First Incidence of CYP9K1, CYP6P4 and CYP6Z1 in Anopheles gambiae ss from Nigeria

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Abstract

Background: Anopheles resistance to insecticides is an impediment to the success of malaria control programmes in sub-Saharan Africa. This study addresses the enzymes that play the key role in insecticide resistance in Kogi State, Nigeria.

Methods: Anopheles immature stages were collected from breeding sites across four LGAs in Kogi State, Nigeria. Adult anophelines were identified morphologically and molecularly to species level using the polymerase chain reaction. Adult Anopheles mosquitoes were exposed to eight different insecticides belonging to organochlorine, organophosphate, carbamate and pyrethroids. Newly developed and integrable to the LabDisk automated diagnostic platform quantitative Reverse Transcription-real-time PCR (qRT-PCR) 3-plex TaqMan® assays were used to quantify 7 detoxification genes’ expression. Fold-changes, 95% CIs and statistical significance were performed according to the Pfaffl methods.

Results: An. gambiae sl were susceptible to pirimiphos methyl in all the LGAs, but resistance to alphacypermethrin, permethrin, DDT, bendiocarb, propoxur, lambdacyhalothrin and deltamethrin. The detoxification genes CYP6P3(1.6), CYP6M2(36 folds), CYP9K1(6.4 folds), CYP6P4(5.3), CYP6Z1(4.8 folds), GSTE2(4.1 fold), CYP6P1(0.50 folds) and CYP4G16 (2.36 folds) were encountered but only CYP6M2, CYP9K1, CYP6P4 and CYP6Z1 were found to be up regulated in the Dekina multi resistant (pyrethroids, DDT, bendiocarb) population compared to two susceptible (Kisumu and G3) laboratory strains. Resistance was observed in seven out of the eight insecticides tested. Very high level of resistance was observed in DDT across the three LGAs. The CYP6M2, CYP9K1, CYP6P4 and CYP6Z1 were up regulated genes that confer resistance to different insecticides.

Conclusion: This study recorded for the first time CYP9K1, CYP6P4 and CYP6Z1 detoxification genes in wild Anopheles gambiae ss populations from Nigeria. In light of the absence of iAche mutations in our results, carbamate (bendiocarb and propoxur) resistance could be associated with the expression of CYP6P4 that has been previously implicated.

Background

Diseases such as malaria, filariasis and dengue transmitted by mosquitoes constitute a threat to
public health and pose major burden to the economy and development across the globe [1]. An estimated 3.3 billion people were at risk of malaria in 2010 with up to 210 million cases and 655,000 deaths recorded mostly in Africa [1]. Malaria contributes to an estimated loss of £7.13 billion Gross Domestic Product (GDP) annually in Africa [2]. World Global Strategy for Malaria Control is targeted at vector control whose objective is to break transmission of malaria parasites through Indoor Residual Spraying (IRS) or Long Lasting Insecticide Nets (LLINs) using pyrethroid [3]. Mosquito vector control accounts for more than half of malaria control costs worldwide. This method is reported to be the most effective against malaria spread [4]. Records from the past decade have shown the major role played by LLINs and IRS in the decrease of malaria incidences [5]. Among the different classes of insecticides only pyrethroids was approved by WHO for malaria control programmes. This is attributed to its safe use, effectiveness, high residual properties and its low mammalian toxicity [6, 7]. Pyrethroid insecticides are the most widely used for indoor control of mosquitoes across the world [8]. The use of ITNs has increased over the years as an estimate of 42% of household own at least one ITN across 44 countries of the sub-Saharan Africa [9]. In order to meet the target of universal access of LLINs, it has been recommended that at least one LLIN be distributed for every two persons at risk of malaria [10]. One of the major limitations of the fight against malaria vectors is the limited arsenal as all the insecticides recommended for public health fall into just four classes (organochlorines, organophosphates, carbamates and pyrethroids). All these four classes are nerve poison and either target acetylcholinesterase in the synapses or the voltage-gated sodium channel of the insect neurons [11]. The arsenal for insecticide impregnated material such as LLINs is even further limited to only one class-the pyrethroids [12].

