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Original Article

SIMULTANEOUS DETECTION OF MULTIPLE POLYMORPHISMS IN *CYP2B6* AND *OPMR1* BY NESTED PCR: A GOOD APPROACH FOR PATIENTS ON METHADONE

AHMAD AL-MEMAN AA[1,2*], RUSLI ISMAIL[2], NURFADHLINA MUSA[3], NASIR MOHAMAD[4]

[1]College of Pharmacy, Qassim University, Unaizah, Al-Qassim, Kingdom of Saudi Arabia, [2]Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia (USM), Kelantan, Malaysia, [3]Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia (USM), Kelantan, [4]Faculty of Medicine and Medical Sciences, Universiti Sultan Zainal-Abidin, Terengganu, Malaysia. Email: ahmadalmeman@gmail.com

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ABSTRACT

Objective: Cytochrome P450 2B6 (CYP2B6) is involved in the metabolism of several therapeutically important drugs and abused intoxicants including methadone. The preferential binding target for methadone is the μ opioid receptor OPRM1. Various SNPs in *CYP2B6* and *OPRM1* may contribute to clinical outcomes in methadone maintenance therapy. The aim of the present study was to develop a consistent and robust assay to identify such genotypic variants.

Methods: We randomly selected patients in the Malay population who were addicted to heroin and had already commenced methadone therapy. We developed a two-step, multiplexed, nested PCR method to simultaneously identify 26 SNPs in *CYP2B6* and *OPRM1*. Three amplicons covering seven regions of interest were amplified in the first PCR step. These amplicons were then used as templates for 11 sets of reactions in the second PCR step.

Results: We successfully amplified DNA fragments containing 26 SNPs of interest from the DNA samples provided by patients receiving methadone therapy: C534T, G779A, G13076A, A454G, G24A, G820A, G942A, G31A, G438A, T106C, C440G, T397A, T15708C, T480C, C17T, G794A, A13072G, A76T, A62T, C64T, A136G, C15618T, G691C, G15631T, T802C, and A118G.

Conclusion: The PCR-based method was sensitive and specific and is the first nested, allele-specific, multiplexed PCR method reported for the simultaneous detection of all 26 clinically important SNPs of *CYP2B6* and *OPMR1*.

Keywords: Nested PCR, CYP2B6, OPMR1, Methadone, Opioids.

INTRODUCTION

Methadone is a μ-receptor agonist valued for its effectiveness in the treatment of opioid dependency and the management of pain. It has high, 70-90%, oral bioavailability and a long plasma elimination half-life when administered in a racemic mixture [1–4], as shown by studies of differential blood-methadone concentrations among samples of the approximately one million people currently on Methadone Maintenance Therapy (MMT)[1] [5–7] and also by pharmacogenomic studies [2,7,8]. Methadone metabolism is attributed primarily to the CYP3A4, CYP2B6, and CYP2D6 proteins in humans [2,9–12]. The detection of various single nucleotide polymorphisms (SNPs) involved in methadone action *in vivo* could help to identify an individual's susceptibility to methadone toxicity and improve toxicological interpretation. There is much variability among individuals in the expression and enzymatic activities of the Cytochrome P450 (CYP) enzymes affecting methadone clearance [13]. The *CYP* genes are highly polymorphic, with interethnic differences in allele frequencies [14].

The importance of pharmacogenomic research for determining patient-specific drug susceptibilities and elucidating drug toxicities is increasing progressively. Hence, genotyping pharmacologically relevant SNPs may be a useful forensic tool [15–17]. It has been shown that most of the pharmacologically important SNPs are located within genetic coding regions (exonic domains), and such regions are ideal candidates to determine differential drug responses [18]. A number of important SNPs have also been identified within critical gene promoters, which are noncoding regions [19]. The differential drug metabolism caused by genetic variation for the CYP enzymes and in the μ opioid-receptor gene *OPRM1* are recognized as key variables determining an individual's sensitivity to opioids [7,20,21]. The *CYP2* gene family has several subfamilies that are located in clusters within the human genome. *CYP2B* is located in one such cluster containing six subfamilies (*CYP2A*, *CYP2B*, *CYP2F*, *CYP2G*, *CYP2S*, and *CYP2T*) on human chromosome 19 [22]. There are two known human *CYP2B* loci: the functional *CYP2B6* and its pseudogene, *CYP2B7*, located inside a block of ~112 kb in the middle of the *CYP2A18P* locus, between 19q12 and 19q13.2 [22,23-25]. *CYP2B6* comprises nine exons encoding a protein of 491 amino acids [24]. In addition to the wild-type allele, several *CYP2B6* allelic variants (C64T, C777A, A785G, C1459T, G516T, A785G, G516T, A785G, C1459T, A415G, and G516T) have been identified [23,26]. Previous reports have found that *CYP2B6**6 (G516T and A785G) was associated with higher (S)-methadone plasma levels and a prolonged QTc interval but did not have a major influence on the methadone dose requirement [7,10]. Furthermore, those results were specific to (S)-methadone, which is known to contribute only scarcely to the activation of OPRM1. Hence, the *6/*6 genotype did not impact on clinical outcomes [7,10]. Genetic variation within the opioid receptors may also affect methadone pharmacodynamics. OPRM1 is of particular interest, because it is the preferential binding target of methadone. A number of *OPRM1* variants have been identified, and the A118G missense mutation in exon 1 has been linked to significant reductions in β-endorphin binding [27], increased morphine requirements [28], protection from morphine-6-glucuronide (M6G)-induced toxicity [21], and increased susceptibility to drug addiction [29]. Therefore genetic variants, such as the *OPRM1* A118G variant, could affect drug binding and patient responses.

