7 were not used, to avoid excessive repetition of DDT.

Acetylcholinesterase Resistance (Ace-1R) in *An. gambiae s.l.* by PCR

Primers were used according to Fanello *et al.* (2002). The lyophilized primers were rehydrated by nuclease-free water to 300 µM stock solution, from this stock aliquots 30 µM were used for the primers. Only DNase free reagents were used. DNA extracted samples, all PCR reagents and rehydrated primers were stored at -20^{o}C. In the master mix cabinet, the PCR components mixed included buffers, primers, and enzymes according to the recommended procedure. The procedure was done on ice in a designated clean area. One TaqMan 2X Master Mix (14 µl), EX2AGDIR 1 (Forward primer) (2 µl), EX4AGREV 2 (Reverse primer) (2 µl), Nuclease free water (9 µl), DNA template (3 µl), Total PCR Reaction volume (30 µl). Primer sequence used: EX2AGDIR 1- AGG TCA CGG TGA GTC CGT ACG A; EX4AGREV 2 – AGG GCG GAC AGC AGA TGC AGC GA.

The amplification protocol was adopted and modified from Fanello *et al.* (2002). Sufficient master mix was prepared according to the number of samples tested and two extra reactions to compensate for pipetting errors. The PCR master mix was prepared, thoroughly vortexed and briefly spun down (5-10 seconds) before it was aliquot appropriate amount per well of the PCR strip. The strip was tapped gently on the bench to settle the content. It was then placed in the standard Thermocycler that had been pre-programmed with the appropriate cycling parameters as follows: Hold (95^{o}C for 90 seconds), 35 cycles (94^{o}C for 30 seconds; 68^{o}C for 30 seconds; 72^{o}C for 60 seconds), Hold: Final extension (72^{o}C for 5 minutes). The PCR products were verified by running them on a 2% agarose gel at 120 Volts for a period of 1 hour 30 minutes.

Agarose gel electrophoresis was used for size separation of the PCR product by comparison with a DNA ladder (a molecular weight marker). The DNA molecules had a net negative charge because of their phosphate backbone, so they migrated toward the positive electrode. The shorter the DNA molecules, the faster they can migrate through the gel, while the longer the molecules, the slower they migrate because they are more impeded by the gel. Gel was stained by gel red for 10 minutes, then DNA bands were visualized by ultraviolet (U.V.) trans-illuminator. DNA bands in positive sample indicated successful amplification of the target sequence. Samples of *An. gambiae s.l.* that were resistant and susceptible to various insecticides, were used to determine the presence or absence of ace-1r genes in the *An. gambiae s.l.* and its pattern across the different insecticides tested in the three vegetations. DNA extract from each of the set of mosquito samples tested with a particular insecticide, were put into each well of the agarose gel. Expected Band size - 924bp.

Sequencing of the PCR Amplified Products and Phylogenetic Tree

PCR products were purified prior to sequencing. The evolutionary distances were computed using the Kimura 2-parameter method (Tamura *et al.*, 2004). Evolutionary analysis was carried out on purified products, using MEGA X (Kumar *et al.*, 2018) and evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987).

RESULTS

Determination of Knock Down Resistance (KDR) Pattern of *An. gambiae s.l.* in Lowland Forest Vegetation

The electrophoretic patterns of the PCR products in Figure 2 revealed that for *An. gambiae s.l.* in the lowland forest vegetation, samples 2 and 4 were weakly positive for the presence of KDR genes while samples 1, 3, 5-10 were negative for the presence of the KDR genes.

The mangrove and fresh water swamp vegetations were completely negative for the presence of KDR. The lane L is the DNA molecular weight ladder (molecular marker).