The spread of resistance to the four classes of insecticides, especially to pyrethroid has been attributed to the failure of insecticide treated nets and indoor residual spraying across malaria endemic regions. Pyrethroid efficacy is being threatened by the spread of resistance in target populations. This phenomenon is spreading at a rapid rate [13, 14]. The use of IRS and LLINs in vector control is experiencing a major setback due to insecticides resistance [11]. Sub-Saharan Africa Malaria Vector Control Programmes mostly depend on IRS or Insecticides Treated Nets (ITNs) which
largely rely on the susceptibility status of the vectors to the different classes of insecticides especially pyrethroids [15, 16].

Over the past decade, the main factor driving the emergence and spread of insecticide resistance has been linked to the reliance on a single class of insecticide: the pyrethroids [17]. Pyrethroid has been extensively used in LLINs. The extensive use of agricultural insecticides which also fall into the same class as those of public health have also been attributed to the emergence of resistance [10]. Anopheles gambiae resistance to pyrethroid and DDT is linked to the substitution at a single codon in the sodium channel gene, referred to as knock-down resistance (kdr) gene. There are two common types which include leucine to phenylalanine substitution, referred to as West kdr [18] and a leucine to serine substitution, known as the East kdr [19]. Other forms of resistance have also been observed in Anopheles gambiae across the sub-Saharan Africa [18–20]. Resistant insect vectors have shown one form of resistance mechanism or the other. These include cuticle insensitivity, behavioural, metabolic and altered target-site resistance [21].

Metabolic resistance is commonly associated with elevated activity of one or more members of the three large multigene enzyme families, glutathione S-transferase (GSTs), cytochrome P450 monooxygenase (P450s) and carboxylesterases (COEs) [22]. Metabolic detoxification has been reported to be the most common form of insecticide resistance [8]. Metabolic enzymes in insects are a self defence mechanism against xenobiotics. It is therefore not imperative that metabolic detoxification may be a common mechanism of resistance to insecticide [23]. Several reports of pyrethroid resistance have been reported throughout Africa [18–20]. Elevated levels of cytochrome P450 monooxygenase and esterase activities have been reported in pyrethroid-resistance mosquitoes [8]. The resistance of An. albimanus to permethrin in Guatemala was reported to be associated to elevated levels of P450 monooxygenase and esterase. Increased level of P450 monooxygenase activities has been reported in An. gambiae permethrin resistance population [24]. Higher elevated level of CYP6Z1 has been reported in An. gambiae pyrethroid resistance female [25]. Cytochrome P450 are one of the largest and most diversified classes of enzymes found in nature [26]. CYP6 family has been reported in insecticide resistance more than any other CYP family. CYP4 and
CYP9 families have also been reported in insecticides detoxification roles. Different genes in the cytochrome P450 have been reported in An. gambiae across Africa. In Tanzania and Zanzibar, CYP314A1, CYP12F1 and CYP6Z1 were reported to be involved in the metabolism of DDT in An. gambiae [7]. This phenomenon was also reported in western Kenya [7, 27–29].

In Nigeria and Kenya, CYP325A3 have been reported to metabolize permethrin in An. gambiae [29], while in Ghana, CYP6Z3 was reported in permethrin resistance in An. Gambiae [27]. Investigations from Yaoundé Cameroun also showed the role played by CYP6M2, CYP6P3 and CYP6Z3 in the detoxification of DDT or pyrethroid in An. gambiae [29]. High elevations of CYP6M2 and CYP6P3 in An. gambiae have also been implicated in bendiocarb resistance from Cote d’Ivoire [7, 28]. CYP6M2 gene was reported to be highly expressed in DDT-resistant population of An. gambiae from Ghana [7].

There is no evidence of any study carried out on insecticide resistance on anopheline mosquitoes in Kogi East in Kogi State of Nigeria. The study looked into some mechanisms used by An. gambiae in countering the potency of insecticides in the study area.

Methods

Study area

The study was carried out in three Local Government Areas (LGAs) (Dekina, Ibaji and Omala LGAs) in Kogi East, Kogi State, Nigeria. Kogi East is located in the north-central geopolitical zone of Nigeria which lies in the guinea savannah. The state has two distinct weather; dry season, which lasts from November to March and wet season extends from April to October and annual rainfall ranges from 1016 to 1524 mm. Kogi State has an annual temperature range of 22.8 °C and 33.2 °C. Lokoja, the state capital is moderately hot throughout the year. The vegetation of the state consists of mixed leguminous (guinea) woodland to savannah forest. Kogi State has wide expanse of fadama in the river basin and long stretches of tropical savannah forest in the Western and Southern belt of the state [30]. The state has a population of projection of more 4,473,500 in 2016 [31] and aside civil service, agriculture is the major occupation of the inhabitants.