The second-most-studied SNP in *OPRM1* is C17T. The suggested amino acid change resulting from this SNP is alanine to valine [23]. Several other SNPs in *OPRM1* have been studied, including: G172T, IVS2 G31A, G877A, C2078G, C440G, A454G, G794A, T802C, G1401C, C17T, G24A, G779A, and G942A. Some of these SNPs lead to amino acid changes, and others have not yet been studied [27]. Methods have been developed to investigate *CYP2B6* and *OPRM1* individually. Matrix-assisted laser desorption/ionization time-of-flight spectrometry was used to detect *CYP2B6* alleles, and it proved to be a superior technology [30]. The method is known to be accurate, reproducible, and highly sensitive for detection, but it also is very expensive. In the present study, we aimed to develop a rapid, inexpensive, and robust multiplex PCR method for the simultaneous detection of clinically relevant *CYP2B6* and *OPMR1* polymorphisms.

MATERIALS AND METHODS

The study was granted ethical approval by the ethical committee of the University of Malaya, as a part of a large study of HIV and heroin addiction. This prospective, one-phase experimental clinical pharmacogenetic study was performed by the Institute for Research in Molecular Medicine (INFORMM), Malaysia. Chemicals were obatiend from Promega (Promega Corporation, Madison, WI, U.S.A.) and Sigma (Sigma Chemical Co. Ltd. St. Louis, MO, U.S.A.). Chemicals and equipment are listed in Tables 1 and 2; respectively.

Participants

Patients were selected by convenience sampling from four different clinics: the University Malaya Medical Centre, Insaf Murni Clinic, Dr. Khafiz's clinic, and Klinik Putri. We randomly selected 100 patients who were addicted to heroin and who had been visiting one of the clinics regularly for MMT. Upon understanding and accepting our experimental policy and procedure, the patients were invited to sign an informed consent form. After the patients gave their informed consent, 10 ml of blood was withdrawn from each patient in citrated tubes.

Table 1: Chemicals and Reagents used for DNA extraction

Supplier	Chemicals and Reagents
QIAGEN, Foster City, U.S.A	AW,AE Lysis Buffers
Promega, Promega Corporation, Madison U.S.A	Agarose LE Analytical Grade
Biotool, Biotechnological & Medical Laboratories, S.A. Spain	Biotools® DNA Polymerase (recombinant E. coli), 1U/ul
Promega, Promega Corporation, Madison U.S.A	Boric Acid
Boehringer Mannheim ®, Roche Molecular Biochemicals, GmbH-Germany	Deoxynucleoside Triphosphate set 100 uM
Promega, Promega Corporation, Madison U.S.A	DNA ladder 1kb
Promega, Promega Corporation, Madison U.S.A	DNA ladder 100 kb
Promega, Promega Corporation, Madison U.S.A	Ethanol 96-100%
Promega, Promega Corporation, Madison U.S.A	Ethidium Bromide (10mg/ml) Molecular Biology Grade
Promega, Promega Corporation, Madison U.S.A	Ethylenediamine-tetraacetic acid (EDTA) Ultra Pure Grade
Promega, Promega Corporation, Madison U.S.A	Magnesium Chlorid 50 mM
Sigma Chemical Co. Ltd., St Louis. MO. U.S.A	Potassium Chloride Molecular Biology Grade
Promega, Promega Corporation, Madison U.S.A	Tris base Ultra Pure Grade
Promega, Promega Corporation, Madison U.S.A	Triton X-100 Ultra Pure Grade
Invitrogen, California, U.S.A	PCR primers
Mili-Q® Reagent Water System, Milipore MA, U.S.A	Ultra pure water: Distilled, deionized (Ω 18.2)
QIAGEN, Foster City, U.S.A	QIAGEN Protease