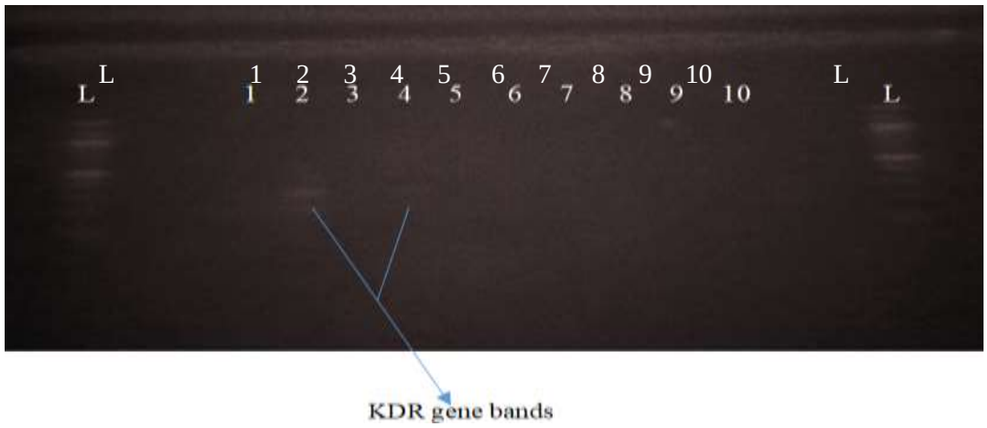

Figure 2. Determination of KDR Pattern of *Anopheles gambiae s.l.* in Lowland Forest Vegetation revealed faint band on sample 2 and fainter band on sample 4.

Determination of Ace-1 Pattern of *Anopheles gambiae s.l.* in Lowland Forest Vegetation

The electrophoretic patterns of the PCR products revealed in Figure 3 that for *An. gambiae s.l.* in the lowland forest vegetation, samples 1-12 were positive for the presence of ace-1r genes at 924 bp while sample 11 was weakly positive for the presence of ace-1r genes. The lane L is the DNA molecular weight ladder (molecular marker).

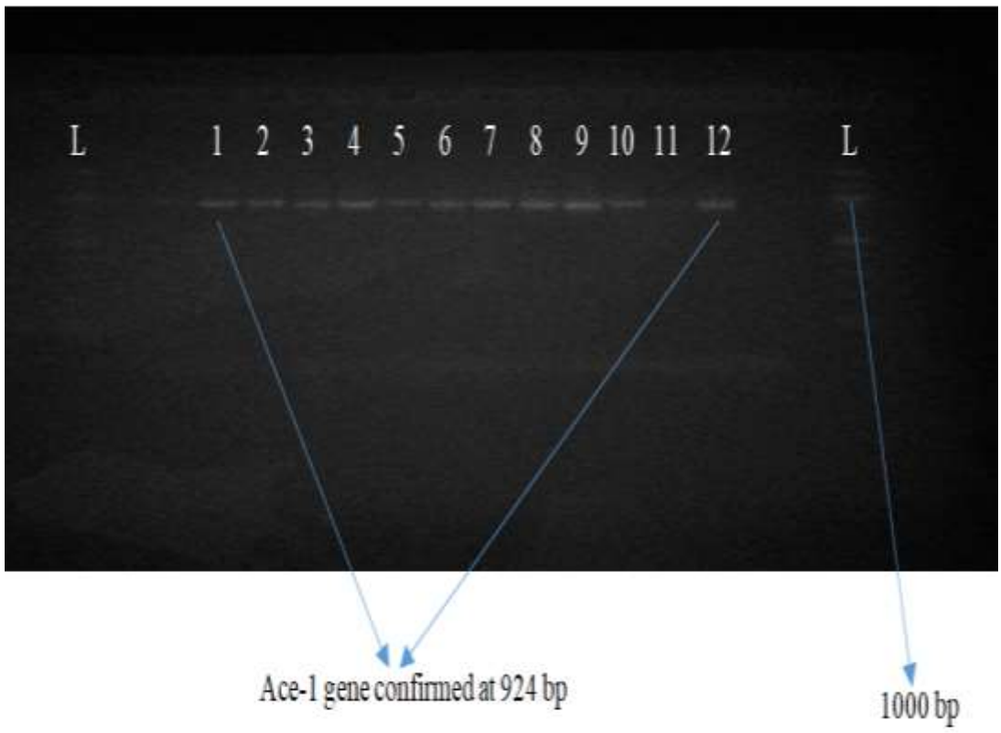

Figure 3. Determination of Ace-1 Pattern of *Anopheles gambiae s.l.* in Lowland Forest Vegetation

