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*Full Length Research Paper*

# Differential expression of cytochrome P450 genes in a laboratory selected *Anopheles arabiensis* colony

**Givemore Munhenga[1,2] and Lizette L. Koekemoer[1,3]***

[1]Vector Control Reference Unit , National Institute for Communicable Diseases, NHLS, Private Bag X4, Sandringham, Johannesburg 2131, South Africa.
[2]School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Johannesburg, South Africa.
[3]Malaria Entomology Research Unit, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa.

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**In southern Africa pyrethroid, resistance in Anopheles *arabiensis* is mainly mediated by cytochrome P450s. The spectra of P450 genes involved are not fully understood. We report on the transcriptional profile of six P450 genes previously implicated in pyrethroid resistance from a laboratory selected permethrin-resistance. Quantification of expression levels of *CYP6Z1, CYP6Z2, CYP6Z3, CYP6M2, CYP6P3* and *CYP4G16* was performed using qPCR from a susceptible and permethrin resistant selected colony. *CYP6Z1, CYP6Z2* and *CYP6M2* were significantly up-regulated in the selected colony with a relative fold over expression of 4.7, 1.7 and 1.4 respectively. Increase in expression levels of three genes in the selected strains suggests their roles in permethrin metabolism. These results provide useful information on future studies to develop new insecticides and tools for detecting and managing insecticide resistance.**

**Key words**: KwaZulu-Natal, *Anopheles arabiensis*, pyrethroid resistance, cytochrome P450, synergist

## INTRODUCTION

*Anopheles arabiensis* remains a very important malaria vector in countries experiencing hot and dry weather conditions such as southern Africa, Ethiopia, Eritrea and Sudan (Coetzee, 2000). This species is the main vector in South Africa and Zimbabwe where vector control strategies mainly rely on the use of insecticides (Maharaj et al., 2005; Masendu et al., 2005). Pyrethroids are the preferred insecticide in these two countries. However, intensive use of insecticides in both public health and agriculture has led to the development of resistance in various mosquito vector species (Ellisa et al., 1993; Awolola et al., 2002; Stump et al., 2004; Munhenga et al., 2008, Mouatcho et al., 2009) and is a cause of concern in any malaria control programme where insecticides are a corner stone for vector control.

Insecticide resistance to pyrethroids is mainly through target site insensitivity and or metabolic detoxification of the insecticide by enzymes. Target site resistance to pyrethroids and DDT termed knockdown resistance, has been thoroughly studied and is due to a substitution at a single codon in the sodium channel gene (Martinez et al., 1998; Ranson et al., 2000). Understanding the molecular basis of target site resistance led to development of sensitive diagnostic tools (Martinez et al., 1998, Lynd et al., 2005; Bass et al., 2007, Vezenegho et al., 2009). Resistant allele frequency is determined with these tools thereby making it possible for vector control managers to monitor and determine the impact of resistance. However, the same cannot be said of metabolic based resistance mechanisms. In metabolic resistance, when an insect is exposed to insecticide, this results in either an overproduction of specific enzymes, leading to increased metabolism or sequestration, or secondly, an alteration in the catalytic centre of the enzyme unit that metabolizes the insecticides and this results in production of enzymes which can efficiently detoxify the insecticide (Li et al., 2007). The enzymes responsible for detoxification of insecticides are transcribed by three members of large multigene enzyme systems: monooxygenases

*Corresponding author. E-mail: givemorem@nicd.ac.za or lizettek@nicd.ac.za.

(P450's), non-specific esterases (NSE), and glutathione S-transferases (GST's), (Hemingway and Ranson, 2004). The intricate mechanisms involved are, however, not fully understood. Through biochemical and synergist assays it has been established that P450s play a central role in conferring insecticide resistance in insect species (Scharf et al., 1997; Brooke et al., 2001; Awolola et al., 2009; Mouatcho et al., 2009). In mosquito species, increased activities of P450s have been associated with pyrethroid resistance (Ellisa et al., 1993; Etang et al., 2007; Munhenga et al., 2008). However, this alone is not informative enough as cytochrome P450s are known to consist of multigene superfamilies of enzymes playing different roles in oxidative metabolism of endogenous compounds (Mansuy, 1998; Feyereisen, 1999), and only a few being attributed to insecticide detoxification. With increased threat on malaria vector control caused by insecticide resistance attributed to P450s, there has been an interest in understanding the role of individual P450s genes involved in insecticide resistance. It is envisaged that identification of these candidate genes will be useful in development of more sensitive diagnostic tests for effective monitoring of metabolic based resistance development.