Table 2: Instruments for PCR Genotyping

Supplier	Instruments
Primo Submarine Gel System, ThermoQuest, E-C apparatus Division, Holbrook, New York.	Electrophoresis Tank Mini (EC320 and Maxicell® (EC340)
ThermoQuest, E-C apparatus Division, Holbrook, New York.	Electrophoresis Power Supply EC 250-90
Vilber Lourmat, Cedex, France	Gel Documentation System Bio-Vision 1000
Perkin Elmer Applied Biosystems, Roche MolecularSystems, Inc. and F.Hoffman-La Roche Ltd, U.S.A	GeneAmp PCR System 9700®
Eppendorf, Hamburg, Germany	Micropipette
Eppendorf, Hamburg, Germany	Minicentrifuge, temperature controlled
IKA®-Worke, GmbH and Co.Germany.	MS1 Minishaker
Thermo Fisher Scientific, U.S.A	NanoDrop
Mettler-Toledo GmbH, Process Analytics, Industrie Nord, Urdorf	pH meter MP320U
Shimadzu Corporation, Japan	Spectrophotometer Shimadzu UV-1601
Grant Instruments Ltd, England	Temperature Controlled Water Bath Grant Y28
QIAGEN, Foster City, U.S.A	QIAmp DNA Mini Kit
Germany	Pulse-Vortexing
Sartorius, Goettingen, Germany	Weighing scale CPA6202S

DNA Extraction

Genomic DNA was extracted from the blood samples and purified by spin protocols using the QIAamp DNA Mini and Blood Mini Purification Kit® following the manufacturer's protocols (QIAGEN, Foster City, CA, U.S.A).

Determination of DNA Concentration and Purity

The extracted DNA was quantified and tested for purity using a spectrophotometer (NanoDrop, Thermo Fisher Scientific, MA, U.S.A.) prior to PCR amplification. The concentration of the genomic DNA was standardized to 100ng/µl. The purity was estimated from the ratio of absorbance readings at 260 nm and 280 nm. Distilled water was used to initiate the testing process, and 1µl AE buffer ((QIAGEN, Foster City, CA, U.S.A)) was used as a blank. Duplicates of the readings were obtained for all samples, and mean values were recorded. The purity was accepted if the 260/280 ratio was from 1.7 to 1.9. The concentration of the DNA in ng/µl was calculated by the NanoDrop software.

Determination of DNA Integrity

To test the integrity of the extracted genomic DNA, 5ml of the sample was separated by gel electrophoresis at 50V for approximately 2h or until the loading dye was 1cm away from the bottom of the gel. The process was confirmed by the appearance of the relevant DNA bands with the expected sizes (30–50 kb).

PCR-Based Detection of *CYP2B6* and *OPRM1* Polymorphisms

A PCR protocol using specifically designed primers was developed to detect the *CYP2B6* alleles *1, *2, and *9. Similarly, a separate PCR protocol was developed to detect 17 SNPs in *OPRM1*. Then, a two-step, multiplexed PCR protocol was developed to simultaneously detect all 26 SNPs in both genes combined. Samples were tested in batches, 20 each, according to the PCR feasibility. Every patient wastested for all the SNPs. In the first step, three amplicons covering seven regions of interest containing the SNPs were created. In the second step, 11 additional sets of reactions amplified each of the 26 SNPs (Fig. 1). The amplicons created by the first PCR served as templates for the second PCR. In the second PCR, specific primers were used to detect the SNPs of interest in the amplicons.

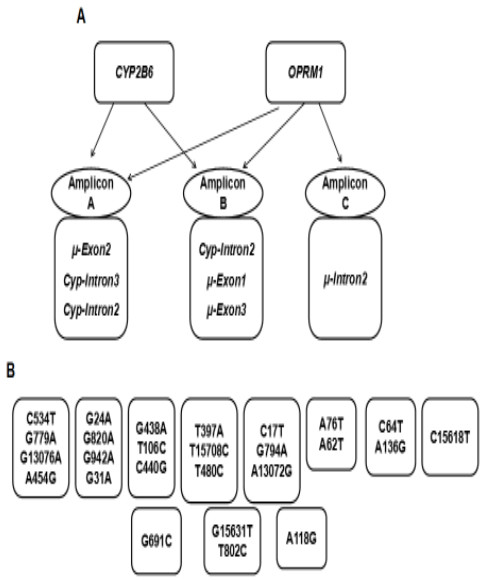


Fig. 1: Schematic design for the optimization of nested PCR reactions. (A) Amplification sites of amplicons A, B, and C covering regions of CYP2B6 and OPRM1 in the first PCR multiplex reactions. (B) Expected SNPs within CYP2B6 and OPRM1 amplified in the second PCR.

Sequence Alignments and Primer Design

The reference sequences used for CYB2B6 and OPRM1 were retrieved from the online National Center for Biotechnology Information (NCBI) database (accession number: M29874 and AH006705.1; retrospectively). Specific primers were designed manually to detect each of the 26 SNPsin a two-step nested PCR. The primers used in the first PCR were designed to amplify three amplicons (A, B, and C), which contained all of the required regions of both genes (Table 3). The primers used in the second PCR were designed to have specific 3´ ends that could distinguish single-nucleotide variations of interest (Table 4). The BLAST program at the NCBI website was used to align the primers to the reference sequences to check for primer specificity.

