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Over expression of a Cytochrome P450 (CYP6P9) in a Major African Malaria Vector, *Anopheles Funestus*, Resistant to Pyrethroids

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Abstract

***Anopheles funestus* Giles is one of the major African malaria vectors. It has previously been implicated in a major outbreak of malaria in KwaZulu/Natal, South Africa, during the period 1996 to 2000. The re-emergence of this vector was associated with monooxygenase-based resistance to pyrethroid insecticides. We have identified a gene from the monooxygenase CYP6 family, CYP6P9, which is over expressed in a pyrethroid resistant strain originating from Mozambique. Quantitative Real-Time PCR shows that this gene is highly over expressed in the egg and adult stages of the resistant strain relative to the susceptible strain but the larval stages showed almost no difference in expression between strains. This gene is genetically linked to a major locus associated with pyrethroid resistance in this *A. funestus* population.**

Keywords: P450, insecticide resistance, mosquito.

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Introduction

Malaria remains one of the devastating diseases in Africa and malaria control programmes rely extensively on insecticide-based vector control. Insecticides are mainly employed in indoor residual spraying (IRS) or applied to bednets (insecticide treated nets/ITNs). The importance of indoor residual spraying to control vector mosquitoes was reiterated recently by W.H.O (www.who.int/mediacentre/news/releases/2006/pr50/en/). Pyrethroid insecticides remain the first choice of chemical for IRS due to their low mammalian toxicity, rapid breakdown in the environment and efficacy and are also the only class of chemicals allowed for use on impregnated bednets. However, pyrethroid resistance in vector mosquitoes has recently been reported throughout Africa including South Africa (Hargreaves *et al*., 2000), Nigeria (Awolola *et al*., 2002), Cameroon (Chouaibou *et al*., 2006), Cote d'Ivoire (Tia *et al*., 2006), Ghana (Coetzee *et al*., 2006) and Benin (N'Guessan *et al*., 2007).

Because of the rapid and widespread development of resistance to insecticides in target vector populations, insecticide resistance management is becoming an integral part of vector control. This strategy is designed to monitor and circumvent the development of insecticide resistance in affected populations as well as to prevent the development of resistance in unaffected populations. In the absence of resistance management, the impact of insecticide resistance on malaria transmission can be severe. Such an impact was clearly demonstrated in KwaZulu/Natal, South Africa, when pyrethroid resistant *Anopheles funestus* Giles re-emerged during the late 1990's leading to a severe malaria epidemic during the period 1996 to 2000. This occurred despite ongoing control efforts using pyrethroid insecticides (Hargreaves *et al*., 2000; Coetzee & Fontenille, 2004). *A. funestus* is the nominal member of the funestus group and, along with *Anopheles arabiensis* Patton, is a major vector of malaria in the southern African region. *A. funestus* is highly anthropophilic and endophilic making it especially vulnerable to control by IRS assuming effectiveness of the insecticide employed (Gillies & DeMeillon, 1968).

Pyrethroid resistance can arise due to mutations in the target site, the sodium ion channel gene, or up-regulation of metabolic enzymes, mainly monooxygenases which detoxify the insecticide. There are no reports of target site resistance in *A. funestus* to date. Detoxification of pyrethroids in insects is mainly accomplished by monooxygenases, also called P450 enzymes. Over expression of one or more of these P450 genes has been associated with pyrethroid resistance in house flies (Kasai & Scott, 2000), mosquitoes (Nikou *et al*., 2003; David *et al*., 2005) and many other insects. Majority of these genes are from four main families, CYP4, 6 and 9 (Feyerseisen *et al*., 1989). During the last 18 years since the isolation of the first insect P450 in the housefly *Musca domestica* (Feyereisen *et al*., 1989), over 300 P450s have been identified in insects (http://p450.antibes.inra.fr/). Insect P450s are distributed across 48 CYP families which include CYP4, CYP6, CYP9, CYP12, CYP18, CYP28, CYP49 and CYP301-341 (http://drnelson.utmem.edu/CytochromeP450.html). Identification of this P450 diversity in insects was accelerated by the genomic sequencing of *Drosophila melanogaster* and *A. gambiae* (Tijet *et al*., 2001; Ranson *et al*., 2002). In *A. funestus*, 31 partial P450 genes were isolated using degenerate primers based on *A. gambiae* P450 sequences (Amenya *et al*., 2005).

