Cloning Of Anopheles Gambiae CYP6M2 Gene Promoter And Construction Of Its Luciferase Reporter System

Balarabe R. Mohammed, Craig S. Wilding, PhillipJ. Collier, Yusuf Y. Deeni

Abstract: *CYP6M2* is a Cytochrome P450 gene involved in the detoxification of multiple classes of public health insecticidesin the malaria mosquito *Anopheles gambiae*. Some P450 genes are known to be up regulatedby the transcription factorsCnCC/ Keap 1 and (ss)/ (Tgo) in *Drosophilamelanogaster*. Whether this regulatory mechanism is involved inthe regulation of P450s in *Anopheles gambiae* is yet to be identified..In this study, we investigated cloning of 896 bp *CYP6M2* promoter and construction of its luciferase reporter system in *Anopheles gambiae*. Bioinformatics tools were used to search for 5' up stream region of *CYP6M2* promoter sequence and specific primers designed. The promoter region of *CYP6M2* (896 bp) was amplified using isolated the designed primers and isolated genomic DNA from insecticide resistant Tiassale and susceptible Kisumu strains of *An. gambiae*. The PCR products were cloned intopJET1.2/blunt vector. The resultant plasmid DNA was transformed into *Escherichia coli* XL1-Blue competent cells for propagation and purification, the plasmid constructs isolated and sequenced. Both cloned pJET1.2 and pGL3-Basic vectors were digested with *Bgl*II, purified and then ligated into the luciferase expression vector pGL3-Basic vector. The designed *CYP6M2* promoter constructs were confirmed by sequencing. Results revealed that the retrieved *CYP6M2* gene promoter sequence has 96% and 95% similarity for Kisumu and Tiassale cloned sequences respectively.Cloning of *CYP6M2* gene promoter and construction of its dual luciferase reporter system were successfully established, this will be essential in further studies aimed at understanding the regulatory mechanisms involved in insecticide resistance, which is important in the control of malaria.

Index terms: *Anophelesgambiae*,*CYP6M2*, *Drosophilamelanogaster*, malaria, insecticide resistance, PCR, P450s

1 INTRODUCTION

Cytochrome P450 (CYP) is one of the largest and most functionally diversified classes of heme-containing enzymes found in nature [1], [2]. These P450s constitute one of the oldest enzyme superfamilies'and are present in all living organisms including mammals, plants, bacteria, and insects [3], [4],[5]. Cytochrome P450s are found in almost all tissues and are important in the biosynthesis of several endogenous compounds, as well as metabolism of xenobiotics [6]. Insecticides are used widely in control of insects of public health importance including control of mosquito vectors of malaria [7].Increased insecticide detoxification mediated by over-expressedP450s is a common mechanism of insecticide resistance [8].

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Only a limited numberof Cytochrome P450s have been identified through microarray studies ofthe insecticide resistance phenotype as being repeatedly implicated in the resistance phenotype. With the increased threat tomalaria vector control caused by insecticide resistance attributed to P450s, there has been an interest in understanding the role and underlying mechanisms of this resistance [9] which will be useful in the development of more sensitive diagnostic tests for effective monitoring of metabolic based resistance development. In *Anopheles gambiae* genome alone,there are 111 annotated Cytochromes P450 [10], [11], [4]. Studies revealed that CYP6M2 gene in *Anopheles gambiae* is established to directly involved in the acquirement of insecticide resistance [12], [11],[13],[14],[15]. Whilst P450s such as CYP6M2 have been identified as having a role in resistance, the regulatory mechanisms underpinning the over-expression of this gene (and other P450s) is yet to be ascertained.Some Cytochrome P450 genes are known to be up regulated by Cap 'n' Collar isoform C(CnCC)/Kelch-like ECH-associated protein 1(Keap 1) and/Spineless (ss)/Tango in *Drosophilamelanogaster* [16],[17]. These are orthologs to Nuclear factor erythroid 2- related factor 2 (Nrf2)/ Kelch-like –ECH protein 1 (Keap 1) and Aryl hydrocarbon nuclear translocator (ARNT) signalling pathways in mammals respectively [18], [19], [20], [21]. This mechanism is yet to be identified in *Anophelesgambiae*. Previous studies in insects revealed the use of Luciferase assay to demonstrate this up regulation of Cytochrome P450 genesinvolved in insecticide resistance [22], [23]. In the present study, as a pre- requisite towards understanding these molecular mechanisms involved in insecticide resistance in *Anophelesgambiae*, we investigated the cloning of CYP6M2 gene promoter and construction of its luciferase reporter assay system. These are quintessential tools in the dual luciferase reporter assays.