Mosquito collection and rearing

Eggs, larvae and pupae of An. gambiae were collected during the wet season from April to October
2017. The immature were collected from 228 natural breeding sites (tyre tracks, rice fields, puddles etc) across the three LGAs using soup ladle, dippers and pipette. These larvae and pupae were transported in containers containing the waters from where they were collected to the insectary of the Department of Biological Sciences, Kogi State University, Ayingba, Kogi State, Nigeria. They were reared to adults under standard protocols as described in Nkya et al. [32]. The larvae were fed daily with a mixture of finely ground fish food and brewer’s yeast, while pupae were transferred into pupae cups and placed in cages. Emerging adults were maintained on 5% sugar solution until they were used for insecticide susceptibility test. Kisumu strain of An. gambiae collected from National Arbovirus and Vector Research Centre, Enugu, Nigeria was used as the reference strain.

**Insecticide susceptibility test**

Adult susceptibility tube bioassays were carried out with eight insecticides representative of all four classes of insecticides available for use in public health. This was done using WHO established methods [33]. WHO insecticide treated filter papers with diagnostic doses recommended by WHO were used [33]. The insecticides included 4% DDT (organochlorine), alphacypermethrin 0.5%, permethrin 0.5%, lambdacyhalothrin 0.05%, deltamethrin 0.05%, (pyrethroid), bendiocarb 0.1% (carbamate) and pirimiphos-methyl 0.25% (organophosphate). Tube bioassay was conducted using 3 to 5 days old non blood fed adult female mosquitoes.

An adult mosquito was considered alive if it is able to fly, regardless of the number of legs remaining. Any knocked-down mosquitoes, whether or not they have lost legs or wings, were considered moribund and were counted as dead. On completion of the susceptibility test, a subset of mosquitoes from each collection area were properly preserved in RNAlater and SCB solution and send with ice packs using WMX box to the Laboratory of Institute of Molecular Biology and Biotechnology, Heraklion, Greece for molecular analysis. Further investigations were carried out for the identification of resistance mechanisms as noted above if on the diagnostic concentrations a significant number of survivors were found (more than 2%). Further tests were conducted in order to determine the underlying genetic mechanisms responsible for the observed resistance. These investigations included identification of the survivors, and at least 20% of dead mosquitoes, in order to identify in
which species the signs of resistance are present. This information was used in assessing the likelihood of cross-resistance between insecticides classes, and also provided valuable information about the potential for spread of resistance in vector populations [33].

**Morphological identification**

Adult anophelines were identified morphologically with the aid of a light microscope and taxonomic keys of Gillies and De Meillon [34] and Gillies and Coetzee [35].

**DNA extraction from single mosquitoes**

DNA was extracted from individual mosquitoes preserved in ethanol using the DNAzol™ Reagent (Invitrogen), for isolation of genomic DNA from solid and liquid samples according to the manufacturer’s instructions. The pellet containing DNA was dissolved in 15.0 μL of DEPC treated water.

**Total RNA and DNA extraction from mosquito pools**

Total RNA and DNA were extracted using a magnetic beads-based approach using the MagaZorb kit (Promega). The quantity and purity of DNA and total RNA were assessed spectrophotometrically (Nanodrop). The quality of RNA was assessed by 1.0% w/v agarose gel electrophoresis.

**Specific identification**

Each mosquito was identified to species level using the polymerase chain reaction (PCR) assay of Scott *et al.* [36] and slightly modified by Van Rensburg *et al.* [37].

**Genotyping of mosquito samples and multiplex RT-qPCR for gene expression analysis**

Species ID and target site mutation determination were performed using the assays described in the IVCC Vector Population Monitoring Tool (VPMT) Protocol [38] with minor modifications.