Cytochrome P450 enzymes confer insecticide resistance via increased levels of P450 activity resulting from elevated expression of P450 genes. This up-regulation has been recorded in 25 P450 genes, belonging to four families; *CYP4*, *CYP6*, *CYP9*, and *CYP12* (Feyereisen, 1999; David et al., 2005). Detailed studies in *Anopheles gambiae* have shown that there is a cluster of cytochrome P450 genes located in the chromosome arm 3R associated with pyrethroid resistance (Ranson et al., 2004). This locus consists of several P450 genes of which CYP6Z1 (Nikou et al., 2003; David et al., 2005), CYP6Z2 (Muller et al., 2007a), CYP6Z3 (Muller et al., 2007b) and CYP6M2 (Muller et al., 2007a; Djouaka et al., 2008) have been implicated in pyrethroid resistance. While progress has been made in understanding candidate P450 genes putatively involved in pyrethroid resistance in *A. gambiae* and *Anopheles funestus*, there is limited information on the role of individual P450s in insecticide resistant *A. arabiensis* despite its equally important role in malaria transmission.

Here, we report the transcriptional analysis of six P450s genes from a permethrin-resistant *A. arabiensis* laboratory strain which is under continuous permethrin selection pressure. Previous analysis implicated elevated cytochrome P450 enzyme activity as the main pyrethroid resistant mechanism in this strain (Mouatcho et al., 2009).

## MATERIALS AND METHODS

### Insect strains

Two *A. arabiensis* laboratory colonies, designated KWAG and KWAG-Perm, maintained in the Botha DeMeillon insectary (Vector Control Reference Unit, South Africa) were used in this study. KWAG originated from Mamfene, KwaZulu-Natal, and was colonized in 2005 from a wild population showing permethrin resistance (78%) (Mouatcho et al., 2009). This colony reverted back to fully permethrin susceptible in the absence of selection pressure. However, a subpopulation of the same colony was placed under permethrin pressure and resulted in a pyrethroid resistant colony called KWAG-Perm (details on colony can be found in Mouatcho et al., 2009).

### Insecticide susceptibility test

The standard WHO susceptibility tests for adult mosquitoes was carried on KWAG and KWAG-Perm using test-kits and insecticide-impregnated filter papers supplied by the WHO (WHO, 1998). Three day old adults reared from the two colonies were exposed to 0.75% permethrin. Each test consisted of 25 mosquitoes per tube with two controls. Four replicates were done for each colony. All filter papers were tested; both prior to and after exposure to an insecticide susceptible *A. arabiensis* colony (KGB) in order to confirm insecticidal activity. For each bioassay, knockdown of mosquitoes was recorded after 60 min and mortality scored after 24 h. Each exposure tube was allowed 24 h recovery during which time 10% (w/v) sugar solution was available. Population susceptibility was classified according to the WHO criterion, which considers mortality above 98% and below 80% representative of susceptible and resistant populations, respectively (WHO, 1998).

### Synergist analysis

Synergistic assay using piperonyl butoxide (PBO) was conducted on the permethrin selected colony to confirm involvement of P450s in permethrin resistance using the method described in Mouatcho et al. (2009).

### P450 gene quantification

#### RNA extraction

Total RNA was extracted (Paton et al., 2000) from three day old adult mosquitoes from both the unselected (also called baseline colony) and the permethrin resistant selected colony. To minimize gene expression variations, RNA was extracted from 10 mosquitoes per treatment for each of the three biological replicates. For each biological repeat, adult males and females from the baseline and permethrin selected colony were collected simultaneously and immediately used for RNA extraction. After extraction, RNA quality and quantities were assessed using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Oxfordshire, UK) at 230, 260 and 280 nm.

#### cDNA synthesis

Synthesis of cDNA was carried out on 2 µg of total RNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, Forster City, CA, USA; Cat no. 4387406) following the manufacturer's instructions. Total cDNA was quantified using a Nanodrop spectrophotometer.

#### Primer design

The full length CYP6Z2, CYP6Z3 and CYP4G16 gene sequence of *A. gambiae* deposited on NCBI website, (http://www.ncbi.nlm.