2 EXPERIMENTAL APPROACHES

2.1 Mosquito strains

The Kisumu strain of *Anophelesgambiae*, a laboratory insecticide susceptible strain originally colonised from Kisumu, Western Kenya in 1953 [23] anda wild multiple insecticide resistant Tiassalekro (Tiassale) [24] strain originally collected from northwest of Abidjan in Ivory Coast [25] were used in this study.

2.2 Sequence Retrieval of CYP6M2 upstream region

In order to design the primers, the sequence of *Anophelesgambiae* CYP6M2 gene upstream regionwas identified from the VectorBase (Bioinformatics Resource for Invertebrate Vectors of Human Pathogens)(https://www.vectorbase.org/).All sequence 5' of the transcription start site and up to the large transposable element ≈900bp upstream was extracted. We hypothesised that all regulatory promoter elements would be limited to this region.

2.3 Designing primers for PCR

In order to isolate the putative promoter sequence, specific primers were designed and analysed for physical properties using primer 3.0 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and OligoAnalyzer 3.1 (Integrated DNA technologies, IDT) https://eu.idtdna.com/analyzerApplications/OligoAnalyzer/)in order to ensure stringent primerdesign parameters were utilised. The Nucleotide BLAST tool from VectorBase was used to perform a genome wide homology and alignment searches using ((https://www.vectorbase.org/clustalw)to verify the specificity of the designed primers to the *Anophelesgambiae* CYP6M2 upstreamsequence.

2.4 Computer based sequence analysis

A wide range of web-based tools were used for the CYP6M2 gene putative promoter sequences. The upstream sequences of CYP6M2 were submitted for analysis through the BLASTX.BLAST was accessed through NCBI (http://www.ncbi. nlm. nih.gov/) to search for sequence similarity. To compare reverse and forward strand sequences, the reverse strand was reverse complemented using the reverse and complement program web site (http://www.Bioinformatics.org/sms/revcomp.html) and the pairwise alignment undertaken using the VectorBase data(https://www.Vector base. org/search/site/CYP6M2)respectively.

2.5 Isolation of genomic DNA and Polymerase Chain Reaction (PCR)

The mosquito strains were kindly provided by Liverpool insect testing establishment (LITE) unit of the Liverpool School of Tropical Medicine (LSTM). Genomic DNA was extracted from both Kisumu and Tiassale strains of the *Anophelesgambiae*using the Qiagen DNEasy kit (Cat. # 69561, Qiagen, Valencia, CA, and USA). The concentration and purity of the isolated DNA were analysed using Nanodrop 1000 Spectrophotometer. The designed primers forthe upstream of CYP6M2 region were used to amplify this regionfrom the genomic DNA ofsusceptible Kisumu(Kis) and resistant Tiassale (Tias) strains of *Anophelesgambiae* (Table 1). The PCR conditions using HOT start DNA polymerase (Cat. # F-549S/L, Thermoscientific, Pittsburgh, PA, USA) were as follows: Initial denaturation at 98^0C for 30s, followed by 30 cycles at 98^0C for 10s, 57^0C for 15s, and 72^0C for 30s with a final 5 min extension at 72^0C.

