Post-mortem Molecular Investigation:

Exploring Genetic Variation in CYP2D6 in Deceased Individuals at Salt River Mortuary

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List of Abbreviations/Symbols

A_{230}	Absorbance at 230 nm
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
ADR	Adverse Drug Reactions
BLAST	Basic Local Alignment Search Tool
Bp	Base Pair
c.	Coding DNA Reference
CNS	Central Nervous System
CNV	Copy Number Variation
CoD	Cause of Death
CYP	Cytochrome P450
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxynulceotide Triphosphate
EDTA	Ethylene-diamine-tetraacetic Acid
EM	Extensive Metaboliser
G/C/A/T	Guanine/Cytosine/ Adenine/Thymine
HREC	Human Research Ethics Committee
IM	Intermediate Metaboliser
Indels	Insertion/Deletions
LC-MS/MS	Liquid Chromatography tandem Mass Spectrometry
mg	Milligram
MWM	Molecular Weight Marker
MoD	Manner of Death
n	Number
ng	Nanograms
nm	Nanometer
NTC	No Template Control
PCR	Polymerase Chain Reaction
PM	Poor Metaboliser
qPCR	Quantitative Polymerase Chain Reaction
rpm	Revolutions Per Minute
SNP	Single Nucleotide Polymorphism
SRM	Salt River Mortuary
SSRI	Selective Serotonin Reuptake Inhibitor
SWGTOX	Scientific Working Group of Forensic Toxicology
TBE	Tris-Borate-EDTA
TCA	Tricyclic Antidepressants
TE	Tris-EDTA
THC	Δ^9-tetrahydrocannabinol
T*m*	Melting Temperature
UM	Ultra-rapid Metaboliser
UNODC	United Nations Office on Drug and Crime
USA	United States of America
UV	Ultraviolet
µl	Microliter
µM	Micro Molar
°C	Degrees Celsius
%	Percentage

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Abstract

Drug use is a major burden in Cape Town, South Africa, and at times may be fatal. Individuals suspected to have demised from drug intoxication are referred for medico-legal investigation, in order for cause of death to be determined. Sometimes, it remains ambiguous as to whether the drug intoxication was suicidal or accidental, even after a full post-mortem examination. Literature has shown that molecular analysis of genetic variants in genes encoding for drug metabolising enzymes may provide insight into the manner of death. At Cape Town's Salt River Mortuary, numerous toxicological-related cases yield ambiguous results, which may potentially be resolved with molecular analyses. However, no optimised molecular assay to sequence drug metabolising enzymes currently exists in a local context. The aim of this project was to design and optimise a molecular-based assay to sequence the drug metabolising enzyme, CYP2D6. Subsequent to primer design, exons in *CYP2D6* were amplified and sequenced. The optimised assay was then applied to DNA from two decedents suspected to have demised from drug intoxication. Following a toxicological drug screen, certain drugs metabolised by *CYP2D6* were reported. The assay revealed genetic variants within *CYP2D6*; both individuals were heterozygous for 138insT, rendering one allele in each individual defective. While one decedent also exhibited variants with normal and unknown haplotypes, the other decedent was homozygous for *17 (decreased functionality), overall making the former an intermediate (altered) or extensive (normal) metaboliser and the latter, an intermediate metaboliser of specific drugs. Quantitative toxicological results were unavailable; consequently, the contribution of the metabolism phenotype on death in these cases could not be established. However, the genetic variants, combined with the presence of these drugs in each case, suggests altered drug metabolism, which should be investigated further and interpreted within each case context. These findings would also be beneficial to the decedents' living relatives, who may also carry these variants. Overall, this study demonstrates the value of molecular analyses in forensic investigations of toxicological-related fatalities, and lays the foundation for additional future research, particularly since the molecular assay has now been successfully optimised.

1. Introduction

1.1 Literature review

1.1.1 Background

To date, literature reviews have not collated information on the variations of the *CYP2D6* gene from a forensic post-mortem perspective for indigenous Black African populations. Given the diversity observed in indigenous Black African populations and the importance of identifying the association between drug-metabolising enzymes and forensic post-mortem cases, it is important to review the literature and collate what is already known about identified *CYP2D6* variants in order to reveal the gaps requiring further study (Gaedigk and Coetsee, 2008).

Pharmacogenetics, is the study of genetic polymorphisms in drug-metabolising enzymes and is a science that has been thoroughly explored globally. One of the most highly polymorphic and studied of these drug-metabolising enzymes is CYP2D6 (Alessandrini *et al.,* 2013). Many studies have investigated the *CYP2D6* variants associated with adverse drug reactions (ADRs). When compared globally, however, there have been relatively few studies performed on indigenous Black African populations (Alessandrini *et al.,* 2013).

Toxicogenetics has derived from pharmacogenetics and is defined as the study of genetic variations that cause variable drug responses through polymorphisms of drug transporters, drug metabolising enzymes and drug receptors (Severino and Del Zompo, 2004). It focuses on how variations are manifested, how they interact to produce specific phenotypes and how these phenotypes affect drug response (Severino and Del Zompo, 2004). This discipline is not yet fully utilised in the South African Forensic Medicine context.

South Africa has witnessed an exponential growth of drug abuse to epidemic proportions – widely attributed to the relatively cheap costs and increased availability – which may have contributed to an increase in toxicological-related deaths (Dada *et al.,* 2015). Using toxicological analyses to determine the manner of death (MoD) in such instances, is not always straightforward. In ambiguous cases, for instance when high concentrations of drugs are present in the blood, despite therapeutic doses, or when metabolites are present in biological specimens in a manner inconsistent with prescription history or reported consumption, the MoD remains 'undetermined'. Since these ambiguous cases are often not resolved by current

analytical practices, expanding post-mortem investigations to include a genetic component, 'Molecular Autopsies', is being explored (Sajantila *et al.,* 2010).

The limited knowledge of polymorphisms and their impact in indigenous Black African populations may underestimate the importance of their forensic application in toxicogenetics (Matimba *et al.,* 2009).

1.1.2 Substance Use and Abuse

Widespread concern regarding the rise in substance use and abuse has given rise to a global 'war on drugs'. It was estimated that around 250 million people (5% of the world's population) used an illicit drug at least once in 2015 (UNODC, 2017). Most countries have experienced an exponential increase in drug use and this has been witnessed, particularly, amongst the youth (Crouch and Wegner, 2014). Developing countries, in particular, are vulnerable to the increase in drug use, which places pressure on their health systems, society and economy (Crouch and Wegner, 2014). Factors including informal settlements, poverty, crime, unemployment, drug-dependent social groups and social uncertainty create a fecund environment for substance abuse (James, 1999). Drugs are popularly used and abused, which has been reported to fabricate an entire drug culture, particularly amongst the impoverished (Newcomb and Bentler, 1989; Abadinsky, 2017).