Determination of Ace-1 Pattern of *Anopheles gambiae s.l.* in Mangrove Vegetation

The electrophoretic patterns of the PCR products revealed in Figure 4 that samples 1-3 and 6 were positive for the presence of ace-1r genes in *An. gambiae s.l.* while sample 4 and 5 were weakly positive and very weakly positive respectively and sample 7 was completely negative to the presence of ace-1r genes at 924 bp, in the mangrove vegetation. The lane L is the DNA molecular weight ladder (molecular marker).

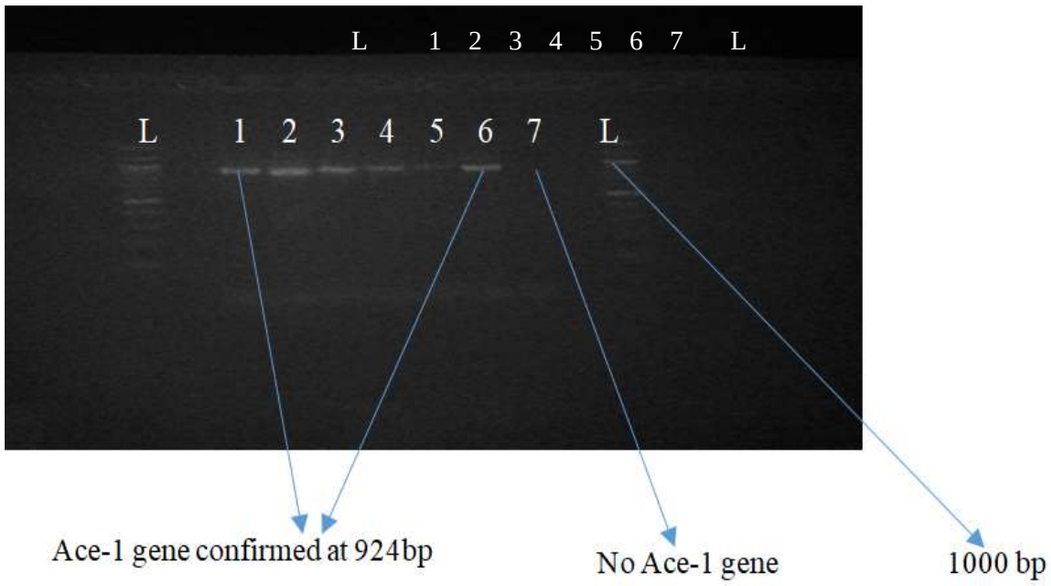

Figure 4. Determination of Ace-1 Pattern of *Anopheles gambiae s.l.* in Mangrove Vegetation

Determination of Ace-1 Pattern of *Anopheles gambiae s.l.* in Fresh Water Swamp Vegetation

The electrophoretic patterns of the PCR products revealed in Figure 5 that samples 1- 7 were positive for presence of ace-1r genes in *An. gambiae s.l.* at 924 bp though sample 1 was extremely weakly positive to the presence of ace-1r genes, in the fresh water swamp vegetation. The lane L is the DNA molecular weight ladder (molecular marker).