2.6Cloning and sequencing of CYP6M2 promoter

PCR products were purified using a Thermo fisherDNA purification kit (Cat. #K0701, Thermoscientific, Pittsburgh, PA, USA) and ligated into CloneJET™ PCR cloning system (Cat. #K1231, Pittsburgh, PA, USA) and sequenced. Ligation reactions were carried out by incubating 10 µl of 2X reaction buffer, 1 µl vector (50 ng/µl), 2 µl purified PCR product (100 ng/µl), 1 µl T4 DNA ligase and 6 µl sterile distilled water (to 20 µl) at 22^0C for 30 min. Ligation mixtures were transformed intoXL1-Blue competent cells (Cat. # 200249, Stratagene, Santa Clara, CA, USA) and positive colonies were identified by colony PCR using vector specific primers. Sequencing was undertaken by Source Biosciences. Consensus sequences for both strains were aligned using EMBOSS (http://www.ebi.ac.uk/emboss/align/).

2.7 Construction of luciferase reporter system of CYP6M2 promoter

The CYP6M2_Kis and CYP6M2_Tias inserts were cut from the pJET1.2/blunt vector by digestion with B*gl*II restriction enzyme (Figure 2) on the bench after an overnight Methylene blue staining (MT-Blue staining) and gel purified (Cat. # 28704, Qiagen, Hilden, Germany) before ligation into pGL3-basic vector (Cat.#E1741,Promega,Madison, WI, USA).

2.8Preparation of PGL3-Basic Vector

Recombinant pGL3-Basic vector was transformed into*E.coli*and spread onto 5 mL LB agar containing 100 µg/mL ampicillin plates and incubated at 37^0C overnight for colonies to grow, then cultured overnight in LB broth containing 50 µg/mLampicillin at 37^0C with shaking in order to grow up for storage as glycerol stock.

2.9 Restriction digests of CYP6M2_Kis, CYP6M2_Tias and pGL3-Basic vector

The pGL3- Basic vector was digested using *Bgl*II restriction enzyme (Figure 2). The digested pGL3 -Basic vector was then treated with calf intestinal alkaline phosphatase (CIAP) (Cat. #F-201L, Thermoscientific, Pittsburgh, PA, USA) which dephosphorylates the 5' end so that plasmid self-ligation will not occur. This will enhance the obtention of positive clones as only bacterial transformants harvesting a plasmid with an insert will grow in ampicillin containing medium.Vector was purified using Qiagen mini-prep kit (Cat. #28704, Qiagen, Hilden, Germany) before ligation with the digested CYP6M2_Kis and CYP6M2_Tias inserts. The resultant construct was confirmed by *Bgl*II enzyme restriction digestion and the sequence analysed using the RVprimer3 and GLprimer2.

2.9 PCR Colony screening

In order to ascertain the presence of the insert within the pGL3-Basic vector and in the correct orientation, RVprimer3 (Reporter Vector Primer 3) (5' CTA-GCA-AAA-TAG-GCT-GTC-CCC 3') (Promega, Madison, WI, USA), a sequencing primer designed for use with pGL3 Luciferase vector.

3 RESULTS

3.1Sequence identification of CYP6M2_Kis and CYP6M2_Tias promoter

The sequence of CYP6M2(Accession number AGAP008212) putativepromoter was retrieved from VectorBase. Due to the presence of a large transposable element upstream of CYP6M2 only a total of 896 bp was considered as putative upstream regulatory region. Using Primer3 primers to amplify this upstream region from Wild and lab colonies of *An. gambiae* were designed. Table 1 showed the specific forward (tRNA-1) and reverse primers (CYP6M2_START) designed using primer3 (version 0.40).

Table 1: Primer Design – CYP6M2 896 bp promoter

Primer	sequence	Length (bp)	GC (%)	Tm (^{0}C)
tRNA-15'	GAACCCACGACCCTGAGAT 3'	19	57.9	56.6
START	5' ATTTTTGGAACGCGAGGAG 3'	19	47.4	53.3

3.2 Polymerase chain reaction (PCR) and DNA sequencing of CYP6M2 upstream region from susceptible and resistance *An. gambiae* strains

PCRproducts amplified from the Kisumu and Tiassale strains were 896 bp and 930 bp respectively (**Figure** 1).