**Table 1.** Primer pair sequences of oligonucleotide primers and annealing temperatures used for P450 gene quantification.

| Gene | Accession number | Primer | Sequence (5'TO 3') | Transcript length | Annealing temperature (°C) |
|---|---|---|---|---|---|
| CYP6Z1 | AF487535 | CYP6Z1_qF<br>CYP6Z1_qR | TTA CAT TCA CAC TGC ACG AG<br>CTT CAC GCA CAA ATC CAG AT | 146 bp | 56.6 |
| CYP6Z2 | | CYP6Z2_F<br>CYP6Z3_R | ATC GCT TCG GTG TTC TTC<br>AAT CAA TTC AGG CTG GAG AG | 182 bp | 53.9 |
| CYP6Z3 | | CYP6Z3_F<br>CYP6Z3_R | CAA CAA CCT GTA CCA CAA GTC<br>GGA TCG TGC TCT TCA TTG C | 162 bp | 53.9 |
| CYP6M2 | AY193729 | CYP6M2_F<br>CYP6M2_R | GTA TGA TGC AGG CCC GTA TAG<br>GCC ATA ATG AAA CTC TCC TTC G | 112 bp | 55.3 |
| CYP6P3 | | CYP6P3_F<br>CYP6P3_R | AGC TAA TTA ACG CGG TGC TG<br>AAG TGT GGA TTC GGA GCG TA | 121bp | 53.2 |
| CYP4G16 | | CYP4G16_F<br>CYP4G16_R | TAG AGC GGT GCC TTA TGG<br>CGA TTC CAA GCG GTG AAG | 158 bp | 53.9 |
| 18S | | 18S_F<br>18S_R | TAC CTG GGC GTT CTA CTC<br>CTT TGA GCA CTC TAA TTT GTT C | 130 bp | - |
| S7 ribosomal | AY380336 | S7_F<br>S7_R | GTG CCG GTG CCG AAA CAG AA<br>AGC ACA AAC ACT CCA ATA ATC AAG | 472 bp | - |
| rpL8 | | rpL8_F<br>rpL8_R | CAT CAG CAC ATC GGT AAG<br>ACA GAG CAC TCA CTA CTC | 162 bp | - |
| bactin | | bactin_F<br>bactin_R | ACC AAG AGC CTG AAG CAC<br>CGA GCA CGA CAC ACT ATA TAC | 123 bp | - |
| tbp | | tbp_F<br>tbp_R | GAC ATC GTC ATC AAC AAC<br>CCG TAC AGG TAA TCT TCC | 181 bp | - |
| Gapdh | | gapdh_F<br>gapdh_R | GAC TGC CAC TCG TCC ATC<br>CCT TGG TCT GCA TGT ACT TG | 139 bp | - |

nih.gov/), were used to design the specific primers (Table 1), using the Beacon Designer 3.0 software (Biorad, Hercules, CA, USA). Specificity of the primers was confirmed by sequencing genomic DNA from *A. arabiensis* specimens from the selected cohorts. For *CYP6Z1*, *CYP6M2*, and *CYP6P3*, the primer sequence designed for *A. gambiae s.s* were used (Nikou et al., 2003; Muller et al., 2007a). Specificity of primers was confirmed by sequencing PCR products post amplification.

**Selection of reference genes for gene quantification**

Six reference genes: beta actin (bactin), 18S ribosomal RNA (18S), M2 ribosomal protein L8 (rpL8), tata box binding protein (tbp), glucose-6-phosphate dehydro-genase (gapdh) and ribosomal (S7) were selected for assessment as these genes have previously been used as reference genes by others (Nishimura et al., 2006; Muller et al., 2008). For each gene, full length gene sequence of *A. gambiae* deposited on the NCBI website was used to design specific primer using the Beacon Designer software (Biorad, Hercules, CA, USA). Table 1 summarizes the primer pair sequence of the reference genes assessed. Each gene was amplified in triplicate for the three biological repeats of the two strains KWAG and KWAG-Perm). PCR conditions were optimized and 5 µl of the amplified product were electrophoresed on a 2.5% agarose gel to verify amplicon size. The remainders of the amplicons were sent to Inqaba biochemical industry for sequencing to confirm whether the right amplicon was amplified. Threshold values (Cq) were directly used to compare differences in expression of each reference gene between the susceptible and resistant samples.

**Relative quantification of P450 genes**

Quantification of expression levels of each gene (*CYP6Z1*, *CYP6Z2*, *CYP6Z3*, *CYP6M2*, *CYP6P3* and *CYP4G16*) was performed in a CFX 96 real time PCR machine (Biorad, Hercules, CA, USA). 18S rRNA gene was used as the reference gene. Concurrently, a standard curve was generated for both the target and housekeeping genes using a 2 fold dilution series from 80 to 0.076 ng. Each dilution concentration for the standard curve was done in duplicate, while reactions for the target gene and 18S rRNA

**Table 2.** General expression levels of candidate reference genes in *A. arabiensis* KWAG-Perm (selected) and KWAG-base (unselected) colonies.

| Candidate reference gene | KWAG-Perm $F_{12}$ [Cq (mean ± SE)] | KWAG-base [Cq (mean ± SE)] | P value |
|---|---|---|---|
| Bactin | 29.7 ± 0.368 | 23.9 ± 0.133 | 0.000 |
| 18S rRNA | 11.8 ± 0.111 | 12.0 ± 0.121 | 0.052 |
| rpL8 | Failed to amplify | Failed to amplify | - |
| tbp | 36.6 ± 0.485 | 32.9 ± 0.121 | 0.000 |
| gadph | 25.1 ± 0.352 | 18.4 ± 0.182 | 0.000 |
| S7 | 25.6 ± 0.225 | 18.1 ± 0.086 | 0.000 |

were performed in triplicate for each biological sample.