PCR Optimization

Optimization of the First PCR

The first PCR step generated three amplicons (A, B, and C) covering all of the required genomic regions. The conditions for each amplicon were optimized to avoid non-specific bands, gain the highest yield, and acquire a suitable intensity. Amplicons were separated in such a way to ensure optimal compatibility. The optimized PCR included 2µl of the primer mix, 2µl(200ng) genomic DNA and 10µl master mix (6.05µl dH_2O, 2.5µl 1X Biotool® PCR Buffer [B&M Labs, Madrid, Spain] 0.75µl amplicons A and C or 1µl amplicon B, 0.75µl $MgCl_2$, 0.5µl 2mM dNTPs [Promega], primer sets (Invitrogen, Frederick, Maryland, USA), and 0.2µl Biotool® Taq Polymerase [B&M Labs, Madrid, Spain]) (Table 5). The PCR amplification was performed using the Perkin Elmer Gene AmpR PCR System 2400 (Applied Biosystems, Foster City, CA, U.S.A.). The optimal cycling conditions for generating the three amplicons were: pre-denaturation at 94°C for 2min; 35 cycles of denaturation at 94°C for 30s, annealing at 59°C (52°C for amplicon A) for 30s, and extension at 72°C for 2 min. Upon completion of 35 cycles, the reaction was continued at 72°C for another 7 min. The PCR product was stained with ethidium bromide and analyzed by electrophoresis in 3.5% agarose gel at 130V for 1h. The combination of primers in each PCR and the expected fragment size of the products are listed in table 3.

Optimization of the Second PCR

Following a successful application in the first PCR step, the resulting amplicons were used as templates for the second PCR step to amplify the specific SNPs. The reaction mixture (Table 6)for the second PCR step comprised 2.5µl 1X Biotool® PCR Buffer, 0.75µl 2mM $MgCl_2$, 0.5µl 2mM dNTPs, 2 µl primers, 1µl 1U Biotool® Taq Polymerase, and 2µl genomic DNA for a total volume of 10µl. The 11 PCRs for the amplification of all 26 SNPs were performed with the following cycling conditions: pre-denaturation at 94°C for 2min; 15 cycles of denaturation at 94°C for 30s, annealing (at 63°C for set 1; 67°C for sets 2, 5, and 7; 65°C for sets 3, 4, and 10; 50°C for set 6; 58°C for set 8; and 66°C for set 9) for 30s, and extension at 72°C for 1min. A final extension was performed at 72°C for 7min after the completion of 15 cycles. The products were stained in ethidium bromide and resolved by electrophoresis in 3.0% agarose gel at 80V for 1h. If a band was questionable, the PCR was repeated until a clear band was evident. The combination of primers in each PCR and the expected fragment size of the products are listed in Table 4.

The reproducibility and specificity of the PCR were tested following the optimization process. Positive and negative controls were included in routine PCR batches as quality control seeds to avoid potential assay errors. We used autoclaved distilled water as a negative control in our PCR runsto eliminate false-positive results. Samples in which mutations were detected were sequenced and then used as positive controls in subsequent tests. All the positive controls and negative controls were used in routine PCR batches as quality control seeds to avoid any potential assay errors. It should be noted that no internal control was used in our study.

Interpretation of the PCR bands

Each sample was amplified twice in parallel. Each paired reaction included a common primer and either a wild-type primer or a

mutant-type primer. Upon successful amplification, the size of the amplicon was determined by gel electrophoresis. If a single band appeared in the reaction containing the wild-type primer, the allele was considered to be wild type. If a single band appeared in the reaction containing the mutant primer, the allele was considered to be mutant type. Both alleles were considered homozygous if the relevant band appeared in only one of the paired reactions. If the relevant bands appeared in both paired reactions, the allele was considered heterozygous (Fig. 2).

DISCUSSION

Being the most clinically related to methadone, CYP2B6 and OPRM1 are always of concern when therapeutic optimization is an issue. Both genes may contribute to the variability in dosing and hence the success of the treatment plan. The availability of both in one test could ease the genetic testing and make it attractive for clinicians (18–21). Expecting the optimal dose may help the clinicians to decide the duration for escalating the dose and hence improve the patient's compliance.