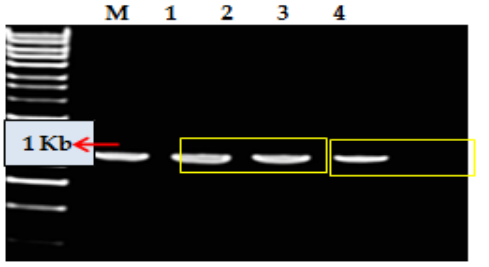

Fig.1: 1% Agarose gels showing the amplification of CYP6M2 promoter using genomic DNA from *Anophelesgambiae* 5µl each of (Kisumu -L2 & L3 (White box) and Tiassale- L4& L5 (Yellow box) mixed with 1 µl of loading dye (Bioline) all with a molecular weight of

3.5 Cloning of CYP6M2 putative promoter

In order to propagate the cloning of CYP6M2_Kis (896 bp) and CYP6M2_Tias (930 bp) PCR products, the promoter sequences were respectively ligated into the Clone JET PCR Cloning Vector.

3.4 DNA SEQUNCING

The PCR products were cloned, purified and sent with pJET1.2 forward and pJET1.2 reverse primers for DNA sequencing and the cloned sequences were analysed using NCBI blast tool. Two sequences from the Kisumu strain and two from the Tiassale strain were generated. These sequences varied in length and sequence from that of the PEST strain of *An. gambiae* retrieved from VectorBase with CYP6M2_Kis 1(914 bp, 94%), CYP6M2_Kis 2(878 bp 91.5%) CYP6M2_Tias 1 (928 bp 92.6%) and CYP6M2_Tias 2 (928 bp, 92.6%) similarity to *Anopheles gambiae* chromosome 3(3R).

3.5 Construction of Luciferase reporter vectors

*Bgl*II restriction enzyme was used to perform a single enzyme digest of both the pJET-CYP6M2_Kis and pJET-CYP6M2_Tias gene construct and pGL3-Basic vector (Figure 2). This was possible owing to multiple *Bgl*II recognition sites on both sides of the multiple cloning regions in the CloneJET PCR cloning Vector and pGL3 - Basic Vector.

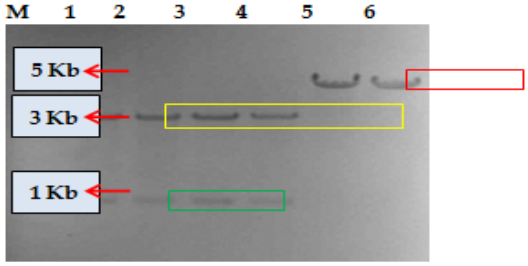

Fig.2: 1% Agarose gel showing *Bgl*II restriction digest of the purified pJET1.2 2974 bp (Yellow box), The insert (L1-L2-CYP6M2_Kis-1-2(White box) and L3-L4 CYP6M2_Tias-1-2(Green box). L6-L7 (Red box) is digested pGL3- basic vector (4818bp) respectively using the MT blue staining protocol. M is 5µl of the molecularweight maker (10 Kb from Bioline) loaded. **The gel was run for 40 min at 120V.**

The cloned CYP6M2 gene promoter fragments were excised from their respective gels on the bench followingmethylene blue (MT) Blue staining and purified. High cloning efficiencies are attained when cloning vectors are treated with alkaline phosphatase following *Bgl*II restriction digestion. Singly-cut vector will be recircularized during the ligation step. Dephosphorylation of the 5' phosphate group prevented this process thereby increasing the efficiency of the insert ligation in the plasmid [21]. *Bgl*II restriction digest of the pGL3-CYP6M2_Kis andpGL3-CYP6M2_Tias gene constructs was carried out on the Midiprep DNA samples to further verify the successful ligation and amplification of gel purified DNA. This was made possible due to the presence of multiple *Bgl*II recognition sites on both sides of the multiple cloning. The concentration of each plasmid was adjusted to 600 ng/ µl using a Nanodrop Spectrophotometer.