Recreational substance use is defined as the general use of any psychoactive substance that usually starts on an experimental basis (Brook *et al*., 2006). In some instances, experimentation leads to substance abuse, which is defined as the routine use of a substance, consumed in excess of the therapeutic amount or with methods that are dangerous to themselves or others (Orwa, 2014; Crouch and Wegner, 2014). Substances of abuse are often categorised according to the drug's medical use and dependency potential (US Department of Justice, United States Drug Enforcement Administration, 2013). In South Africa, the primary consideration in classifying a drug, according to the Medicines Control Council, is the drug's safety profile regarding the therapeutic indications for its use. Drugs are listed into schedules based on route of administration, strength, dose, duration of treatment or a combination of these factors (Medicines Control Council, 2014).

Although the emphasis of substance abuse normally pertains to illicit drugs, over-the-counter and prescription medication (therapeutic drugs) are also abused (Crouch and Wegner, 2014). Commonly abused illicit drugs include substances in the cannabis, opioid, cocaine or

amphetamine-type stimulant groups, while commonly abused prescription medications include opioid analgesics, stimulants and sedative hypnotics (Orwa, 2014). The substances abused are often a result of what is commonly available, which may depend on the socio-economic status of the individual or the community. Some of these substances are produced within the borders of a country, while many others are shipped in from other parts of the world. According to the United Nations Office on Drugs and Crime (UNODC), South Africa features as an international transit country and regional hub for illicit drugs entering Southern Africa (Orwa, 2014).

1.1.3 Commonly Abused Substances in the Western Cape

As observed globally, drug abuse in the Western Cape is ever increasing and the pattern of commonly used drugs is constantly changing (Odejide, 2006). Currently, uncontrolled substance abuse in the Western Cape is adversely affecting not only adults and adolescents but the youth as well (Odejide, 2006).

The most commonly abused psychoactive substances in the Western Cape include alcohol, cannabis, methamphetamine and methaqualone (Parry *et al.,* 2004; Dada *et al.,* 2015). Methaqualone, commonly known by its trade name Mandrax, was previously – from data collected in a study by Parry *et al.* (2004) – the second most frequently reported substance of abuse, after cannabis, in adolescents in Cape Town between 1997 and 2001. Mandrax is a sedative and hypnotic drug which is often mixed with cannabis, as it is said to enhance the psychoactive effects of the active ingredient in cannabis, Δ^9- tetrahydrocannabinol (THC) (da Silva *et al.,* 2007). Mandrax is known to have highly addictive properties, producing both physical and psychological dependence (da Silva *et al.,* 2007).

Since 2005, the Cape Town area has seen a sudden increase in the level of abuse of methamphetamine hydrochloride, which is believed to be smuggled in from China (Odejide, 2006; Orwa, 2014). Methamphetamine – with its numerous forms and street names – is easily administered through smoking or intravenous injection and is a powerful addictive stimulant that produces heightened energy levels, alertness, hyper-sexuality and an intense euphoric sensation (Orwa, 2014; Panenka *et al.,* 2013; Meade *et al.,* 2015). The bitter white powder can be manufactured locally from commonly available household reagents. Methamphetamine abuse in the Western Cape has grown to epidemic proportions, widely attributed to the relatively cheap costs and increased availability (Dada *et al.,* 2015). Between 2005 and 2009,

methamphetamine was documented as the primary drug of abuse in the Western Cape, having overtaken Mandrax and cannabis (van Heerden *et al.,* 2009).

Historically, substance abuse data in South Africa has been limited and has largely been derived from rehabilitation centres (Dada *et al.,* 2016). This information, although not an ideal representation, does highlight worrisome statistics. Given the lack of accessibility to intervention by much of the population, it has become a crisis in which death may often be the consequence (Crouch and Wegner, 2014).

1.1.4 Medico-Legal Forensic Investigations

In South Africa, the provincial Department of Health's Forensic Pathology Service performs medico-legal investigations of deaths, which were or may have resulted from unnatural causes (Vincent *et al.,* 2015). These examinations are conducted primarily in terms of the Inquest Act (Act no. 58 of 1959) and may only be performed by authorised medical practitioners (primarily Forensic Pathologists) (du Toit-Prinsloo and Saayman, 2012). Since, these medico-legal examinations are mandated by law, they can be performed without the consent of the next-of-kin (du Toit-Prinsloo and Saayman, 2012). These investigations include the examination of a body with the purpose of establishing the cause of death (CoD) and/or circumstances surrounding the death (National Health Act, Act no. 61 of 2003).

The National Health Act (Act no. 61 of 2003), regards the following to be deaths from unnatural causes: (i) "any death due to physical or chemical, direct or indirect, and/or related complications"; (ii) "any death, which in the opinion of the medical practitioner, has resulted from an act of omission or commission, which may be illegal/criminal in nature"; (iii) "any death as outlined in the Health Professions Amendment Act (Act no. 29 of 2007)"; and (iv) any death that is sudden and unexpected, or unexplained" (National Health Act, Act no. 61 of 2003).

Salt River Mortuary (SRM) and academic centre is a medico-legal facility that serves the West Metropole of the City of Cape Town and investigates more than 3500 cases per annum (M6 graded). Due to the high case load, additional ancillary investigations which are not crucial to the determination of CoD cannot be supported in this resource-limited setting. In cases where the death is thought to be due to substance use or abuse, a medico-legal investigation is performed (National Health Act, Act no. 61 of 2003), which includes a comprehensive toxicological analysis. However, in cases involving violence, whereby the CoD is clear (e.g.

gunshot) then ancillary toxicological investigations are not performed in this setting, even though the use of drugs may contribute towards circumstances surrounding death.

According to the Scientific Working Group for Forensic Toxicology (SWGTOX), forensic toxicology is the study of the adverse effects of drugs and chemicals on biological systems, whereby the adverse effects have administrative or medico-legal consequences (Forensic Toxicology Council, 2010). Toxicological-related cases require further investigation with forensic pathologists and toxicologists needing to establish the role of the substance in the causation of, or contribution to, death. Interpretation of forensic toxicological results is a task requiring knowledge of several aspects of pharmacology and analytical toxicology (Musshoff *et al.,* 2010). In addition, post-mortem toxicology carries with it inherent challenges associated with analysis and interpretation in a deceased individual. For example, a number of drugs are unstable in a post-mortem environment and require careful consideration when interpreting their significance (Drummer, 2004). Drugs are also prone to degradation and redistributive processes, which may significantly alter their concentration (Drummer, 2004). These processes, together with individual factors such as the development of tolerance for a drug or genetic alteration in drug disposition, indicate that toxicological results should not be interpreted in isolation and a thorough investigation of the relevant circumstances is needed before a definitive conclusion of the possible effect of the substance on the body can be made (Drummer, 2004).

One of the more common applications of post-mortem toxicology is the determination and interpretation of drug concentrations in the body, which is important in establishing the role of the drug in death in specific cases (Drummer, 2007). These concentrations may lie within therapeutic, toxic or 'lethal' levels. The term 'therapeutic' implies a concentration wherein the drug is expected to be effective without causing any toxic effects (Schulz *et al.,* 2012). Conversely, 'lethal' concentrations are often suggested to contribute to death; however, these are largely based on reported fatal case studies. It is imperative that given individual and drug factors that alter the nature and extent of inter-individual toxicity, post-mortem toxicology should be interpreted in light of scene, medical and social history as well as autopsy and other investigative findings. While there have been substantial improvements in the understanding of the significance and application of forensic toxicology over the last 20 years, forensic pathologists and toxicologists are still faced with ambiguous cases in which toxicological results are either inconsistent with death or do not correspond to prescription or drug use history (Drummer, 2007). The challenges associated with the interpretation of post-mortem

toxicological results require consideration and strong understanding so as to apply to such ambiguous cases.