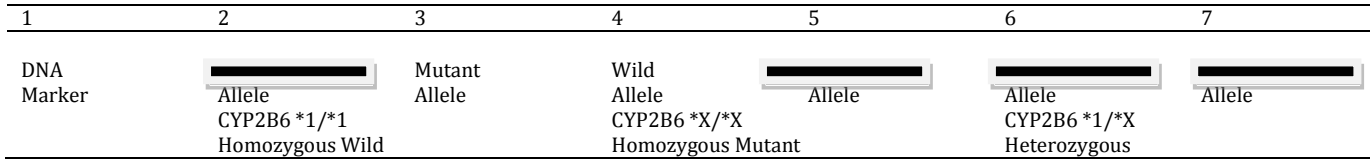


Fig. 2: Interpretation of Gel electrophoresis

RESULTS

The following tables show the results of the optimized method and the electrophoresis details:

Table 3: Primer sequence of first PCR

Amplicon	Primer Name	Primer Sequence	Optimized Primer Concentration (pmol)	Fragment Size (bp)
	Fw: µEx2	5'-TTC TCA CTC TTC TTC CTT TAT CTC-3'	0.4	
A	Rv: µEx2	5'-GAC TAA GAC AAT GGG GCT CTC CA-3'	0.4	483
	Fw: Int3	5'-TAA ACT GGA GCT AAT AAT CAA-3'	0.2	
	Rv: Int3	5'-CTC AGT CTC TCT CTG ACT CTA-3'	0.2	616
	Fw: Int1	5'-CAG GAC CAT GGA ACT CAG CGT-3'	0.01	
	Rv: Int1	5'-GTA CCA AGG CAA GAA GCA GCT-3'	0.01	230
	Fw: Int2	5'-AAC TTC TTC TAC AAC CAA CCC-3'	0.3	
B	Rv: Int2	5'-TCA TTG TCT TTC TTT CCT ATT CA-3'	0.3	222
	Fw: µEx1	5'-AAA GTC TCG GTG CTC CTG GCT-3'	0.5	
	Rv: µEx1	5'-TGG GAG TTA GGT GTC TCT TTG TA-3'	0.5	420
	Fw: µEx3	5'-GCC TTA AGT TAG CTC TGG TCA-3'	0.1	
	Rv: µEx3	5'-TGT CAT CCC CAG TAG ATA TAC C-3'	0.1	677
	Fw: µInt2	5'-TAG ATT TCC GTA CTC CCC GAA-3'	0.05	
C	Rv: µInt2	5'-CGC AAG ATC ATC AGT CCA TAG-3'	0.05	1020

Table 4: Primer sequence of second PCR

No	Primer Name	Primer Sequence	Fragment Size (bp)	SNPs	Set
1	Fw: 454A	GGT GAA TAT GCT GGT GAA CAT GTT	215	A454G	1
2	Fw: 454G	GGT GAA TAT GCT GGT GAA CAT GTC			
3	Fw: 534C	GGT TCA TTT TTG TTC CCT GAA C	397	C534T	
4	Fw: 534T	GGT TCA TTT TTG TTC CCT GAA T			
5	Fw: 779G	GCT ATG GAC TGA TGA TCT TGC G	448	G779A	
6	Fw: 779A	GCT ATG GAC TGA TGA TCT TGC A			
7	Fw: 13076G	GGA CTT CGG GAT GGG AAA GCG	120	G13076A	
8	Fw: 13076A	GGA CTT CGG GAT GGG AAA GCA			
9	Rv: 24G	ATG AGT GCA ATT GCT GGC GTT C	102	G24A	2
10	Rv: 24A	ATG AGT GCA ATT GCT GGC GTT T			
11	Rv: 820G	GAT CCT TCG AAG ATT CCT GTC	312	G820A	
12	Rv: 820A	GAT CCT TCG AAG ATT CCT GTT			
13	Rv: 942G	TGC CAA GAA ACA GTC TGG AAC	434	G924A	
14	Rv: 942A	TGC CAA GAA ACA GTC TGG AAT			
15	Rv: 31G	AAC ATA TCA GGC TGT GAA CCC	162	G31A	
16	Rv: 31A	AAC ATA TCA GGC TGT GAA CCT			
17	Fw: 438G	AAC ATC CAT TAC CTG GAG CCG	492	G438A	3
18	Fw: 438A	AAC ATC CAT TAC CTG GAG CCA			
19	Fw: 106T	GCC CCA TTG TCT TAG TCA CAT	824	T106C	
20	Fw: 106C	GCC CCA TTG TCT TAG TCA CAC			
21	Fw: 440C	CCA TCC TTT GCA AGA TAG TGA TCT C	330	C440G	
22	Fw: 440G	CCA TCC TTT GCAAGA TAG TGA TCT G			
23	Rv: 397T	GTT TTC ACT TCA TTT TTT GAT GGA	531	T397A	4
24	Rv: 397A	GTT TTC ACT TCA TTT TTT GAT GGT			