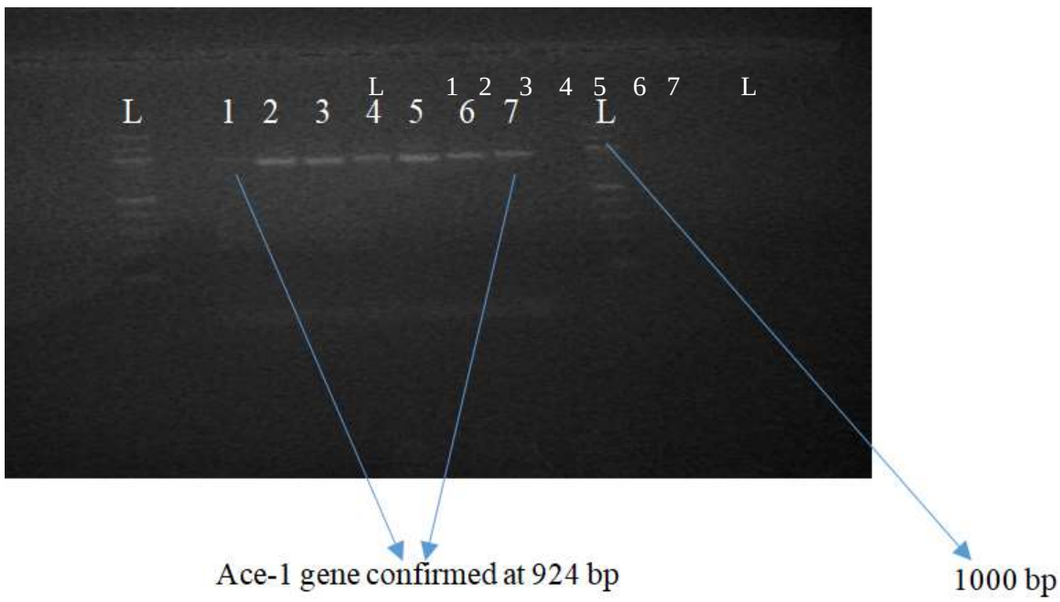

Figure 5. Determination of Ace-1 Pattern of *Anopheles gambiae s.l.* in Fresh Water Swamp Vegetation

Phylogenetic and Sequenced Analysis

The phylogenetic tree was constructed from the *An. gambiae s.l.* genes amplified in this study, from fresh water swamp, lowland forest and mangrove vegetations. The closest evolutionary relative of the sequenced gene on a phylogenetic tree was formed on one branch (Figure 6). The sequenced of the *An. gambiae* genome from this study is closely related to *An. gambiae* (Ace-1) gene at 99.57% identity to accession number KP165373.1, with a max score of 1769 on 100% query coverage and E-value of 0.0.