1.1.5 Ambiguous Toxicity Cases

Inconsistencies in post-mortem toxicology may include cases where high concentrations of drugs or metabolites, despite therapeutic doses, are found or vice versa; or if the concentrations do not appear to correlate to prescription medication history. This is often simply flagged as a reduced or increased functioning of the drug. The difficulty of interpreting such cases intensifies when there are no witnesses to the circumstances that may have led to the death or knowledge of the medical history and where there are inconsistencies with the amount of prescription medication on scene, particularly, when there is a suspected suicide but a history of suicide attempts is unknown (Sajantila *et al.,* 2010).

Literature indicates, in some of these cases, that genetic testing may be of value. In pharmacogenetic studies of neonatal opioid toxicity following maternal use of codeine during breastfeeding, there have been several reported cases of neonate deaths as a result of morphine toxicity from breastfeeding mothers that had consumed codeine prescribed for obstetric pain (Madadi *et al.,* 2009). The cause was based on the mothers being *CYP2D6* ultra-rapid metabolisers and not attributable to negligent use of medication. This genetic predisposition resulted in the prodrug codeine producing excessive morphine concentrations, which may distribute into milk, resulting in toxicity in the breastfed neonates (Madadi *et al.,* 2009).

Another study, by Sallee *et al.* (2000), reported that a 9-year-old boy, previously diagnosed with attention-deficit hyperactivity disorder, obsessive-compulsive disorder and Tourette's Syndrome, died as a result of fluoxetine toxicity. Fluoxetine had been used to treat the child. It was found that a genetic predisposition of the *CYP2D6* gene impairing the metabolism of the drug was responsible for high concentrations of fluoxetine in the blood and not excess administration of the drug (Sallee *et al.,* 2000). Similarly, in a study by Jin *et al.* (2005) discussing 25 fentanyl toxicity cases, it was reported that 24 of the individuals died as a result of fentanyl toxicity whereby the manner of death was deemed accidental in 18 cases. The fentanyl toxicity was as a result of lowered enzyme activity of the *CYP3A4* and *3A5* genes (Jin *et al.,* 2005).

These cases represent the necessity of ancillary investigations in determining CoD in cases where toxicology may be ambiguous. Although these ambiguous cases are often not resolved

by current post-mortem practices, expanding post-mortem investigations to include a genetic analysis as a component is one avenue that is currently being explored (Sajantila *et al.,* 2010).

1.1.6 Molecular Autopsies

Interpretation of post-mortem toxicology in cases resulting from drug abuse or toxicity is complex. Distinguishing chronic from acute use of drugs may be complicated by time- and site-dependent variations in parent or metabolite concentrations as well as variation in metabolising phenotypes (Musshoff *et al.,* 2010). Factors that affect an individual's pathophysiological phenotype, as related to drug efficacy, may include physiological or, environmental factors, developmental age and, more importantly to this review, genetic variations (Sajantila *et al.,* 2010; Agrawal and Rennert, 2012). The term 'Molecular Autopsy' is defined as the medico-legal investigation involving genetic testing and includes genotyping individuals to identify genetic predispositions and contributory factors towards death (Sajantila *et al.,* 2010).

An individual's genetic makeup plays a pivotal role in the metabolism of drugs and often a reduced metabolic capacity, causing accidental toxicity, is a result of genetic variation (Kupiec *et al.,* 2006; Neukamm *et al.,* 2013). ADRs, is one of the leading causes of death in drug-related cases in the United States and is associated with abnormal pharmacokinetics (Jin *et al.,* 2005; Andersen *et al.,* 2013). ADRs not only result from abnormal pharmacokinetics, whereby genetic influences alter drug metabolism; but also, when there are synergistic effects between two or more drugs. ADR-related cases may benefit from the incorporation of molecular autopsies, and concerns regarding the underutilisation of this ancillary investigation, have been raised by the U.S. National Academy of Sciences (Sajantila *et al.,* 2010). This sentiment should be in accordance with the development of the application of toxicogenetics in South Africa, where ADRs in a clinical setting have been reported to occur in 14% of hospitalised patients and result in a five to ten times higher fatality rate (Warnich *et al.,* 2011).

The value of molecular autopsies for unresolved ambiguous cases is threefold: firstly, genetic analyses have shown to resolve the CoD in numerous cases, which not only brings closure to the family of the deceased, but also enables improved targeting of public health resources (Nsubuga *et al.,* 2006); secondly, the mechanisms underlying these deaths can be better understood, furthering research on preventative measures (Axler-DiPerte *et al.,* 2014); and thirdly, genetic information on the risks for sudden unexpected, unexplained deaths or for adverse drug reactions fatal can be of paramount importance to living relatives who may be at

risk of carrying the same genetic mutation (Axler-DiPerte *et al.,* 2014). For these reasons, the aim of a study by Sanchez *et al.* (2016) was to evaluate whether molecular autopsies could increase the identification of the potential aetiology of death. Knowing the aetiology would be an ideal preventative measure that could identify which next-of-kin of the decedent should undergo further clinical genetic evaluation (Semsarian and Hamilton, 2012; Sanchez *et al.,* 2016).

A 2008 study by Carturan *et al.* illustrated how up to 35% of cases sent for post-mortem genetic testing provided informative results, even though post-mortem samples were not collected in a 'DNA-friendly' manner (according to protocols/guidelines), (Carturan *et al.,* 2008). Molecular autopsies may decrease the amount of unresolved, undetermined and/or ambiguous cases, and may also provide a significant amount of genetic information about individual cases.

1.1.7 The Role of CYP2D6 in Drug Metabolic Pathways

The human body has the ability to carry out various chemical reactions using numerous mechanisms as a response to internal stimuli. These internal stimuli can result from the introduction of xenobiotics, such as drugs or toxins, into the body (Motulsky, 1957). The routes by which these drugs are metabolised or bio-transformed are countless and often vary among drug types. Metabolism of these xenobiotics occurs predominantly in the liver in two phases (Gibson and Skett, 1994). Phase I and phase II – of which the latter is often known to occur as a result of phase I metabolism (Gibson and Skett, 1994). Phase I metabolism, utilises enzymes such as Cytochrome P450 (CYP) enzymes, which catalyse more than 90% of the metabolic processes that occur in the liver, intestines and kidney while Phase II involves conjugation of drugs to improve their excretion (Arici and Ozhan, 2016; Tracy *et al.,* 2016). This family of enzymes is responsible for the metabolism of many therapeutic and abused drugs, carcinogens and other endogenous substrates (Alessandrini *et al.,* 2013).