25	Fw: 15708T	CAA GAT CAA GAG TTC CTG AAG AT	315	T15708G	
26	Fw: 15708G	CAA GAT CAA GAG TTC CTG AAG AG			
27	Rv: 480T	CTA GAT CCT CCA TGA GGT CTA	611	T480C	
28	Rv: 480C	CTA GAT CCT CCA TGA GGT CTG			
29	Rv: 794G	CTT TGG AGC CAG AGA GCA TGC	285	G794A	5
30	Rv: 794A	CTT TGG AGC CAG AGA GCA TGT			
31	Fw: 17C	TAC CAT GGA CAG CAG CGC TGC	367	C17T	
32	Fw: 17T	TAC CAT GGA CAG CAG CGC TGT			
33	Rv: 13072A	ACC GCT CCT CCA CAC TCC GCT T	136	A13072G	
34	Rv: 13072G	ACC GCT CCT CCA CAC TCC GCT C			
35	Rv: 76A	TGG TGG GAG GCG GTC ATG GGT	121	A67T	6
36	Rv: 76T	TGG TGG GAG GCG GTC ATG GGA			
37	Rv: 62A	CAT GGG TGT TAG GGT GGC GCT	89	A62T	
38	Rv: 62T	CAT GGG TGT TAG GGT GGC GCA			
39	Fw: 64C	TCT TGC TAC TCC TGG TTC AGC	179	C64T	7
40	Fw: 64T	TCT TGC TAC TCC TGG TTC AGT			
41	Fw: 136A	CTT TTG GGA AAC CTT CTG CAG A	108	A136G	
42	Fw: 136G	CTT TTG GGA AAC CTT CTG CAG G			
43	Rv: 15618C	TAA TGG ACT GGA AGA GGA AGG	290	C15618T	8
44	Rv: 15618T	TAA TGG ACT GGA AGA GGA AGG			
45	Fw: 691G	GCT CTG GTC AAG GCT AAA AAT G	240	G691C	9
46	Fw: 691C	GCT CTG GTC AAG GCT AAA AAT C			
47	Fw: 15631G	GAC CCC ACC TTC CTC TTC CAG	388	G15631T	10
48	Fw: 15631T	GAC CCC ACC TTC CTC TTC CAT			
49	Fw: 802T	TCA AGA GTG TCC GCA TGC TCT	424	T802C	
50	Fw: 802C	TCA AGA GTG TCC GCA TGC TCC			
51	Fw: 118A	CAA CTT GTC CCA CTT AGA TGG CA	267	A118G	11
52	Fw: 118G	CAA CTT GTC CCA CTT AGA TGG CG			

Table 5A:The master mix for amlicon A

MM1	**1 X µl**	**Final concentration/ µl**
dH_2O	6.05	72.6
Buffer	2.5	30
$MgCl_2$	0.75	9
dNTPs	0.5	6
Taq Polymerase	0.2	2.4
TOTAL	10	120

Table 5B: The Master mix for amplicon B

MM1	**1 X µl**	**Final concentration/ µl**
dH_2O	6.05	72.6
Buffer	2.5	30
$MgCl_2$	1	12
dNTPs	0.5	6
Taq Polymerase	0.2	2.4
TOTAL	10	120

Table 5C: The master mix for amplicon C

MM1	**1 X µl**	**Final concentration/ µl**
dH_2O	6.05	72.6
Buffer	2.5	30
$MgCl_2$	0.75	9
dNTPs	0.5	6
Taq Polymerase	0.2	2.4
TOTAL	10	120

Initially, the genomic DNA was extracted and purified using a column-based method and spin protocols. This method was designed for processing up to 200µl human whole blood and simplifies the extraction process (QIAGEN, 2009). In the assay, the DNA binds specifically to the silica-gel membrane in the column, allowing the other contaminants to pass through to the waste tube. Because the DNA is washed several times, proteins and all other PCR inhibitors are removed efficiently from the column, leaving the DNA available to be washed in a specific tube by a particular buffer supplied by the kit. Generally, the extracted DNA should range in size from 200bp–50kb. The kit provides several advantages, such as an optimized lysing buffer, stabilized nucleic acids, and enhanced selective DNA absorption to the silica gel-membrane, and is a rapid and ready-to-use method of obtaining high yields of high-quality DNA with complete removal of contaminants to ensure efficient and pure downstream amplification.

The average yield of the extracted DNA was112 ± 45 µg, and the average absorbance was 1.8 ± 0.23 from 15µl blood, which represented 100% purity. The extracted genomic DNAs were all intact and exhibited a size of more than 10kb, as referenced to the 1-kb DNA marker. Smears appearing at the bottom of the gels emphasized the DNA integrity.

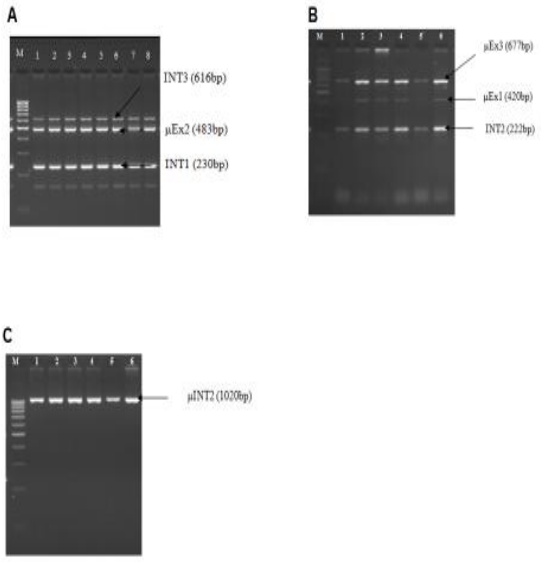
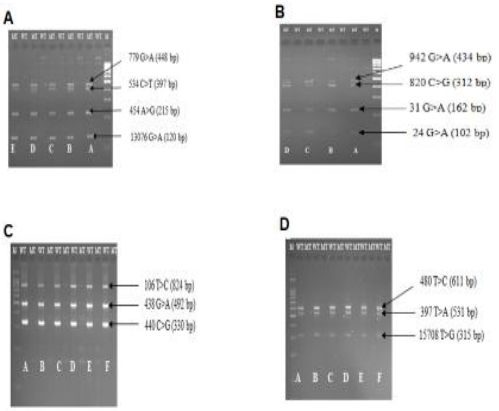