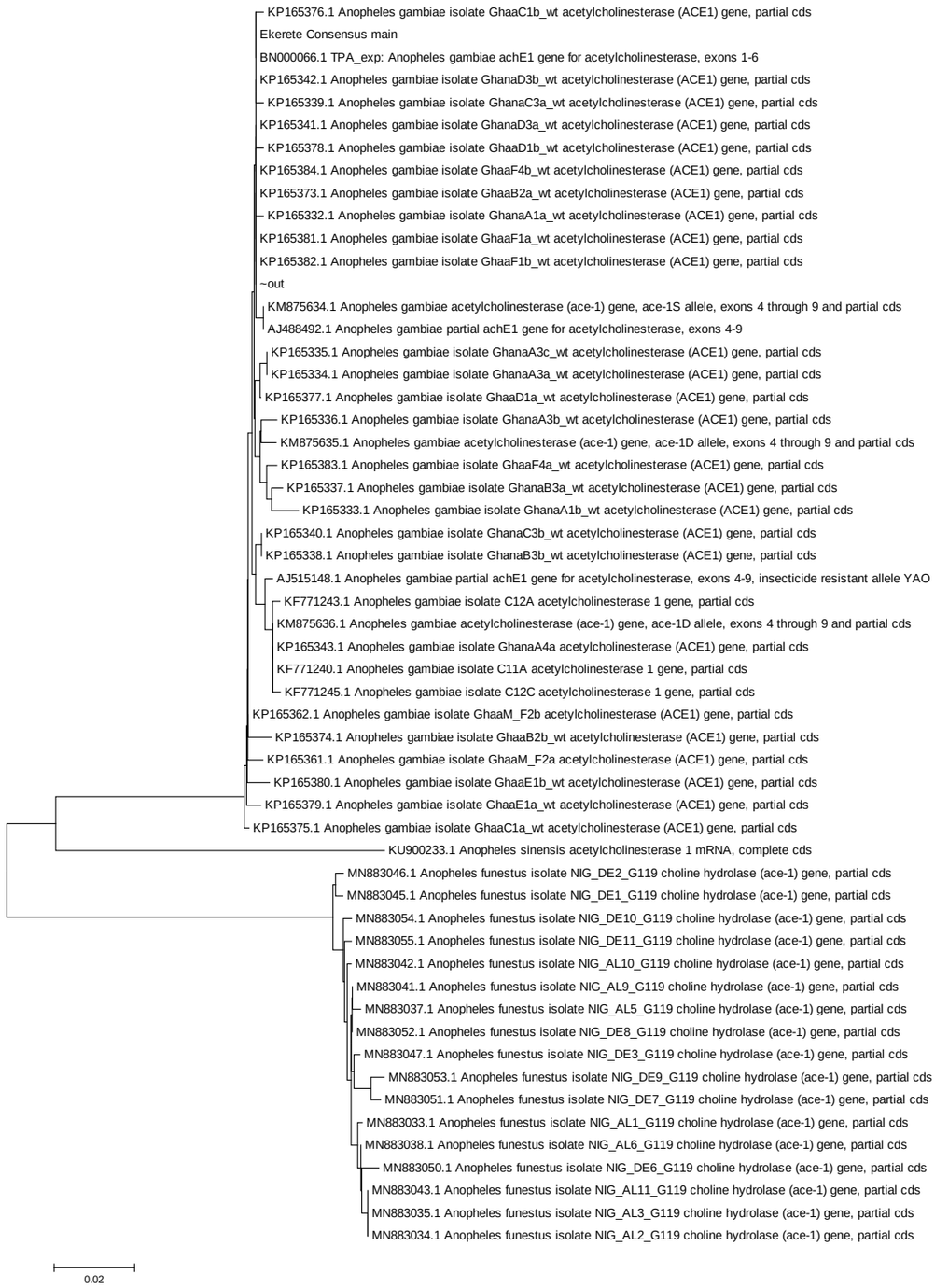

Figure 6. Phylogenetic Analysis of the Ace-1 Resistance in *Anopheles gambiae s.l.* Samples from Fresh Water Swamp, Lowland Forest and Mangrove Vegetations

Sequenced Genome for the Ace-1 Resistance in *Anopheles gambiae s.l.* Samples from Fresh Water Swamp, Lowland Forest and Mangrove Vegetations

CATATATAGGTCACGGTGAGTCCGTACGAATTATAGATGCCGAGTTGGGCAC
GCTCGAGCATGTCCACAGTGGAGCAACGCCGCGGCGACGCGGCCTGACGAG
GCGCGAGTCAAACTCGGGTAAGTACGCGATTGGAAGTGGGGGGACGTTTACC

CTACCGTGTACTACAACGCACTTTACCCCCACGCACACGCACCGGCAGACGC
GAACGACAACGATCCGCTGGTGGTCAACACGGATAAGGGGCGCATCCGCGG
CATTACGGTCGATGCGCCCAGCGGCAAGAAGGTGGACGTGTGGCTCGGCATT
CCCTACGCCCAGCCGCCGGTCGGGCCGCTACGGTTCCGTCATCCGCGGCCGG
CCGAAAAGTGGACCGGCGTGCTGAACACGACCACACCGCCCAACAGCTGCGT
GCAGATCGTGGACACCGTGTTCGGCGACTTCCCGGGCGCGACCATGTGGAAC
CCGAACACGCCCCTGTCCGAGGACTGTCTGTACATTAACGTGGTGGCACCGC
GACCCCGGCCCAAGAATGCGGCCGTCATGCTGTGGATCTTCGGCGGCGGCTT
CTACTCCGGCACCGCCACCCTGGACGTGTACGACCACCGGGCGCTTGCGTCG
GAGGAGAACGTGATCGTGGTGTCGCTGCAGTACCGCGTGGCCAGTCTGGGCT
TCCTGTTTCTCGGCACCCCGGAAGCGCCGGGCAATGCGGGACTGTTCGATCA
GAACCTTGCGCTACGGTAGGTGTCTTTGCATGTGTGAATGAGGGTATAGTATT
CTAACGAGGTGCTCTTCTTCCCATCACTTCTTGGGAGTCAGCTGGGTGCGGGA
CAACATTCACCGGTTCGGTGGCGATCCGTCGCGTGTGACACTGGTCGGCGAG
AGTGCCGGCGCCGTCTCGGTGTCGCTGCATCTGCTGTCCGCCCTTTCCCG