Newly developed and integrable to the LabDisk automated diagnostic platform quantitative Reverse Transcription-real-time PCR (qRT-PCR) 3-plex TaqMan® assays were used for the quantification of 7 detoxification genes’ expression (CYP6P3, CYP6M2, CYP9K1, CYP6P4, CYP6Z1, GSTE2, CYP6P1, CYP4G16) using RPS7 for normalization purposes in each assay. Reactions were performed in the Viia7 Real-Time PCR system (Applied Biosystems) using a one-step RT-PCR mastermix supplied by FTD (Fast-track diagnostics, Luxembourg) in a total reaction volume of 10 uL. The thermal cycle
parameters were: 50 °C for 15 min, 95 °C for 3 min, and 40 cycles of 95 °C for 3 s and 60 °C for 30 s, presenting a sample to result time of ~75 min. Duplicates of samples were amplified and each run always included a non-template control.

**Statistical analysis:** Calculation of fold-changes, 95% CIs and statistical significance was performed according to the Pfaffl method [39]. Graphs were constructed with the SigmaPlot software (v12.0).

**Results And Discussion**

*An gambiae* sl in all the three LGAs (Dekina, Ibaji and Omala) were only susceptible to pirimiphos methyl which recorded 100% mortality but resistance was observed in alphacypermethrin, permethrin, DDT, bendiocarb, propoxur, lambdacyhalothrin and deltamethrin.

**Detox gene expression**

Four detoxification genes (CYP6M2, CYP9K1, CYP6P4 and CYP6Z1) (Table 1) were significantly upregulated in the Dekina muti-resistance mosquito population. The following detoxification genes (CYP6M2, CYP9K1, CYP6P4 and CYP6Z1) were found to be consistently upregulated in the Dekina multi resistant (pyrethroids, DDT, bendiocarb) population compared to two susceptible (Kisumu and G3) laboratory strains (Figure 1). CYP6M2 was previously found overexpressed in pyrethroid-resistant populations from Nigeria [19]. This is the first detection of CYP9K1, CYP6P4 and CYP6Z1 overexpression in multi-resistant *Anopheles gambiae* ss populations in Nigeria. These genes have been found to be up regulated in DDT-resistant (all genes) and bendiocarb-resistant (CYP6P4, CYP6Z1) populations from the neighbouring Cameroon [40].

In contrast, organophosphates and carbamates caused insect death by blocking synaptic neurotransmission through the inhibition of acetylcholinesterase (AChE), encoded by the ACE-1 gene in *An. gambiae*. Indeed, a specific P450 enzyme, CYP6M2, has been demonstrated to metabolize organophosphates and carbamates, suggesting the potential to cause cross-resistance in *Anopheles gambiae*. The presence of some non-silent point mutations in the NaV gene has been reported to be associated with pyrethroid and DDT resistance [27]. The findings of this study is similar to the report of Silva et al. [41], that *An. gambiae* showed high activity of GST genes (GSTE2), P450 (CYP6Z1 and CYP325) and peroxidases in DDT resistant mosquitoes. Genes with anti-oxidizing function (Superoxide
dismutase, GST, Peroxidase and P450) were differently expressed in deltamethrin resistant populations of \textit{An. arabiensis} in Cameroon [23]. High expression of CYP6P3, a gene of the P450 family, has also been observed in permethrin-resistant populations of \textit{An. gambiae} [42]. The finding of this study was similar to the report of Fossog \textit{et al.} [43], as detoxification genes implicated in both M and S forms included CYP6M2, CYP9K1, CYP6P3, CYP6P4, CYP6Z3, GSTD1-6 and GSTD1-4. The majority of these have also been previously associated with resistance to pyrethroids and/or DDT [22, 44]. Two genes, CYP6M2 and GSTD1-6, encode enzymes that have also been demonstrated to metabolize DDT and have been reported to contribute to DDT resistant phenotype in both M and S form of \textit{An. gambiae} in Yaoundé, Cameroon [43]. Several additional detoxification genes such as CYP6P3 and CYP6Z3 are upregulated in the DDT resistant and other CYP6 candidate genes involved in DDT metabolism in M and S form populations included CYP6Z3 [25, 27].

In light of the absence of iAche mutations in our results, carbamate (bendiocarb and propoxur) resistance could be associated with the overexpression of CYP6P4 that has been previously implicated to bendiocarb resistance in Cameroon [27, 40, 43].

Different selective pressures have been fingered to trigger resistance in insects. This varies with time and location. Chemicals used in agriculture and veterinary services have been reported to fall into the same class with those used in public health and this has resulted in selective pressure. As these chemical are reported to be washed into mosquitoes breeding habitats [45].