Monooxygenase-based resistance to pyrethroids in southern African *A. funestus* was first demonstrated by Brooke *et al*. (2001). The controlling resistance factor appears to be inherited as a single locus, autosomal, incompletely dominant phenotype (Okoye *et al*., unpublished data). Quantitative trait locus (QTL) mapping of the phenotype identified a major pyrethroid resistance locus on chromosome arm 2R, coinciding with the location of a cluster of CYP6 genes mapped by *in situ* hybridisation (Wondji *et al*., 2007). These data support the hypothesis that pyrethroid resistance in southern African *A. funestus* is mediated by one or a cluster of P450 enzymes specifically from the CYP6 family. In the current study, we used hybridisation and PCR techniques to identify a specific P450 gene that is over expressed in the pyrethroid resistant strain.

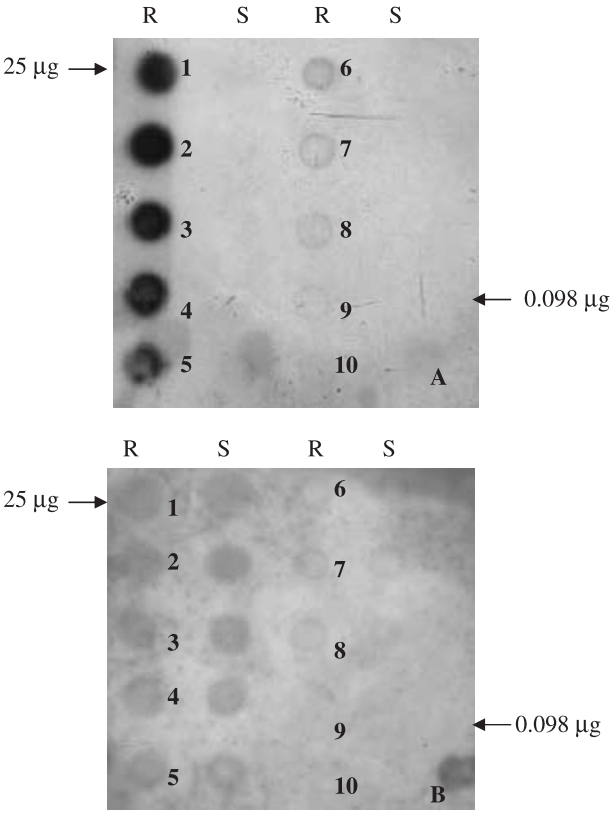

Figure 1. Dot blot analysis of 3-day old adult FUMOZ-R (R) and FANG (S) RNAs. Autoradiogram after 5 h exposure. (A) Autoradiogram showing hybridisation results with cocktail CYP6 probes. (B) Autoradiogram showing hybridisation result with rsp7 probe. RNA concentrations (1 = 25 µg, 2 = 12.5 µg, 3 = 6.25 µg, 4 = 3.125 µg, 5 = 1.56 µg, 6 = 0.78 µg, 7 = 0.39 µg, 8 = 0.195 µg, 9 = 0.098 µg, 10 = 0.049 µg).

Results

In initial experiments, serial dilutions of RNA from the resistant and susceptible strains were hybridised with cocktail probes from the CYP4, 6 or 9 classes. These four classes were targeted based on their association with pyrethroid resistance in other insect species such as *A. gambiae*, *M. domestica* etc. (Feyerseisen *et al*., 1989). The cocktail probes consist of those subfamilies isolated and sequenced from cDNA by Amenya *et al*. (2005) as this paper is an extension of the same project. A detectable signal was only observed with the CYP6 cocktail probe and only in the resistant strain (Fig. 1). Expression of CYP6 P450s was clearly visible with 1.5 µg of the total RNA from the FUMOZ-R strain whereas no signal was observed even at the highest concentration (25 µg) in the susceptible strain. The CYP6 primer cocktail was prepared from the partial sequences of six CYP6 genes from three different subfamilies (CYP6P, CYP6Z and CYP6M). We subsequently prepared single gene probes and used these to compare gene expression between the FUMOZ-R and FANG strains. The starting RNA concentration was increased to 50 µg. When using a probe for the CYP6P9 gene, expression was clearly visible even at low concentrations of FUMOZ-R RNA while remaining almost undetectable in the FANG strain (Fig. 2). No signal was detectable in either strain when using single probes from the remaining five CYP6 genes (data not shown). Over expression of the CYP6P9 gene was confirmed using northern blot analysis (Fig. 3) which showed a single transcript of approximately 2 kb with expression visibly higher in the FUMOZ-R strain.