DISCUSSION

The knockdown resistance (KDR) result in lowland forest vegetation, revealed the presence of the Leu – Phe (L1014F) KDR point mutations. The homozygous resistant genotype (RR) was found in two out of the twelve samples (sample 2 and sample 4 of the insecticide-treated samples of *An. gambiae s.l.*) in the lowland forest vegetation. This agrees with the findings in Ibadan, northern Nigeria, Kilokope in Togo, Mali, Cote d'Ivoire, southeast Benin Republic, Burkina Faso, southwest and northern Cameroon (Awolola, 2007; Namountougou *et al.,* 2012; Cisse *et al.,* 2015; Etang *et al.,* 2016; Yahouedo *et al.,* 2016; Abdu *et al.,* 2017; Camara *et al.,* 2018; Ketoh *et al.,* 2018). The KDR gene band on sample 2 was very faint and on sample 4, it was fainter. This might be due to the low amount (quantity) of the KDR gene present in that particular *An. gambiae s.l.* DNA sample. Samples 1, 3, 5-10 were negative for the presence of KDR gene in the *An. gambiae s.l.* as they did not show the gene band on the agarose gel electrophoresis. There was no gene band at all for KDR in mangrove and fresh water swamp vegetations. They were completely negative to the presence of KDR gene in the *An. gambiae s.l.* population, unlike in the lowland forest vegetation that had some traces of the gene.

The acetylcolinesterase resistance (ace-1r) result in lowland forest vegetation revealed the presence of the ace-1r gene in all the various-insecticide-treated samples of *An. gambiae s.l.* There was a gene band on sample 11, though it was faint. This might have been due to the low amount (quantity) of the gene present in that particular sample. In sample 12, all the *An. gambiae s.l.* were susceptible to primiphos-methyl insecticide and it was added/placed as a control/check sample during the PCR analysis, to guard against only insecticide-resistant samples being used for the bioassay. This sample 12 which was completely susceptible to primiphos-methyl insecticide also emerged positive to ace-1r gene. This may imply that, the gene for the ace-1r was already present in the genome of the *An. gambiae s.l.* but was yet to be expressed phenotypically. This could make clear that phenotypic bioassay (susceptibility test) for insect resistance to insecticides is not conclusive in a population of insects (*An. gambiae s.l.*) since there may exist in the insect population genotypic-resistant and phenotypic-susceptible insects (*An. gambiae s.l.*). Boussougou-Sambe *et al.* (2018) had given a similar report in southwest Cameroon, in which the frequency of the KDR L1014F was higher in resistant mosquitoes (85.38%) than it was found in susceptible mosquitoes (80.26%) and that even the difference was not statistically significant ($p = 0.5622$). This is very vital in control planning for vectors of malaria, so as not to have a false positive result of susceptibility in a population, only to be unprepared against a sudden breakout of resistance in a near future.

The presence of KDR gene was positive in sample 2 and sample 4, in the lowland forest vegetation, also sample 2 and sample 4 of the same lowland forest vegetation were found to be positive for the presence of ace-1r gene. The above comparison may clearly show

that, some population of *An. gambiae s.l.* were positive for the presence of both KDR and ace-1r genes, at the same time. This is similar to the report of Dabire *et al.* (2008), in which multiple resistance in *An. gambiae s.l.* was found in moist savanna of western Burkina Faso, with the concomitant presence of KDR L1014F and ace-1 G119S mutations. It also agrees with the work of Cisse *et al.* (2015) in Mali, in which some mosquitoes were found carrying both resistant alleles, ace-1r and KDR-w, simultaneously.