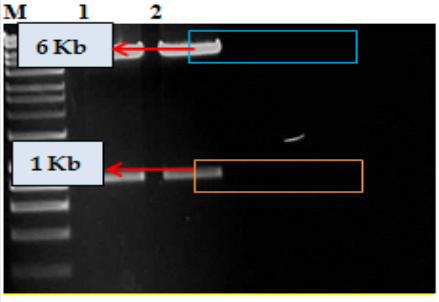

Fig.3: 1% Agarose gel showing the *Bgl*II restriction digests after loading 5 µl LI Kisumu and L2 Tiassale plasmids mixed with 1 µl loading dye. L1 and L2 showed bands of about 1Kb and 6 Kb for the CYP6M2_Kis and CYP6M2_Tias inserts (Orange box) and pGL3-Basic vector (Blue box) respectively. While M is 5µl of the molecular weight maker (10 Kb from Bioline) loadCYP6M2_Tias inserts (Orange box) and pGL3-Basic vector (Blue box) respectively. While M is 5µl of the molecular weight maker (10 Kb from Bioline) loaded. **The gel was run for 40 min at 120 V.**

4 DISCUSSIONS

CYP6M2 has been repeatedly identified as up-regulated in insecticide resistant mosquitoes and demonstrated to be capable of insecticide metabolism leading to insecticide resistance, thereby making this gene an attractive target for further studies. Little is known about the regulatory mechanisms of CYP6M2 gene expression. In this study, 896 bp 5' upstream region of CYP6M2 gene was amplified by PCR using genomicDNA from both Kisumu and Tiassale strains of *Anophelesgambiae*as template for PCR. The concentrationof the genomic DNA was 100 ng/µL and 110 ng/µL for the Kisumu and Tiassale strains respectively, though not always reliable and reproducible using the Nanodrop in comparison to fluorescent nucleic acid stain (Pico Green)[27], but were more than the required 5 to 10 ng/µL for a successful PCR.The vector contains an expanded multiple cloning site, as well as a T7 promoter for in vitro transcription. The blunt ends of the vector contain 5'-phosphoryl groups. This enhances re-circularization (self-ligation) of the plasmids. The terminal 5-phosphate residues from both termini of the plasmid DNA with alkaline phosphatase.*Bgl*II restriction digest of the plasmid DNA in preparation for ligation into pGL3-Basic vector was carried out as shown in Figure **2**. The PCR products were subsequently extracted from gel on the bench after an overnight Methylene blue staining (MT-Blue staining). Exposure to UV light and Ethidium bromide were therefore avoided asoverexposure would result in the degradation of DNA and the formation of pyrimidine dimers while the Ethidium bromide also intercalates with the DNA thereby causing disruption of its integrity [28].In order to confirm the size and orientation of the CYP6M2 promoter, *Bgl*II restriction digests of the pGL3-CYP6M2_Kis and pGL3-CYP6M2 _Tias plasmids were carried out as shown in Figure 3). The respective bands were excised and gel purified in accordance with the previously highlighted protocol in the experimental approach. Gel purified CYP6M2_Kis and CYP6M2_Tias samples were ligated into the respective pGL3-Basic Vectors. Bacterial transformation, clonal selection and Midiprep of the CYP6M2_Kis and CYP6M2_Tias constructs and pRL-CMV using QiaPrep spin columns. Nucleotide sequence analysis of the *Anophelesgambiae* CYP6M2 putative promoter revealed that there are several potential transcription factor binding sites (TFBS).

5. CONCLUSION

The 5' upstream region of CYP6M2 gene fromboth Kisumu and Tiassale strains were successfully isolated from *Anophelesgambiae*. Based on the sequencing result and bioinformatics analysis, several overlapping consensus sequence on the 5' 896 bp CYP6M2 promoter hypothesised to be the main regulator for CYP6M2 expression were identified. The CYP6M2 luciferase reporter systems were also successfully constructed. In the future, luciferase reporter assays can be conducted in order to investigate the effects of endogenous and xenobiotic substances on CYP6M2 up stream region hypothesised to contain the promoter. This will provide information on the understanding of the regulatory mechanisms involved in insecticide resistance in *Anophelesgambiae.*

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