To quantify the difference in expression of CYP6P9 between the two strains primers were designed to amplify

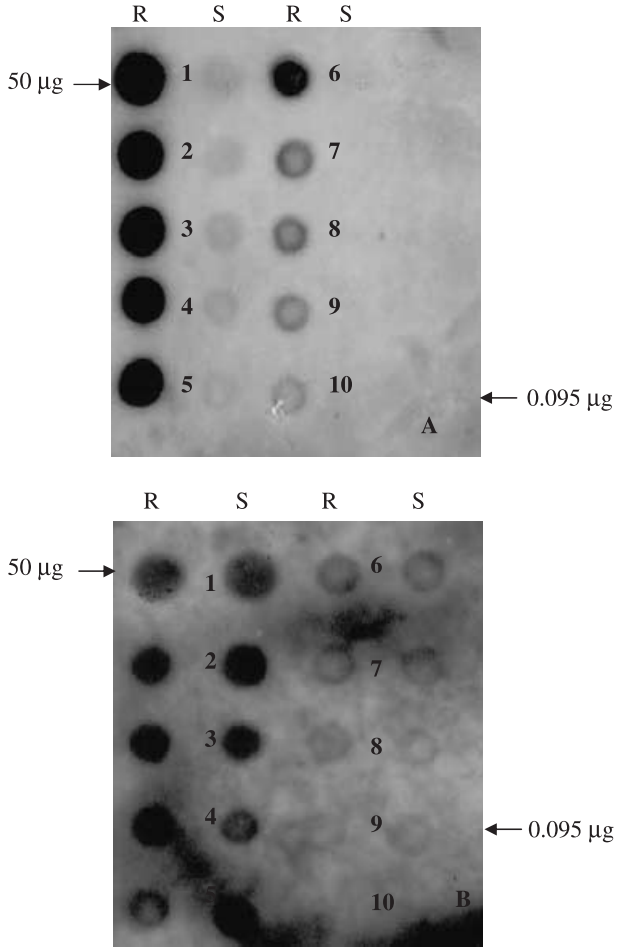

Figure 2. Dot blot analysis of 3-day old FUMOZ-R (R) and FANG (S) RNAs. Autoradiogram after overnight exposure. (A) Autoradiogram showing hybridisation results with CYP6P9 probe. (B) Autoradiogram showing hybridisation result with rsp7 probe. RNA concentrations (1 = 50 µg, 2 = 25 µg, 3 = 12.5 µg, 4 = 6.25 µg, 5 = 3.125 µg, 6 = 1.56 µg, 7 = 0.78 µg, 8 = 0.39 µg, 9 = 0.195 µg, 10 = 0.098 µg).

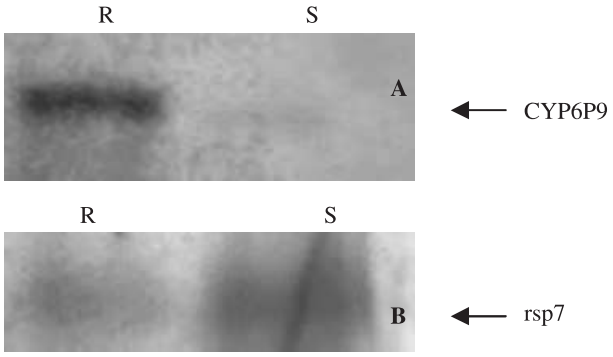

Figure 3. Northern blot analysis of CYP6P9 mRNA levels in FUMOZ-R (R) and FANG (S) strains. RNA (5 µg) was loaded in each lane (A) CYP6P9 probe (B) rsp7 probe.

a 140 bp fragment from the CYP6P9 gene and 135 bp for rsp7 and used in real time PCR reactions using cDNA from both strains as a template. Melting curve analysis showed a single product specific melting temperature of 87.5 °C and 89 °C for CYP6P9 and rsp7 respectively. No primer dimers were observed. The products were sequenced to confirm that the expected products have been amplified.