Cytochrome is derived from the haemoprotein nature of the enzyme, while "P450" is derived from the maximum wavelength at which the complex absorbs light (450 nm) (Jaiswal *et al.,* 1985). CYP450s are conveniently arranged into families and subfamilies, based on percent amino acid sequence identity. Enzymes sharing greater than or equal to 40 percent identity are assigned to a particular family, whereas those sharing greater than or equal to 55 percent identity make up a particular subfamily designated by a letter (Nelson, 2004). There are 57 putatively functional human CYP450s, of which only a small subset within the CYP 1, 2, and 3 families metabolise xenobiotics of interest to forensic toxicologists (Zanger & Schwab,

2013). The expression of these CYP450s are influenced by factors such as polymorphisms, disease state, age, sex, ethnicity and induction or alteration by xenobiotics. The CYP450 drug metabolising enzyme system is a major source of inter-individual variability in drug pharmacokinetics and toxicity (Jamei *et al.,* 2009).

The CYP450 2 family is the largest and most diverse CYP450 family, consisting of 16 genes in 11 subfamilies (Nelson, 2009). CYP2C19 (CYP2C subfamily), CYP2D6 (CYP2D subfamily) and CYP3A4 (CYP3A subfamily) enzymes are considered most pharmacologically significant, with regards to drug metabolism (Guengerich and Cheng, 2011). The CYP2D family consists of a single protein-coding gene, *CYP2D6*, that possesses more than 100 variant alleles (Zanger and Schwab, 2013). This enzyme metabolises more than 25% of clinically significant drugs including antidepressants, antihistamines and antipsychotics (Gardiner and Begg, 2006; Sistonen *et al.,* 2007; Zanger and Schwab, 2013) (Table 1). The polymorphic gene is also known for its ability to metabolise drugs from certain illicit drug groups, namely amphetamine-type stimulants, opioid analgesics and sedative hypnotics (Druid *et al.,* 1999; Jannetto *et al.,* 2002; Kupiec *et al.,* 2006; Koski *et al.,* 2007; Crews *et al.,* 2012; Neukamm *et al.,* 2013). *CYP2D6*-mediated metabolism of these drugs is known to be a major source of variation in pharmacokinetics, and subsequently in drug effects (Jamei *et al.,* 2009).

Table 1: List of substances that are metabolised by the CYP2D6 enzyme.

Drugs Metabolised by CYP2D6 Enzyme		
NARCOTIC ANALGESICS	**ANTIDEPRESSANTS**	**ANTIARRHYTMICS & β-BLOCKERS**
Codeine*/**	Amitriptyline	Alprenlol
Dextromethorphan	Clomipramine	Carvedilol
Ethylmorphine	Desipramine	Clonidine
Hydrocodone*	Doxepin	Debrisoquine
Oxycodone*	Duloxetine	Diltiazem
Tramadol*	Fluoxetine	Disopyramide
Morphine**	Fluvoxamine	Flecainide
Methadone	Imipramine	S-metoprolol
	Maprotiline	Mexiletine
ANESTHETICS	Mirtazapine	Nebivolol
Lidocaine	Nortriptyline	Perhexiline
	Paroxetine	Propafenone
ANORETICS	Trimipramine	Propranolol
Dexfenfluramine	Venlafaxine	Sparteine
		Timolol
ANTIHISTAMINES	**ANTIPSYCHOTICS**	
Chlorpheniramine	Aripiprazole	
Diphenhydramine**	Chlorpromazine	**AMPHETAMINE-TYPE STIMULANTS**
Promethazine**	Clozapine	Amphetamine
	Haloperidol	Methamphetamine**
ANTINEOPLASTICS	Perphenazine	3,4–Methylenedioxymethamphetamine (MDMA)**
Tamoxifen	Risperidone	Paramethoxyamphetamine
	Sertindole	3,4-methylenedioxyamphetamine (MDA)
SEDATIVE HYPNOTICS	Thioridazine	3-methoxy-4,5-methylenedioxyamphetamine (MMDA)
Methaqualone** (Mandrax)		
Glutethimide	**ANTIEMETIC**	
	Metoclopramide	
	Ondansetron	

*Prodrug

**Commonly abused illicitly in South Africa
(Legget 2002; Parry *et al.,* 2004; Ingelman-Sundberg, 2005; Wilkinson 2005; Drugaware, 2006; da Silva *et al.,* 2007; Dasgupta 2010; Peltzer *et al.,* 2010; Dada *et al.,* 2015)

1.1.8 Variations in the CYP2D6 Gene and Resulting Metabolising Phenotypes

The *CYP2D6* gene has been extensively studied in certain population groups as it shows the greatest impact of genetic polymorphisms among all drug metabolising enzymes. This is reportedly due to the vast amounts of variants observed, the very few environmental influences and broad substrate selectivity (Zanger and Schwab, 2013). Genetic variation of pharmacogenetic interest in *CYP2D6* is mainly in the form of single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) (Allorge and Tournel, 2011).

SNPs are defined as single-base variations at specific sites in a sequence. While the majority of SNPs are synonymous and have no significant effect on the functionality of a gene, there are some that occur in functionally important regions of genes that may lead to discernible differences in form and function of gene products (Kupiec *et al.,* 2006; Allorge and Tournel, 2011). CNVs are genomic rearrangements which cause partial or complete gene deletions or duplications of coding regions. They alter approximately 12% of the human genome, leading to gene dosage imbalance and coding sequence disruptions (Allorge and Tournel, 2011). When these genetic variations occur in genes that encode drug metabolising enzymes, they can eliminate, decrease or increase the activity of the enzyme and consequently result in certain phenotypic manifestations (Kupiec *et al.,* 2006).

Although there has not yet been complete characterisation of all the variant alleles of *CYP2D6*, according to Whirl-Carrillo *et al.* (2012), there are 196 variant alleles of the *CYP2D6* gene, which define 163 different haplotypes. A haplotype is a set of DNA variations, or polymorphisms, that tend to be inherited together. Of these, over 15 haplotypes, including the most common *CYP2D6*4* and *CYP2D6*5*, encode non-functional products (Pirmohamed and Park, 2001; Whirl-Carrillo *et al.,* 2012). Other types of haplotypes that alter substrate specificity, increase or decrease activity have also been described (Pirmohamed and Park, 2001).

The polymorphisms in *CYP450* genes are reported to produce distinct pharmacogenetic phenotypes categorised as poor, intermediate, extensive, and ultra-rapid metabolisers (Sajantila *et al.,* 2010; Zanger and Schwab, 2013). The different phenotypes suggest complete lack of function, impaired functionality, normal functionality and gain in functional capacity,

respectively, for oxidation of the drug through manipulation of the genes that code for these enzymes (Zanger and Schwab, 2013). The different phenotypes are inferred from the genotypic data of the genes (Musshoff *et al.,* 2010). For instance, ultra-rapid metabolisers (UM) result from the presence of more than two active alleles encoding for a certain CYP450 enzyme, which increases enzyme activity; conversely, poor metabolisers (PM) (where no enzyme activity is observed) lack functional alleles. Extensive metabolisers (EM) carry two functional alleles, which is representative of the majority of the population. Lastly, intermediate metabolisers (IM) carry alleles that partially decrease enzyme activity due to either complete lack of function in one allele with normal functionality in the other, or where a homozygous form exhibits a reduced function (Musshoff *et al.,* 2010). The highest functioning *CYP2D6* allele typically predicts the phenotypic activity of the individual, based on the assumption of dominance (Owen *et al.,* 2009).