The KDR gene bands in sample 2 and sample 4 of the lowland forest vegetation were faint while the ace-1r bands in sample 2 and sample 4 of the same lowland forest vegetation were clear and bold. The amount of DNA extract added in the master mix in both bioassay was 5µl. Therefore, the difference in the faintness and the boldness of the gene bands could have come from the amount/quantity of KDR or ace-1r genes present in the DNA extract of a particular sample of *An. gambiae s.l.* KDR gene was already low in the *An. gambiae s.l.* population in Rivers state, acknowledging the fact that it was not even found in the mangrove and fresh water swamp vegetations. Even in the lowland forest vegetation, it was found in just two out of ten samples in which the bioassay was conducted. This is an indication of how small the amount in the sample 2 and sample 4 would be and consequently, the level of faintness in the agarose gel electrophoresis. While the opposite applied in the ace-1r gene, which was already high in the *An. gambiae s.l.* population in Rivers state, acknowledging the fact that it was found in six out of seven samples in the PCR result of the mangrove vegetation and also in all the seven samples of the fresh water swamp vegetation. Even in the lowland forest vegetation, it was found in all the twelve samples of the PCR result. This could be an indicator to how abundant the amount of ace-1r gene would be present in sample 2 and sample 4 and consequently, immensely affect positively, the level of clarity and boldness the appearance of ace-1r gene band would show in the PCR result. The boldness or faintness of a gene band in a PCR analysis might be directly proportional to the amount/quantity of gene present in the DNA extract used in the analysis.

The ace-1r result in the mangrove vegetation, revealed the presence of the ace-1r gene in all the various-insecticide-treated samples of *An. gambiae s.l.* except in sample 7. Sample 3 gene band was faint; sample 4 gene band was fainter while sample 5 gene band was the faintest in the agarose gel electrophoresis result for mangrove vegetation. This is similar to sample 11 of the lowland forest vegetation and might be attributed to the amount/quantity of the resistant gene present in the affected samples. There was no gene band in sample 7 of mangrove vegetation. The *An. gambiae s.l.* of sample 7 were completely susceptible to the insecticide, bendiocarb, and it was added/placed as a control/check sample during the PCR analysis, to guard against only insecticide-resistant samples being used for the bioassay, thereby removing any form of bias that could have existed.

Comparing and contrasting sample 12 of the lowland forest vegetation and sample 7 of the mangrove vegetation, shows comparatively that both samples were completely susceptible to different insecticides (primiphos-methyl for sample 12 of lowland forest vegetation and bendiocarb for sample 7 of mangrove vegetation) with which they were treated, respectively. On the other hand, the PCR result contrastingly revealed that primiphos-methyl-susceptible sample 12 of lowland forest vegetation may have possessed recessive ace-1r genes while bendiocarb-susceptible sample 7 of mangrove vegetation, showed no sign, even of the faintest ace-1r gene band, it was negative to ace-1r gene even when compared to sample 5 of mangrove vegetation. This comparative and contrasting points might go on to confirm the fact earlier stated, that phenotypically-susceptible *An. gambiae s.l.* were at the same time found to possess the genes for ace-1r.

The ace-1r result of the fresh water swamp vegetation, shows the presence of ace-1r gene in all the various-insecticide-treated samples of *An. gambiae s.l.* Sample 1 gene band was very faint. This is similar to sample 11 of lowland forest vegetation and sample 5 of mangrove vegetation, and as stated earlier, might be attributed to low amount/quantity of the resistant gene present in the affected sample. The *An. gambiae s.l.* of sample 7 were completely susceptible to the insecticide, bendiocarb, and it was added/placed as a