Fig. 3: Samples of Agarose gel electrophoresis of the products of the first PCR step. (A) Amplicon A; lane M, 100-bp ladder; lanes 1–8, 3 amplified regions [Cyp-Int3 (616bp), µExon2 (483), Cyp-Int1 (230bp)]. (B) Amplicon B; lane M, 100-bp ladder; lanes 1–6, 3 amplified regions [µExon3 (677bp), µExon1 (420bp), Cyp-Int2 (222bp)]. (C) Amplicon C; lane M, 100-bp ladder; lanes 1–6, amplified µInt2 (1020bp).



Overall, we found that the QIAGEN DNA extraction and purification kit was efficient and easy to use, produced high DNA yields, was not labor intensive, and had a low risk of contamination. Also, the extraction and purification could be done in a timely fashion with no risk of confusion, and the removal of all the contaminants could be achieved in a single assay. The drawbacks were that it was expensive and consumed a lot of materials.

Our study is based mainly on a multiplex PCR using primers specifically designed to simultaneously detect the *1, *2 and *9 alleles in *CYP2B6* and 17 SNPs in *OPRM1*. We developed a two-step multiplex PCR method to simultaneously detect all 26 SNPs in both genes. The first and second PCRs amplified three amplicons and 11 amplicons, respectively. The selection of alleles was based on previous studies suggesting potential relationships between the alleles and MMT outcomes. Our approach produced different amplicons of different sizes from the same source DNA.

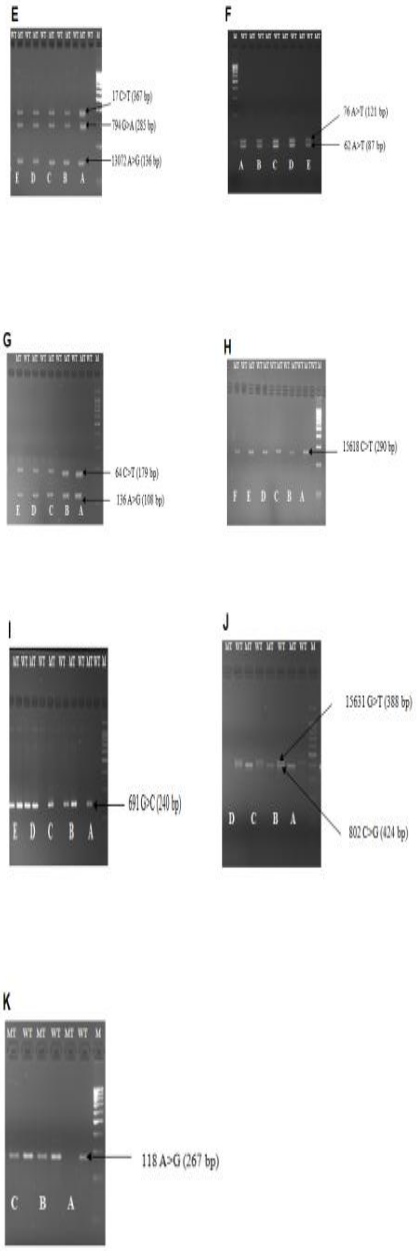


Fig. 4: Samples of Agarose gel electrophoresis of single and multiplex reactions of the second PCR step. Lane M: 100-bp ladder; WT: samples containing wild-type alleles; MT: samples containing mutant alleles. (Panels A-K) Reaction sets 1–11; notations indicate the specific polymorphism and fragment length represented by each band.

Certain reactions could not be mixed with certain other reactions, however, because the specificity was reduced by the appearance of non-specific bands. Specifically, for the C15618T, G691C, and A118G SNPs, a uniplex reaction was used to improve the sensitivity and specificity of detection. The appearance of non-specific bands in multiplex reactions targeting those SNPs might be caused by several things, such as base-pair mismatching or suboptimal $MgCl_2$ or Taq polymerase concentrations. In the end, using the final conditions for the three amplicons and the specific SNPs, we successfully obtained diagnostic gel pictures of the multiplex reactions and the uniplex reaction.