Figure 4 shows the results of the quantitative PCR analysis. CYP6P9 expression was elevated in the resistant FUMOZ-R strain compared to the susceptible FANG strain in the egg and adult stages. However, expression of this gene was approximately equal in the two strains in all larval stages and in the pupal stage (Fig. 4). Elevated expression was particularly distinct in the eggs and 14 day old adults. When comparing the sexes, expression of CYP6P9 was higher in females than males.

The quantitative PCR analysis was also performed using genomic DNA as a template. The ratio of expression between CYP6P9 and rsp7 was approximately 1:1 in both mosquito populations (data not shown) suggesting that the increased production of mRNA in the FUMOZ-R strain is not due to gene duplication.

WHO bioassay exposures using 0.75% permethrin against three day old FUMOZ-R adults showed similar mortality rates between females (22%) and males (28%). However, 14 day old adult mortality was 86% in females and 94% in males. An insecticide dose response comparison between 3–5 day old FUMOZ-R and FANG adult females gave permethrin LD_{50}-values of 141.2 µg/250 ml glass bottle (SE = 17.9) for FUMOZ-R and 1.2 µg/250 ml glass bottle (SE = 0.3) for FANG. These data represent a 118 fold difference in adult response ($P < 0.05$) between the two strains.

Bioassays comparing FUMOZ-R and FANG 4th instar larvae gave permethrin LC_{50}-values of 10.8 µg/ml (SE = 0.17) for FUMOZ-R and 4.3 µg/ml (SE = 0.07) for FANG. This represents only a 2.5 fold difference in response ($P = 0.01$) between the two strains.

Discussion

Investigating metabolic resistance mechanisms in insects is complex owing to the extensive size of the gene families involved and their rapid diversification. Amenya *et al.* (2005) isolated the first 31 partial P450 gene fragments from *A. funestus* in order to study pyrethroid resistance in this species. We have used these partial gene fragments to identify potential genes over expressed in the pyrethroid resistant strain, FUMOZ-R.

Twelve partial CYP4 genes and five partial CYP9 genes did not show any over expression in FUMOZ-R compared to FANG. This indicates that these genes do not associate with the resistance phenotype and are probably not involved in detoxification of pyrethroids in the FUMOZ-R strain.

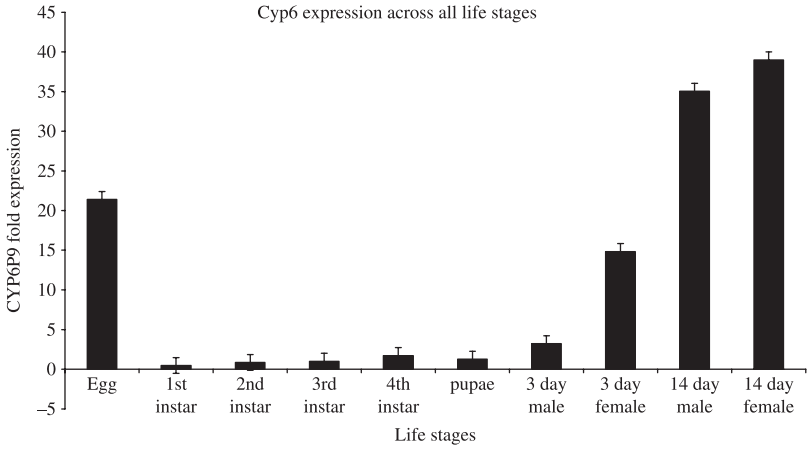

Figure 4. Quantitative PCR analysis CYP6P9 expression in *A. funestus* from the FUMOZ-R (pyrethroid resistant) and FANG (susceptible) strains. For each strain expression was normalised against rsp7 and the ratio of the normalised CYP6P9 expression is plotted. Significant differences in expression between the strains were observed in eggs ($t = 6.31$, $P < 0.05$); 4th: fourth instar larvae ($t = 8.61$, $P < 0.05$); 3 day old males ($t = 22.76$, $P < 0.05$); 3 day old females ($t = 4.72$, $P > 0.05$); 14-day old males ($t = 23.16$, $P < 0.05$) and 14 day old females ($t = 7.46$, $P < 0.05$).