All amplification reactions were carried out in a total volume of 25µl containing 12.5 µl 2X iQ™SYBR® Green Supermix (Bio-Rad, Hercules, CA; Cat No. 170-882) , 200 mM of each specific primer pair specific for each gene and 100 ng of cDNA template. The qPCR cycling conditions consisted of: initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 94°C for 15 s; annealing was varied from 53.2 to 56.6°C for 30 s for each gene (Table 1), primer extension at 72°C for 25 s and a final auto extension at 72°C for 5 min. Acquisition of data was carried out at each cycle immediately after the extension step. A final auto extension step was incorporated at 72°C for 25 s. After the cycling protocol, a final step was applied to all reactions by continuously monitoring fluorescence through the dissociation temperature of the PCR products at a temperature transition rate of 0.5°C/s to generate a melt curve. Melt curve and agarose gel analysis were conducted for each gene to ensure that a single amplicon was amplified. Relative expression levels of each gene were calculated using the comparative cycle threshold method described by Pfaffl (2001). Briefly, amplification efficiencies for the target and housekeeping gene were automatically calculated by the CFX software manager (Bio-Rad, Hercules, CA, USA), with relative gene quantities normalized against the 18S ribosomal RNA (18S). Expression levels between the baseline (calibrator) and permethrin selected colony (sample) were statistically analyzed using the CFX software manager (Biorad). Statistical difference in expression levels was analyzed using REST 2008 statistical package (Corbett LifeSciences).

## RESULTS AND DISCUSSION

WHO susceptibility tests carried out simultaneously on unselected (KWAG) and permethrin selected colony (KWAG-Perm) showed that the selected strain was resistant to permethrin (42% mortality, n = 100) while the baseline colony showed an average mortality of 97.8% (n = 100). These results confirmed the level of pyrethroid resistance in KWAG-Perm as reported by Mouatcho et al. (2009).

Synergist assays performed using PBO, an inhibitor of monooxygenase showed that susceptibility to permethrin was restored in the permethrin selected colony. Mortality 24 h post-exposure of synergized samples was 98.3% (n = 200) while unsynergized samples recorded a mortality of 41.8% (n= 200). The differences in mortality 24 h post exposure between synergized and unsynergized samples using PBO was statistically significant ($\chi^2$=0.4, DF = 4, P < 0.05). This strongly suggests that pyrethroid resistance in this colony is mediated by monooxygenases.

Six genes were evaluated as reference genes and Table 2 shows the mean real-time PCR threshold cycle (Cq) values of genes tested. Of the six, only 18S showed no variation in general expression levels between the selected and unselected samples. Therefore, it was chosen as the reference gene in this investigation.

Quantification analysis of P450 gene transcription levels revealed that only three P450 genes, *CYP6Z1*, *CYP6Z2*, and *CYP6M2* were up regulated in a permethrin resistant *A. arabiensis* strain (Figure 1). *CYP6Z1* showed the highest level of transcription with a relative fold over expression of 4.7. There was a statistically significant difference in the mRNA expression level between the two strains (KWAG and KWAG-Perm) (P<0.001). The second highest gene transcription was obtained from *CYP6M2*, showing a statistically significant fold over increase of 2.2 (P<0.001). *CYP6Z2* was significantly up-regulated in the KWAG-Perm with a fold increase of 1.7 (P = 0.002). Results for *CYP4G16* showed a fold over change of 1.4 which was statistically on the border line (P = 0.05). There was no statistical difference in the expression levels of *CYP6Z3* (P = 0.372), and *CYP6P3* (P = 0.798) between the selected and unselected colonies.