Individuals with PM associated variants for *CYP2D6* may be at increased risk of suffering adverse side effects from increased levels of the drug in the body or from experiencing therapeutic failure due to the inability to effectively metabolise a prodrug to the active metabolite (Musshoff *et al.,* 2010). In contrast, UMs experience significantly increased enzyme activity resulting either in the drug not remaining in the system long enough for the desired therapeutic effect or excessive production of an active metabolite following prodrug administration (Musshoff *et al.,* 2010).

Some haplotypes such as *CYP2D6*1, CYP2D6*2* and *CYP2D6*35* are known to exhibit normal enzyme activity while others such as *CYP2D6*1xN* and *CYP2D6*2xN* encode enzymes with increased activity and *CYP2D6*10, CYP2D6*17, CYP2D6*29* and *CYP2D6*41* with decreased activity (Masimrembwa *et al.,* 1996; Marez *et al.,* 1997; Zhou *et al.,* 2017). *CYP2D6*4, CYP2D6*5 and CYP2D6*6* where entire alleles are absent exhibit complete lack of function (Zhou *et al.,* 2017).

It must be noted that the association between genotype and phenotype is not straightforward (Sajantila *et al.,* 2010). There are numerous allelic variants with unknown or uncertain *in vivo* function which could contribute to any metabolic phenotype. A study by Hicks *et al.* (2014), highlighted that although alleles are assigned a value of classification – no, decreased, normal or increased function – they do not take into account the degree of increased or decreased function or substrate-specific activity of the allele (Hicks *et al.,* 2014). Based on phenotype studies, only individuals with lack-of-function alleles (PM) are significantly associated with an

expected phenotype (Sajantila *et al.,* 2010). Overlapping of phenotypes is apparent and inter-individual, inter-ethnic and other possible attributing factors should not be disregarded.

1.1.9 CYP2D6 Variants in African Populations

Population studies of drug metabolism have shown significant inter-population variance in polymorphic distribution of *CYP2D6* activity and actively-related genetic materials (Abraham and Adithan, 2001). Few studies have been performed on the *CYP2D6* variants in African populations, often leading to allelic frequencies from African-American populations being used to infer allelic frequencies in African populations (Alessandrini *et al.,* 2013). A study by Alessandrini *et al.* (2013) illustrated why population-specific allelic frequencies should not be used interchangeably: while the frequency of *CYP2D6*5* observed in Caucasian, Asian and African-American populations is below 10%, the frequency of this allele is noticeably higher in Mixed-Ancestry South African (17.7%) and South African Bantu (18.8%) populations (Lim *et al.,* 2011). Further variation can be observed by comparing various African populations. For example, the frequency of the *CYP2D6*5* allele in the South African Bantu populations is 18.8%, which is reportedly more than three times that observed in the Kenyan Bantu populations (4.2%) (Alessandrini *et al.,* 2013). For that reason, using the frequency of this PM allele in African-American or Kenyan Bantu populations is not a suitable proxy for the South African Bantu population.

Studies in African populations have also yielded varied frequency results, suggesting regional variation among populations with a prevalence of poor metabolisers ranging from 0-19% (Abraham and Adithan, 2001) (Table 2). The wide variation observed in the *CYP2D6* phenotype in various African populations contradicts the previously, long-standing assumption that these populations were genetically homogenous (Masimirembwa *et al.,* 1996). The influence of these genetic polymorphisms on CYP450 expression and function has made differentiating between suicide and/or accidental death, as well as acute versus chronic intoxication challenging.

Studying genetically diverse populations can contribute immensely to advancements in the field of toxicogenetics and there are a number of benefits that research based on South African populations could offer to future studies (Warnich *et al.,* 2011). These genetically diverse and admixed population groups, often a result of factors including non-random mating and genetic drift, should be utilised to elucidate the genetic variability contributing to the different types of drug responses observed (Warnich *et al.,* 2011).

Table 2: Frequencies from several different studies of *CYP2D6* haplotypes observed in African populations. (Amended from Alessandrini *et al.*, 2013).

			Haplotype	*1 WT	*2 EM	*3 PM	*4 PM	*5 PM	*6 PM	*10 IM	*17 IM
			Variant location	None	rs1058164 rs16947 rs1135840	rs35742686	rs1065852 rs28371703 rs28371704 rs28371705 rs1058164 rs3892097 rs1135840	Gene deletion	rs5030655	rs1065852 rs1058164 rs1135840	rs28371706 rs1058164 rs16947 rs1135840
			Publisher	Kimura *et al.,* 1989	Johansson *et al.,* 1993	Kagimoto *et al.,* 1990		Gaedigk *et al.,* 1991	Saxena *et al.,* 1994	Yokota *et al.,* 1993	Masimirembwa *et al.,* 1996
			Type of Study	Functional	Association	Functional		Association	Association	Functional	Functional
Country	**Population**	**Number of Subjects**	**Frequency (%)**								
South Africa	Bantu (Sistonen *et al.,* 2007)	8	Predicted	37.5	12.5	0	0	18.8	0	0	25.0
	Mixed Ancestry (Gaedigk & Coetsee, 2008; Ikediobi *et al.,* 2011)	99, 67	Predicted, Association	26.77	15.2	0	1.0-7.1	17.17	0	2.5	12.6
	Venda (Dandara *et al.,* 2001; Matimba *et al.,* 2008, 2009)	9, 76, 81	Predicted, Association, Functional	50.0	17.8-44.0	0	0-3.3	4.6	-	12.0-19.0	19-24.0
	Xhosa (Ikediobi *et al.,* 2011; Wright *et al.,* 2010)	53, 109	Association, Predicted	23.6	12.3	-	0-1.9	14.2	-	1.9	13.2
	South African (Dodgen *et al.,* 2015)	100	Predicted	29.1	13.8	-	3.1	8.7	-	-	19.4
Namibia	San (Sistonen *et al.,* 2007)	7	Predicted	0	64.3	0	0	14.3	0	0	7.1
Zimbabwe	San (Matimba *et al.,* 2008, 2009)	64	Association, Functional	-	-	-	9.0	-	-	-	22.0
	Bantu (Masimirembwa *et al.,* 1996; Matimba *et al.,* 2008, 2009)	23, 80	Predicted, Association, Functional	-	63.0	0	0-2.5	3.8	-	0-5.6	20.0
	Zimbabwean (Dandara *et al.,* 2001)	114	Predicted	47.0	13.0	0	2.0	4.0	-	-	34.0

"-" = Undetermined WT = Wildtype EM = Extensive Metaboliser IM = Intermediate Metaboliser PM = Poor Metaboliser UM = Ultra-rapid Metaboliser

Table 2 continued: Frequencies from several different studies of *CYP2D6* haplotypes observed in African populations. (Amended from Alessandrini *et al.*, 2013).