We developed a nested, multiplexed PCR method to simultaneously detect 26 SNPs in *CYP2B6* and *OPMR1* to facilitate more rapid and sensitive detection of clinically relevant polymorphisms in those genes. Developing nested PCR methods is both tedious and time-consuming, however; because it involves the use of multiple primer pairs to amplify different loci. The design and optimization of multiplexed PCR methods usually focus on primers and experimental conditions such as annealing temperature and concentrations of $MgCl_2$, dNTPs, reaction buffer, and DNA polymerase [31,32]. In designing the primers, it is important to make sure that all of the primers have similar melting temperatures and do not interact significantly with themselves, each other, or unwanted regions of the template [33]. To improve the sensitivity and specificity of the test, we optimized the buffer solutions, annealing temperature, and concentrations of primers, dNTPs, and $MgCl_2$. Thus, we applied different conditions in the multiplex or uniplex reactions when required (Viljoen, 2007).

Keeping these views in consideration, we designed a set of primers to specifically amplify a particular part of *CYP2B6*, effectively slicing our regions of interest out of the genome, an approach known to be sensitive, specific, and reproducible [24,34–36]. We found our primers to be compatible with each other, and we used them to produce three different amplicons in a single PCR step. We found that the optimization of annealing temperature during amplification strongly affected the PCR specificity and relative product yield. We increased the annealing temperature step by step from 48°C to 60°C (data not shown) to eliminate the nonspecific products and at the same time avoid false-negative results. We found that the critical parameter for the success of the reaction was the primer concentration. We varied the concentrations of each set of primers in steps (data not shown) until we obtained maximum sensitivity and specificity. The optimum parameters for the multiplexed PCR producing amplicons A, B, and C were thus determined to be a mixture of 1.5mM $MgCl_2$, 2mM dNTPs, 1U Taq DNA polymerase, primers as described in Table 1, and 2.0µl (1:10) template for a final volume of 10µl. The optimized method was found to be reproducible and specific when tested against multiple DNA samples. Samples of the amplified regions are shown in Fig. 3 and Fig. 4 for the first PCR and second PCR; respectively. The gel electrophoresis of the products of the optimized, first-step, multiplexed PCR showed three amplified regions within amplicon A: *CYP2B6* intron 3, *CYP2B6* intron 1, and *OPRM1* exon 2 (Fig. 3A). Likewise, gel electrophoresis showed three amplified regions within amplicon B (*CYP2B6* intron 2, *OPRM1* exon 1, and *OPRM1* exon 3; Fig. 3B) and a single amplified region within amplicon C (*OPRM1* intron 2; Fig. 3C).

We optimized the second PCR step to specifically detect 26 SNPs by dividing it into 11 sets of multiplexed reactions, except for reaction sets 8, 9, and 11, which were uniplexes (Table 2). The second-step PCRs were optimized to 15-cycle reactions containing 1.5mM $MgCl_2$ for sets 1, 3, 5, 6, 7, 8, 9, 10, and 11, or 2.0mM $MgCl_2$,for sets 2 and 4. We designed the primers to match the polymorphic sites, keeping in consideration the homology of the primers with their target sequences and the lengths and GC contents of the primers. Using these primers and the products of the first PCR step, each reaction set in the second PCR step successfully amplified the expected SNPs: G779A, C534T, A454G, and G13076A; G942A, C820G, G31A, and G24A; T106C, G438A, and C440G; T480C, T397A, and T15708G; C17T, G794A, and A13072G; A76T and A62T; C64T and A136G; C15616T; G691C; G15631T and C802G; and A118G (Fig. 3A-K). Mismatches at the 3'end of a primer can cause PCR failures; however, the multiplexed PCR primer sets were successful, as no significant primer dimers or large allelic noise bands were observed.

CONCLUSION

In this study, a nested PCR method for the simultaneous detection of 26 allelic variants of *CYP2B6* and *OPMR1* was developed successfully. The selection of alleles was based on previous studies suggesting potential relationships among the selected alleles. The first step of the allele-specific,nested PCR increases the specificity of the simultaneous amplification of *OPMR1* exon 2 and *CYP2B6* introns 1 and 3 in amplicon A, *OPMR1* exons 1 and 3 and *CYP2B6* intron 2 in amplicon B, and *OPMR1* intron 3 in amplicon C. The products of the first PCR step are then used as templates for the second, multiplexed PCR step, in which 26 SNPs are amplified in eleven sets of reactions. This method can be used as a platform to investigate genetic polymorphisms in *CYP2B6* and *OPRM1* among patients receiving MMT, providing insight into the possible genetic factors affecting the patients' clinical outcomes.

ABBREVIATIONS

PCR, Polymerase Chain Reaction; MMT, Methadone Maintenance Therapy; SNP, Single-Nucleotide Polymorphism; CYP, Cytochrome P450; OPRM1, Opioid Receptor µ 1; dNTP, di-Nucleotide Triphosphate

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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