The strong overexpression of *CYP6Z1* reported in this study is consistent with previous studies where it was 3.5 fold up regulated in adult females of a permethrin resistant laboratory (RSP) strain of *A. gambiae* collected in West Kenya (Nikou et al., 2003). Increase in CYP6M2 and CYP6Z2 recorded in this study is similar to the results obtained by Muller et al. (2007a) using permethrin-resistant adult males and females of *A. gambiae* from Ghana. *CYP4G16* was included in this study as it was previously identified by Muller et al. (2007b) to be associated with deltamethrin resistance in *A. arabiensis* from a study in Cameroon where it showed a 4.5 fold overexpression. The expression level of *CYP4G16* in this study was marginally higher in the selected cohorts. Independent records of overexpression of CYP4G16 in two different geographical locations might indicate possible association of this gene in pyrethroid resistance in *A. arabiensis.*

The absence of increased gene transcription of *CYP6P3* and *CYP6Z3* probably indicates that these genes do not play a role in pyrethroid resistance in *A.*

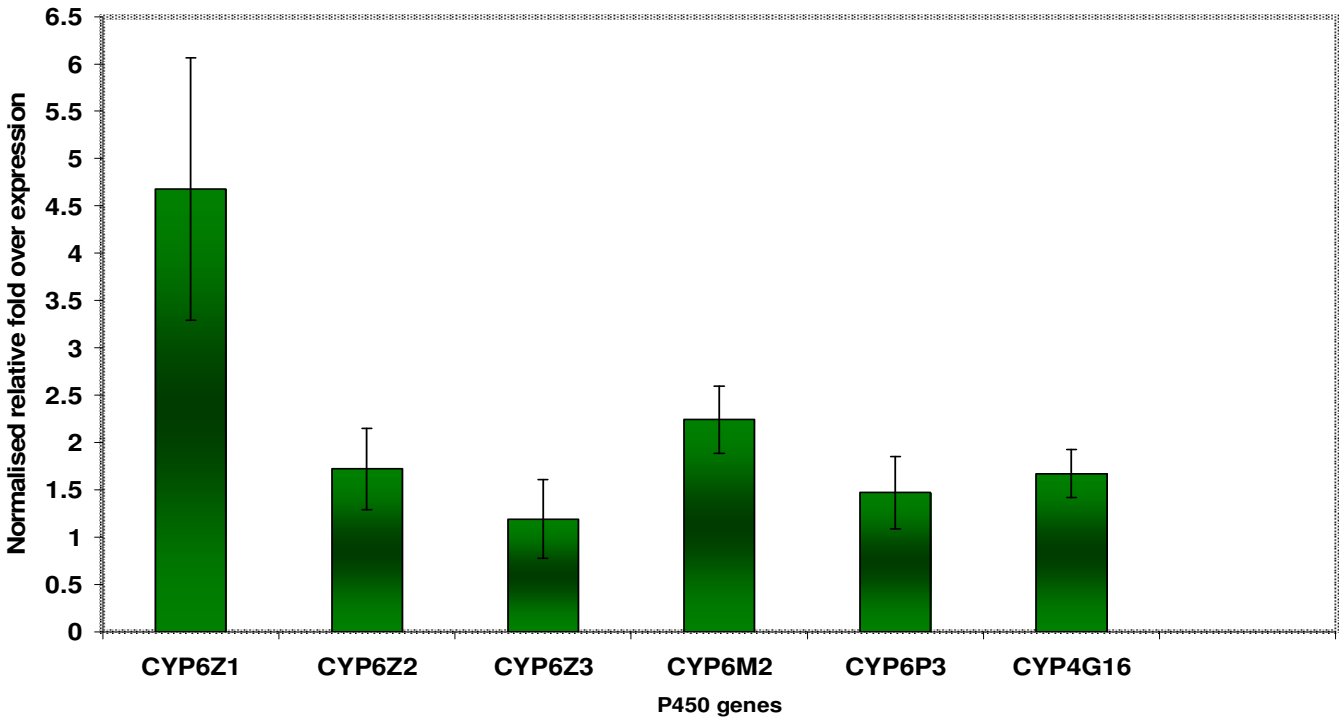


**Figure 1.** Constitutive expression of the six P450 genes in permethrin *A. arabiensis* selected strain (KWAG-Perm) normalised to 18S ribosomal RNA in susceptible (base) and resistant (selected) adult females. Data are presented as mean ± SE of three replicates.

*arabiensis* from South Africa although they have been associated with pyrethroid resistance in other malaria vectors (Muller et al., 2007a; Muller et al., 2008).

## Conclusions

These three genes identified are most likely not the only genes involved in pyrethroid resistant *A. arabiensis* from South Africa and a large scale approach such as microarray analysis will provide additional information on this complex resistance mechanism. Once these genes have been identified, a field trial study will be conducted to investigate if these genes can be used for "early detection" of pyrethroid resistance in *A. arabiensis* from South Africa. This will provide an additional tool to the National Malaria Control Program (NMCP) that might be used in annual surveillance activities.

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