Genes in the monooxygenase CYP6 family have been implicated in insecticide resistance in other insects including *M. domestica* (Feyereisen *et al*., 1995; Liu & Scott, 1998; Kasai & Scott, 2000), *A. gambiae* (Nikou *et al*., 2003), *Anopheles albimanus* (Rongnoparut *et al*., 2003) and *Anopheles minimus* (Rodpradit *et al*., 2005). Identification of the actual gene that was over-expressed in the pyrethroid resistant strain of *A. funestus* was achieved by probing each of the six genes comprising the CYP6 cocktail (CYP6P1, CYP6P2, CYP6P9, CYP6Z1, CYP6M8 and CYP6M7) separately. Only one of these, CYP6P9, was expressed at elevated levels in the resistant strain. While it is recognised that the transcription profile of only a small subset of the CYP6 family has been examined so far (*A. gambiae* contains 30 CYP6 genes) the very high level of over expression of CYP6P9 in the resistant strain (up to 38-fold in 14 day females) and the genomic location of this gene (see below) suggests that expression of this P450, which is an ortholog of CYP6P3 in *A. gambiae* (Amenya *et al*., 2005), is associated with resistance to permethrin in *A. funestus.*

The over expression of CYP6P9 is confined to the eggs and adult stages. There was no significant over-expression in the larvae. Hence if enhanced detoxification of pyrethroids by CYP6P9 is the major causative factor of pyrethroid resistance in the FUMOZ-R strain, the resistance phenotype would be expected to be manifested in the adult stage only. Stage specificity of the resistance phenotype is supported by bioassays against larvae in which there are low levels of variation at permethrin LC_{50} concentrations between the FUMOZ-R and FANG strains. On the other hand, dose-dependent permethrin bioassays against adult females showed an enormous difference in response between the resistant and susceptible strains. Furthermore, the FUMOZ-R colony was under selection at the adult stage only which may account for the development of an adult specific protection mechanism. If resistance is only present in adult mosquitoes in field populations this may suggest that elevated expression is being selected for by use of insecticides in malaria control as opposed to contamination of breeding sites by agricultural insecticides. The expression of CYP6P9 in adult mosquitoes is dramatically increased between the ages of three days old and 14 days old and the effect is most pronounced in females. This is in contrast to bioassay results published by Hunt *et al*. (2005) where mortality following permethrin exposure was significantly higher in 14-day old adults. The biological material used for this study was selected through an additional 25 generations since the original characterisation was carried out and the current bioassay analysis supports the data of Hunt *et al*. (2005). The complexities of age-dependent differentials in enzyme expression may account for variation in the expression of the resistance phenotype with age. Mapping of permethrin resistance QTL's by Wondji *et al.* (2007) shows that the chromosomal position of CYP6P9 associates with permethrin resistance and accounts for the bulk (> 60%) of the resistance phenotype but also implies that other factors play a minor role as well.

Nikou *et al*. (2003) demonstrated that within the CYP6Z subfamily, various genes (CYP6Z1, CYP6Z2 and CYP6Z3) were found to be differentially expressed in the life stages of a pyrethroid resistant strain of *A. gambiae* from Kenya. These genes share a high identity at the amino acid level indicating a recent duplication event and may play a role in the detoxification of insecticide at these specific life stages. In adult *A. gambiae*, a CYP6Z1 gene was found to be over-expressed in the pyrethroid resistant strain.

We are currently investigating the role of CYP6P9 in the detoxification of pyrethroids in order to clarify the role of this enzyme in resistance. Given that other genes may also play a role in this complex resistance mechanism, studies are ongoing using microarrays to identify additional genes involved. The report by Wondji *et al*. (2007) of the presence of a major QTL on chromosome 2R conferring pyrerthoid

resistance and containing a cluster of CYP6 genes including CYP6P9 supports this gene's association with pyrethroid resistance in *A. funestus*. If the upregulation of this enzyme is responsible for detoxification of pyrethriods the resistance mutation is likely to be a change in a promoter or *cis*-acting regulatory region.