			Haplotype	*22 UNK	*27 EM	*29 IM	*41 IM	*43 EM	*45 EM	*1xN UM	*2xN UM
			Variant location	rs138100349	rs769157652	rs61736512 rs1058164 rs16947 rs59421388 rs1135840	rs28735595 rs28624811 rs28633410 rs1080995 rs1080996 rs74644586 rs76312385 rs75276289 rs28695233 rs1081000 rs1058164 rs16947 rs28371725 rs1135840	rs28371696	rs28371699 rs28371701 rs28371702 rs1058164 rs28371710 rs28371718 rs76015180 rs16497 rs28371726 rs1985842 rs28371730 rs4987144 rs1135840	CYP2D6 CNV	CYP2D6 CNV rs1058164 rs16947 rs1135840
			Published	Marez *et al.*, 1997			Raimundo *et al.*, 2000	Marez *et al.*, 1997	Gaedigk *et al.*, 2005	Dahl *et al.*, 1995	Johansson *et al.*, 1993
			Type of Study	Functional			Association	Functional	Association	Functional	Association
Country	**Population**	**Number of Subjects**	**Frequency (%)**								
South Africa	Bantu (Sistonen *et al.*, 2007)	8	Predicted	-	-	6.3	0	-	-	0	0
	Mixed Ancestry (Gaedigk & Coetsee, 2008; Ikediobi *et al.*, 2011)	99, 67	Predicted, Association	-	-	4.6	3.5	-	-	-	1.0
	Venda (Dandara *et al.*, 2001; Matimba *et al.*, 2008, 2009)	9, 76, 81	Predicted, Association, Functional	0	0	6.0	-	0	17.0	-	-
	Xhosa (Ikediobi *et al.*, 2011; Wright *et al.*, 2010)	53, 109	Association, Predicted	-	-	13.2	1.9	0.9	10.4	0	-
	South African (Dodgen *et al.*, 2015)	100	Predicted	0.5	-	3.6	3.6	1.5	-	-	0.5
Namibia	San (Sistonen *et al.*, 2007)	7	Predicted	-	-	0	0	-	-	14.3	0
Zimbabwe	San (Matimba *et al.*, 2008, 2009)	64	Association, Functional	0	-	2.0	-	-	-	-	-
	Bantu (Masimirembwa *et al.*, 1996; Matimba *et al.*, 2008, 2009)	23, 80	Predicted, Association, Functional	0	0	17.0	-	3.0	0	-	2.5
	Zimbabwean (Dandara *et al.*, 2001)	114	Predicted	-	-	-	-	-	-	-	-

"-" = Undetermined WT = Wildtype EM = Extensive Metaboliser IM = Intermediate Metaboliser PM = Poor Metaboliser UM = Ultra-rapid Metaboliser

1.2 Rationale

Fatalities suspected to be due to adverse drug reaction is a prevalent problem faced in the Western Cape (van Heerden *et al.,* 2009). This toxicity may be accidental, due to inadequate or enhanced metabolism/excretion. In cases such as these, drug toxicity may directly cause, or contribute to, death.

Analyses requested by forensic pathologists within the Western Cape do not incorporate a molecular-based analysis component. Furthermore, there are currently no optimised and validated molecular-based assays designed for identifying genetically altered *CYP450* genes in the local Forensic Pathology context. This makes it difficult to understand the influence genetic polymorphisms have on *CYP450* expression and functionality among the deceased.

Molecular assays to investigate genetic variation in *CYP2D6* have been developed in other settings before and this has shown value in clinical applications with regards to pharmaceutical drugs (McElroy *et al.,* 2000).

A molecular assay to sequence genetic variants in *CYP2D6* is hypothesised to also have value in a forensic post-mortem setting as many of the drugs in the Western Cape are metabolised by *CYP2D6*. A molecular autopsy, in which the molecular assay is utilised, may help resolve ambiguous toxicity cases and possibly provide more detailed reports that are able to help magistrates determine a manner of death. Before such an assay can be utilised by Forensic Pathology Services in the local context, an assay needs to be designed, optimised for use on post-mortem samples, thoroughly investigated through research, and then extensively validated, before it is carried out as a service.

Drugs, therapeutic or illicit, which are commonly abused in the Western Cape will be the focus in assay development. While it is acknowledged that many enzymes contribute towards the metabolism of certain drugs, this is the first study in our context and will focus on one enzyme, CYP2D6. This may assist in interpretation of certain fatal drug intoxication cases, such as whether it was a suicide or accidental slow gradual intoxication due to an ineffective drug metabolising enzyme, in particular the CYP2D6 enzyme coding genes. Some cases of drug-related fatalities admitted to SRM, may be the result of accidental adverse drug reactions attributed to polymorphisms observed in the encoding regions of enzymes that metabolise drugs.

Although little research has been conducted on African populations in a forensic setting, it has been reported that South Africans have a unique distribution of polymorphisms in *CYP2D6* (Warnich *et al.,* 2011). The current genomic challenge is to ascertain information on genetically diverse understudied populations, to translate this knowledge into forensic applications that can be applied to post-mortem investigations and to point out the challenges that can impact on the realisation of the benefits of toxicogenetics in South Africa (Warnich *et al.,* 2011). This highlights the importance of designing an experimental assay, by drawing on literature of similar nature, to enable the sequencing of coding regions of *CYP2D6,* which will allow the interpretation of metabolising phenotypes in the local context.

1.3 Aims and Objectives

The aim of this study was to therefore design and optimise a molecular-based assay to evaluate genetic variation in toxicological-related fatalities. This assay hopes to eventually aid the procedures surrounding molecular autopsies for specific toxicity cases associated with CYP2D6 drug metabolism fatalities.

To this end, the objectives were to:

- Design an assay to amplify and sequence the exons of *CYP2D6,* by drawing on similar studies;
- Optimise the assay in a local setting on DNA from healthy, control individuals;
- Set up a standard operating procedure and analysis pipeline using the results generated from optimisation for in-house research use;
- Apply the assay on DNA from two decedents admitted to Salt River Mortuary and interpret the findings within the case context, to demonstrate a proof of concept.

2. Materials and Methods

2.1 Study design and ethics approval

The research was a laboratory-based, prospective, cross-sectional study with a quantitative paradigm. This pilot study was focussed around design and optimisation, following the application of the optimised assay to demonstrate a proof of concept on a case example.

The University of Cape Town Faculty of Health Sciences Human Research Ethics Committee (HREC) granted ethics approval for this study (HREC REF: 110/2017) (Appendix 1).

An overview of the process by which designing and optimising the molecular-based assay took place, as well as its application, is depicted below (Figure 1).