control/check sample during the PCR analysis, to guard against only insecticide-resistant samples being used for the bioassay, thereby eliminating any form of bias that could have existed. Sample 7 which was completely susceptible to the insecticide, bendiocarb, in the fresh water swamp vegetation, was revealed to be positive to the presence of ace-1r gene through PCR analysis. Sample 7 gene band was clearly bold and not faint in any way as would be compared to sample 1 which was resistant to the insecticide, lambda-cyhalothrin. Again it is observed here, that though sample 1 was resistant to lambda-cyhalothrin, its gene band was very faint and sample 7, though was completely susceptible to bendiocarb, the gene band was clearly bold and not faint to any observable extent, when compared to sample 2-6. This could be stated again that the faintness or boldness of gene bands is directly proportional to the amount/quantity of the gene present in the sample at the time the PCR analysis is carried out.

Comparing and contrasting sample 7 of the mangrove vegetation and sample 7 of the fresh water swamp vegetation, shows comparatively that both samples were completely susceptible to the same insecticide, bendiocarb but in different vegetations (mangrove and fresh water swamp), respectively. On the other hand, the PCR result contrastingly revealed that bendiocarb-completely-susceptible sample 7 of mangrove vegetation was negative to ace-1r gene whereas the same bendiocarb-completely-susceptible sample 7 of fresh water swamp vegetation was clear and boldly positive to ace-1r genes. This might confirm earlier stated fact that resistant genes can be recessive in some insects (*An. gambiae s.l.*) within a population, at the time of testing for resistance, while awaiting a time in the future in which the gene will become dominant and be expressed phenotypically.

Comparing and contrasting sample 12 of the lowland forest vegetation and sample 7 of the fresh water swamp vegetation, shows contrastingly that both samples were completely susceptible to different insecticides (primiphos-methyl for sample 12 of lowland forest vegetation and bendiocarb for sample 7 of fresh water swamp vegetation) with which they were both treated, respectively. On the other hand, the PCR result comparatively shows that both (primiphos-methyl-susceptible sample 12 of lowland forest vegetation and bendiocarb-susceptible sample 7 of fresh water swamp vegetation), were positive for the presence of ace-1r genes. From the repeated occurrences in this present study, it shows that *An. gambiae s.l.* that were completely susceptible to different insecticides from different classes, were found to possess ace-1r gene in their genome.

The significance of these resistance genes being studied, is geared toward having successful malaria control programmes which depend on a good knowledge these resistance genes, their mechanisms and sites of actions. This would afford the researcher, the opportunity of working to alter or block such target sites that are responsible for the observed resistance, as well as to stop metabolic reactions responsible for other types of resistances (Ekerette and Ebere, 2022a; 2022b). Overall, this will cause the malaria vectors to lose their resistance status and become susceptible once again, to the available insecticides, thereby achieving the global malaria elimination goal.

RECOMMENDATIONS

Genotypic-based bioassay for example using PCR should be conducted on the susceptible insects (*An. gambiae s.l.*) after the phenotypic-based bioassay (susceptibility test). This is very necessary in control planning for vectors of malaria, so as not to have a false positive result of susceptibility in a population, only to be unprepared for a sudden breakout of resistance in a near future. There should be enough amount/quantity of DNA extract used, during gene analysis which require samples to be run on agarose gel electrophoresis, so as to produce clear/bold bands on the agarose gel rather than faint bands.

CONCLUSION

Knockdown resistance (KDR) gene was found scantily only in the lowland forest vegetation and was completely absent in both the mangrove and fresh water swamp

vegetations, respectively. Acetylcholinesterase resistance (ace-1r) gene was abundantly found in the mangrove, lowland and fresh water swamp vegetations. Some *An. gambiae s.l.* that were completely susceptible during the susceptibility bioassay, were found to have ace-1r genes when they were analysed along with the insecticide resistant *An. gambiae s.l.* using PCR. The faintness or clarity (boldness) of gene bands on agarose gel electrophoresis may be directly proportional to the amount/quantity of target gene present in the DNA sample used for the analysis. Some population of *An. gambiae s.l.* were positive for the presence of both kdr and ace-1r genes, simultaneously.

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