Experimental procedures

Biological material

Two laboratory colonies of *A. funestus* were used in this study: FANG originates from material collected in southern Angola and is susceptible to all insecticides (Hunt *et al.*, 2005). FUMOZ-R originates from material collected in southern Mozambique and has been selected for resistance to permethrin (Hunt *et al.*, 2005). These strains are kept at the Vector Control Reference Unit of the National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa. They are maintained in standard insectary environment of 25 °C with 80% relative humidity and 12 h day/night, 45 min dusk/dawn lighting cycle.

WHO insecticide susceptibility assays

Newly emerged FUMOZ-R male and female adult mosquitoes were separated into cages containing a 10% sugar solution. Ten replicates of 25–30 adult mosquitoes each, at either day 3 or 14 after emergence, were exposed to 0.75% permethrin treated papers for 1 h according to the standard WHO bioassay protocol (WHO, 1998). The 60 min knock-down and final mortalities 24 h post exposure were recorded.

Insecticide dose-response analysis

Cohorts of 3–5 day old adult females were collected from the FUMOZ-R and FANG strains and exposed to a series of permethrin concentrations using a method based on the CDC bottle bioassay procedure of Brogdon & McAllister (1998). Sets of 250 ml treated bottles were produced containing the following series of permethrin concentrations (μg active ingredient per 250 ml bottle): 0.1 μg; 1 μg; 10 μg; 25 μg; 50 μg; 100 μg; 250 μg; 500 μg and 1000 μg. Exposures at each concentration lasted 1 h. 25–30 females were exposed per bottle through 8 replicates per set of bottles. Final mortalities 24 h post exposure at each concentration was recorded. The results were converted to a logarithmic scale and the 50% lethal dose (LD_{50}) was calculated for each cohort. Mean LD_{50} values were compared between the FUMOZ-R and FANG strains using a 2 sample *t* test.

Cohorts of 4th instar larvae were collected from the FUMOZ-R and FANG strains and exposed to a series of permethrin concentrations according to the standard WHO method (WHO, 2005). Sets of bowls containing 100 ml distilled water were treated with permethrin using the following range of concentrations: 990 μg/ml; 19.4 μg/ml; 9.7 μg/ml; 0.37 μg/ml; 3.6 ng/ml. Exposures at each concentration lasted 24 h. 25–30 larvae were exposed per bowl through 5 replicates for FANG and 6 replicates for FUMOZ-R per set of bowls. Final mortalities 2 h post exposure at each concentration was recorded. The results were converted to a logarithmic scale and the 50% lethal concentration (LC_{50}) was calculated for each cohort. Mean LC_{50} values were compared between the FUMOZ-R and FANG strains using a 2 sample t test.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from ten three-day-old adult FUMOZ-R and FANG strains of *A. funestus* using Tri-reagent (Sigma, MO, USA). The RNA was deoxyribonuclease (DNAse)-treated. RNA extractions were repeated on the treated samples and the pellets were re-suspended in 20 μl of DEPC-treated water. The extracted RNA was quantified using a GeneQuant *pro* Spectrophotometer (semi-quantitative PCR) or NanoDrop® Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and stored in aliquots (5 μl) at –70 °C. To prepare cDNA, total RNA was primed with 500 μg oligo (dT) adaptor (5′-GACTCGAGTCGACATCGA-3′) (Amenya *et al.*, 2005) and reverse transcribed using Superscript III (Invitrogen, CA, USA) according to manufacturer's instructions.