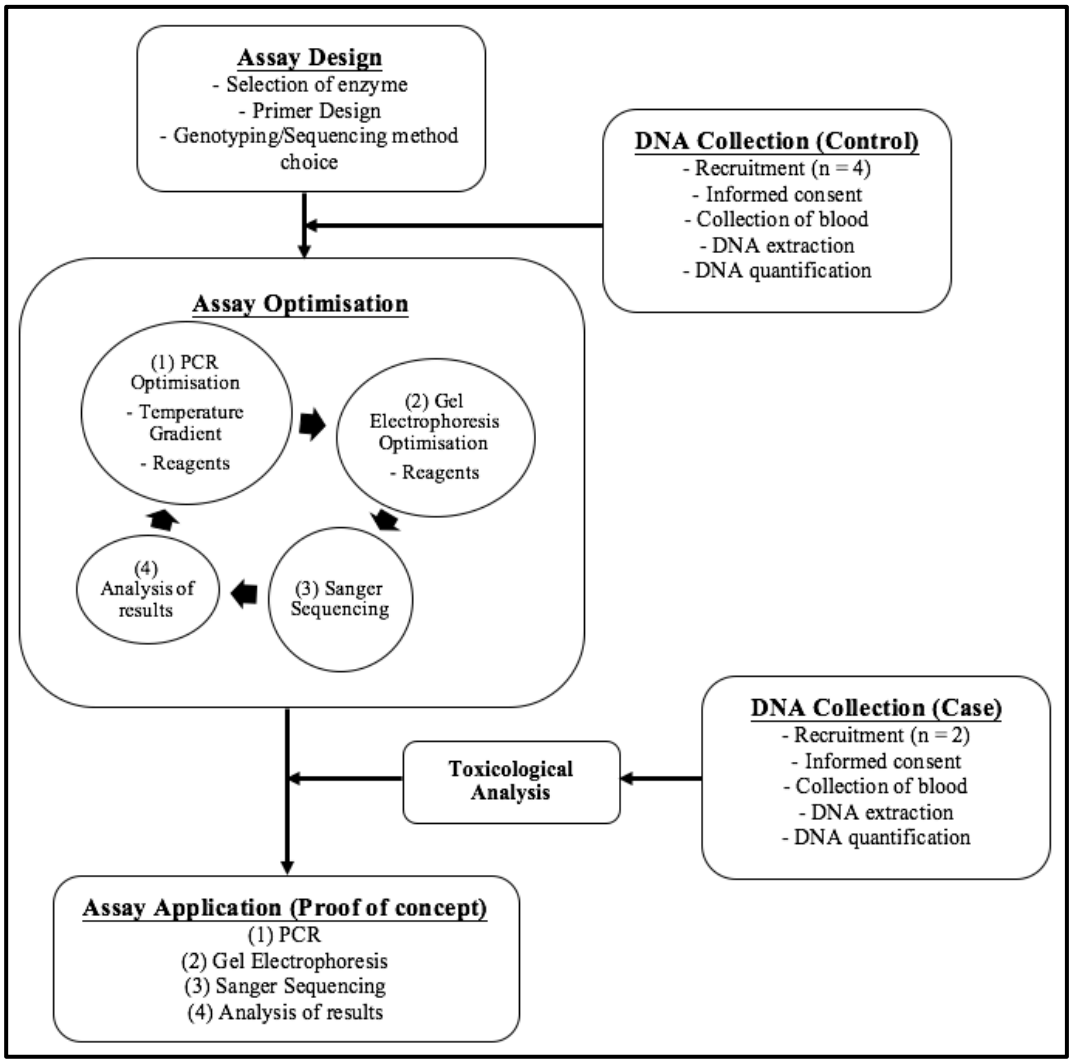

Figure 1: Flow diagram illustrating the dynamic process undertaken to optimise and design the molecular-based assay. The assay was applied to two case examples from Salt River Mortuary.

2.2 Participants and sampling

2.2.1 Control Participants

For the study, a convenience sampling method was employed, whereby biological samples from four living, healthy participants of different populations groups based on race were used for the assay optimisation. This was deemed sufficient for several reasons, including: (i) the amount of DNA extracted from biological samples of a single individual would be enough for optimisation purposes; (ii) having more than one participant as a control highlights reproducibility; and (iii) emphasises the ease of accessibility to different population groups within South Africa in which several different variations are observed. Using DNA from living individuals, as opposed to deceased individuals, was deemed appropriate for the designing and optimisation of the molecular-based assay, due to ethical reasons – as obtaining informed consent and biological samples from the vulnerable deceased population needs to be strongly justified and was unnecessary for the optimisation phase of this study. Participation from control participants was voluntary and volunteers gave informed consent to participate (Appendix 2). Participants were all over the age of 18 years and each self-reported that they belonged to a different population group.

2.2.2 Case Examples

A biological sample from two decedents was sufficient to demonstrate a proof of concept. The identification process of suitable cases was performed in conjunction with a forensic pathologist, whereby the cause of death was associated with possible drug use/abuse. Informed consent from the decedent's next-of-kin was received before any sample collection took place (Appendix 3).

2.2.3 Sample Collection

From the four control participants and the two decedents, a sample of 4 ml of blood was collected by using a venipuncture method and placing the blood into a purple top blood collection tube (BD Vacutainer®, New Jersey, USA) containing ethylene-diamine-tetraacetic acid (EDTA) and stored at 4 degrees Celsius until further processing the same day.

2.2.4 DNA Extraction

Once the samples had been obtained, DNA extraction took place using the QIAamp® DNA

Investigator Kit (QIAGEN, Hilden, Germany). The manufacturer's protocol used, pertains to the isolation of total DNA from small volumes of whole blood (1 – 100 µl). A volume of 100 µl of blood was initially used and in step two of the protocol 10 µl of ATL buffer was added, creating a final volume of 110 µl. In step nine, the MiniElute Column was centrifuged at 10,000 revolutions per minute (rpm) for 1 minute 30 seconds. Lastly, in step 15, 50 µl of ATE buffer was added to elute the DNA from the membrane.

2.2.5 DNA Quantification

Nanodrop spectrophotometry was used to quantify the amount of total nucleic acids in the samples of interest (Desjardins and Conklin, 2010). A 2 µl sample of each DNA extract was pipetted onto the lower pedestal. The absorbance of light at 260 nm was measured and according to Beer's Law, was proportional to the concentration of the sample medium (Gallagher, 1989). This enabled the quantification of the DNA present in the sample as well as the 260/230 and 260/280 purity ratios. This was carried out using the NanoDrop 2000 spectrophotometer (Thermofisher Scientific, Massachusetts, USA).

Quantitative PCR (qPCR) (Applied Biosystems 7500), was performed in-house in a parallel study, using the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, California, USA), in order to establish whether degradation of the samples occurred. It was performed according to the manufacturer's protocol. The trio kit quantifies a short and large fragment of DNA and by comparing the concentration of each fragment in a ratio (short/large), the degradation can be assessed. Degradation scores greater than 1 (>1) are representative of degraded samples.

2.3 Assay Design

2.3.1 Selection of Enzyme and Primer Design

The CYP2D6 enzyme was selected as the enzyme of choice based on its relevance in the metabolism of drugs. Before primers could be designed, the *CYP2D6* gene sequence was downloaded from Ensembl genome browser (http://www.ensembl.org) (Ensembl release 90, August 2017) – a website of an automated annotation of the human genome which provides a range of data that expands to include comparative genomics, variation and regulatory data. It identifies possible variants within coding and/or non-coding regions that can affect primer

design and primer suitability. Ensembl was used to locate the nine different exons within the *CYP2D6* gene.

Primer design is aimed at obtaining a balance between two definitive objectives – specificity and efficiency of amplification (Dieffenbach *et al.,* 1993). Primers were designed in order to amplify and subsequently analyse the coding regions of the *CYP2D6* gene. In order to target the exons within the gene, primers were designed so that they flanked the regions of interest, allowing for the exons to be amplified.