Studies that suggested the effect of agricultural chemicals in selecting for resistance ranged from Nigeria [19], Burkina Faso [12], Benin [10, 46, 47], Cote d’Iviore [5], Cameroon [32] and Ethiopia [48]. This may also be a contributory factor for \textit{An. gambiae} development of resistant against pyrethriod, DDT and carbamates as observed in this study. The study area largely depends on agriculture as means of livelihood especially the Ibaji LGA. Market survey from local dealers across the three LGAs studied in Kogi East showed that the agricultural chemicals used were mostly of the pyrethroids and organophosphates classes. Abdoulaye \textit{et al.} [49] also pointed on excessive use of household chemicals such as coils, fumigation bombs or sprays as selective pressure for insecticides resistance. Pollutants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, heavy
metals, human drugs such as contraceptives and painkillers have also been reported to affect mosquito detoxification enzymes and their response to pyrethroids [50]. Though the scope of this study did not include analysis of the water holding the larvae but it could also be a reason since most of the larvae were collected from tyre tracks and were mostly around human dwellings. There’s therefore tendency that pollutants of such which are mostly produce from pollution caused by vehicles are washed into breeding habitats and since human dwellings were close to larvae habitats, there is also the chance of indiscriminate disposal of such drugs which might find their way into the breading sites.

Studies by Djouaka et al. [18] and Li et al. [45] suggested that plant material that dissolve in water can also give rise to resistance. These plant materials are reported to contain allelochemicals such as alkaloids, cyanogenic glycosides, coumarins, terpenoids, phenolic compounds and tannins [51]. In response to these phytochemicals, insects have evolved various adaptive strategies, including the diversification of their detoxification system [52]. Although the scope of this study did not cover phytochemicals in mosquito habits water, does not mean it should be overlooked as selective pressure promoting resistance in mosquitoes.

The extensive use of ITNs has also been attributed to reasons why pyrethroid has failed since LLINs are impregnated with pyrethroids and pyrethroids are also mostly used for IRS. These have been reported to lead to control failures in Bioko Island, Sri Lanka and Sudan [53].

The contribution of LLINs to selective pressure could also arise if they are abused as observed during our study as they are used for other purposes: such as protecting gardens against rodents and other animals. There is tendency of the impregnated chemicals been washed into mosquitoes breeding sites and pre-exposing the vector larvae to the insecticide.

Conclusion

This study looked at the resistant status of An. gambiae sl from Kogi East to the four classes of insecticides. An. gambiae sl from Kogi East were only susceptible to perimiphos-mythyl out of the eight insecticides tested. Different detoxifying genes were also investigated in the different populations. CYP6M2, CYP9K1, CYP6P4 and CYP6Z1 genes were significantly upregulated and ≥3 fold
in *An. gambiae* ss. An important result of this study is the first report of CYP9K1, CYP6P4 CYP6Z1 detoxification genes in wild *Anopheles gambiae* s.s populations from Nigeria. In light of the absence of iAche mutations in our results, carbamate (bendiocarb and propoxur) resistance could be associated with the overexpression of CYP6P4 that has been previously implicated to bendiocarb resistance in Cameroon. There is need for pre-survey before the distribution of LLINs or Indoor Residual Spraying. There is also need for constant vector monitoring in Nigeria. Sensitization on protective measure against malaria vectors will go a long in reducing the transmission of malaria.

Declarations

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**Availability of data and materials**

Data supporting the conclusions of this article are included within the article. The datasets used and/or analysed during the present study are available from the first author upon request.

**Authors’ contributions**

ASH and JEE conceptualized and designed the study. KM and JV performed the molecular aspect of the experiments and ASH did the statistical analysis. ASH and JEE drafted manuscript with input from all authors.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

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Table
Due to technical limitations, Table 1 is only available for download from the Supplementary Files
Expression analysis of detoxification genes in the Dekina mosquito population (unexposed to insecticides). Error bars indicate 95% CIs. Stars denote genes that showed statistical significant and >3-fold upregulation compared to both Kisumu and G3 susceptible strains. i.e. CYP6M2, CYP9K1, CYP6P4 and CYP6Z1.

Supplementary Files
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