Dot blot analysis

Dot blot analysis was carried out in order to identify the family of P450 that was over expressed in the resistant strain FUMOZ-R. Serial dilutions of RNA from both FUMOZ-R and FANG strains (from 25 μg to 0.049 μg) were spotted onto BioBond™- plus nylon membrane (Sigma, USA). The membranes were UV cross linked and hybridised using DNA probes synthesised by PCR and labelled with DIG according to manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). The templates used for preparing DIG probes were from plasmids containing the genes of interest and were confirmed by sequencing (Amenya *et al.*, 2005). Each gene was separately labelled with DIG followed by denaturing the labelled probe (25 ng) with 1 ml of hybridisation solution (Roche Diagnostics). Cocktail probes were prepared in a final volume of 10 ml hybridisation solution. Hybridisation was carried out independently with either one of the cocktails of probes for CYP4, CYP6 or CYP9 genes. The CYP4 cocktail probe consisted of 12 genes (CYP4C25, CYP4C41, CYP4C40, CYP4C36, CYP4D26, CYP4D27, CYP4D25, CYP4G21, CYP4H14, CYP4J11, CYP4J9 and CYP4J12). The CYP6 cocktail probe consisted of 6 genes (CYP6P1, CYP6P2, CYP6P9, CYP6Z1, CYP6M8 and CYP6M7) while the CYP9 cocktail contained 5 genes (CYP9J12, CYP9J13, CYP9J11, CYP9L2/L3 and CYP9M3). After hybridisation, membranes were washed twice in low stringency wash solution (2 × SSC, 0.1% SDS) at room temperature for 5 min and washed twice at high stringency (0.1 × SSC, 0.1% SDS) at 68 °C for 15 min. After washes, the anti-DIG antibody was used to bind to the labelled probes and antibody binding visualised using chemiluminescent substrate, CSPD (Roche Diagnostics). Each membrane was mounted onto an exposure cassette and a Kodak® Bio Max light film, Light-1 (Kodak, FL, U.S.A.) was placed on top and developed.

To determine the specific gene that was over expressed, FUMOZ-R and FANG, RNA were serially diluted from 50 μg to 0.098 μg and probed with individual CYP6 genes. The level of expression of each gene was compared between the two mosquito strains.

A cDNA coding for the ribosomal protein S7 (rsp7) (Salazar *et al.*, 1993) was cloned from *A. funestus* and sequenced (Accession number EF450776). The plasmid containing the partial fragment of rsp7 was DIG labelled and used as an internal standard for RNA loading in dot blot and northern blot analysis. The membranes were stripped and re-hybridised using the rsp7 probe. The dot blots were repeated four times with independent RNA preparations.

Northern blot analysis

Over expression of CYP6P9 was validated using northern blot analysis. The CYP6P9 probe was used to probe RNA on the membrane. RNA (5 μg) was electrophoresed on a 1.2% formaldehyde agarose gel and transferred by capillary action as described by Sambrook *et al*., (1989). The membrane was hybridised as described in dot blots with the rsp7 gene used as an internal standard.

Quantitative Real-Time PCR (Q-PCR)

Q-PCR was used to validate the northern analysis. Products were sequenced to confirm the identity of the amplified products. The sequence of the CYP6P9 from both strains was deposited in genbank (Accession numbers FANG: EF152577, FUMOZ-R: DQ324779.

Primer design

The full-length CYP6P9 gene sequence (Matambo *et al*., unpublished data) was used to design the specific primer using the Beacon Designer 3.0 software (Biorad, Hercules, CA, USA). The primer sequences are as follows: Cyp6real Fwd1 (5′-GAGGAAGTGAAGAAGCGACATC-3′) and Cyp6real Rev1, 5′-TGACGGTGAGAAGCGGAAC-3′). Rsp7 (EF450776) was used as a reference gene to normalise data (primer sequence as described above) and annealed to both FUMOZ-R and FANG.

Quantification of CYP6P9 expression using real-time PCR

The fold expression of CYP6P9 was quantified in triplicates using the iCycler IQ™ (Biorad, Hercules, CA, USA). RNA and cDNA synthesis for adults and early life stages was prepared as describe above. Each 25 μl reaction contained 12.5 μl SYBR Green supermix (Biorad) and 4 μl of both primers (25 μM), 2.5 μl cDNA (60–80 ng). The PCR program consisted of an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s. Data was collected during each extension cycle. This was followed by an auto-extension cycle of 72 °C for 4 min. The last step included a melting curve analysis. A standard curve was generated using 2-fold serial dilutions of cDNA (1 μg to 0.15 μg). The slope of the standard curve was used to validate the curve and those samples that did not attain the recommended slope of 3.3–3.8 and PCR efficiency of 90–105% were discarded (http://www.Qiagen.com). Fold over expression of CYP6P9 was calculated using the method described by Pfaffl (Pfaffl, 2001). Student *t*-test was used to validate significance of fold over expression.

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