The suitability of the primers was assessed using online primer design tools that included: (i) Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Accessed: 29th January 2017) – a bioinformatic tool that identifies possible primers according to, parameters established, and the insertion of a region of interest; (ii) OligoAnalyzer 3.1 (http://eu.idtdna.com/calc/analyzer) (Integrated DNA Technologies Inc., USA) – a software application that determines the properties of any oligo sequence as well as facilitate the intelligent design of assay conditions; and (iii) primer BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Accessed: 29th January 2017) – which assesses the primer pair specificity against a selected database.

Exon 1 of the *CYP2D6* gene was 1169 base pairs (bp), therefore, three sets of overlapping primers were designed (Exon 1 part 1, 2 and 3), such that each target amplicon length was less than 600 bp; as target DNA between 150 and 600 bp is one of the ideal primer properties in primer design for standard PCR (Dieffenbach *et al.,* 1993). The forward primer for exon 1 part 2 was chosen from the primer sets designed by Matimba *et al.* (2009). Exons 2 – 9 were all shorter than 600 bp; therefore, they were of an ideal length. All primers were designed such that their length was 18-24 bp, they contained a 50 – 65% GC content and maintained a melting temperature (T*m*) between 50 – 60°C. Limited strong secondary structures, avoiding the presence of any heterodimers and avoiding false priming by verifying the primers specificity using Primer BLAST, such that the ideal parameters for primers were met (Vet and Marras, 2005).

Ensembl was used to explore the SNPs observed in the primer binding regions that have either been identified, or flagged as possible SNPs, in previous literature. Any SNPs that were found to be present in the population of interest at a frequency higher than 1%, were noted and the primer was shifted in order to exclude these variants. Once the primers met the ideal parameters, specificity was assessed using primer BLAST and primer sets were deemed

adequate only if the amplicon of interest would be amplified, with no problematic non-specific products being produced (Ye *et al.,* 2012).

The primers were ordered (IDT, Cape Town, South Africa) and were re-suspended in 1 x Tris-EDTA (TE) buffer (Sigma-Aldrich, St. Louis, USA) to a stock concentration of 100 µM.

Table 3: Primer sets designed to amplify the exons in *CYP2D6*.

Primer Set	Primer Direction	Sequence (5' – 3')	Length (bp)	GC content (%)	Predicted T*m* (ºC)	Amplicon size (bp)
1. Exon 1 part 1	Forward	GCCATCATCAGCTCCCTT	18	55.6	54.9	439
	Reverse	CCCAAACCTGCTTCCCCTT	19	57.9	57.9	
2. Exon 1 part 2	Forward	CCCTACCAGAAGCAAACA (Matimba *et al.*, 2009)	18	50.0	52.0	597
	Reverse	CCTATTTGAACCTTGGACGA	20	45.0	52.1	
3. Exon 1 part 3	Forward	CTTCCACCTGCTCACTCC	18	61.1	55.3	314
	Reverse	TCTGTCTCTGTCCCCACC	18	61.7	56.1	
4. Exon 2&3	Forward	GTGGATGGTGGGGCTAAT	18	55.6	54.6	483
	Reverse	ACTCCTCGGTCTCTCGCT	18	61.1	57.7	
5. Exon 4	Forward	CCCGTTCTGTCTGGTGTAG	19	57.9	54.9	266
	Reverse	AGCCTCCCCTCATTCCTC	18	61.1	56.3	
6. Exon 5&6	Forward	GTTCTGTCCCGAGTATGC	18	55.6	52.7	334
	Reverse	CCTGACACTCCTTCTTGC	18	55.6	52.9	
7. Exon 7	Forward	CATAGGAGGCAAGAAGGAG	19	52.6	52.1	382
	Reverse	TGGTGGCATTGAGGACTA	18	50.0	53.1	
8. Exon 8	Forward	ATCCTAGAGTCCAGTCCC	18	55.6	52.3	534
	Reverse	ACTACCACATTGCTTTATTGTAC	23	34.8	51.0	
9. Exon 9	Forward	TATCACCCAGGAGCCAGG	18	61.1	56.3	520
	Reverse	CCCACATGCCAGGACAAT	18	55.6	55.4	

***** Primer design tools included: (i) Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Accessed: 29th January 2017); (ii) OligoAnalyzer 3.1 (http://eu.idtdna.com/calc/analyzer) (Integrated DNA Technologies Inc., USA); and (iii) primer BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Accessed: 29th January 2017).

2.3.2 Sanger Sequencing as a Method of Choice

Genotyping is the process of determining genetic variations at specific loci within an individual. Sequencing, is the process of identifying the exact sequence of a length of DNA

(Sanger *et al.,* 1977). Sequencing was the method of choice as the focus of interest was not just certain genetic variants that are found in commonly studied populations but the entire sequence of understudied populations. In order to have identified genetic variants that may not be found in other population groups but are common in the population group of interest, Sanger sequencing was considered ideal.

2.4 Assay Optimisation

2.4.1 PCR Optimisation

PCR optimisation was initially performed by use of a temperature gradient PCR to establish the optimal annealing temperature for each primer set. The reaction components for each PCR included 1 X *Taq* polymerase 2X Ready Mix, 0.2 μM of each of the forward and reverse primer and 50 ng of DNA template in a final volume of 25 μl. AccuGene® molecular biology grade water (Lonza, Rockland, USA) was added to the negative control (no template control - NTC) instead of template DNA.

Three different *Taq* polymerase ready mixes were also investigated whereby temperature gradient PCRs for part 1 of exon 1 were set up using each of the following *Taq* polymerases respectively; (i) *Taq* DNA Polymerase Master Mix Red kit (AMPLIQON, Odense, Denmark); (ii) EmeraldAmp® MAX HS PCR Master Mix (Takara Biotechnology, Mountain View, USA); and (iii) GoTaq® Green Master Mix (Promega, Madison, USA). The GoTaq polymerase ready mix was selected for the subsequent PCR reactions as it produced PCR products with the best yields and with the least non-specificity over the range of annealing temperatures.

PCR was performed in the Bio-Rad T100 Thermocycler (Bio-Rad Laboratories, USA) and thermal cycling was according to standardised cycling conditions: initial denaturation step at 95°C for 5 minutes; then for 30 cycles thereafter, a three-step process consisting of denaturation at 95°C for 30 seconds; annealing at temperatures ranging from 49°C - 65°C for 30 seconds and elongation at 72°C for 30 seconds was completed. A final elongation step at 72°C for 5 minutes was completed accounting for any remaining single-stranded DNA still needing to be extended. The lowest annealing temperature in the gradient was established by subtracting 5°C from the predicted T*m* of the primers within the primer set.

If non-specific products were observed, the PCR was further optimised by repeating the temperature gradient at higher annealing temperatures. The final annealing temperature

selected for each primer set was based on yield and specificity of the PCR products at each of the temperatures within the gradient. For exon 1 (part 1 and 3) and exon 9, a final annealing temperature of 62.9°C was used, while the rest used a final annealing temperature of 55.3°C.

2.4.2 Agarose Gel Electrophoresis

Subsequent to each PCR, agarose gel electrophoresis was performed to visualise PCR products. A 1% (w/v) agarose gel was made by dissolving agarose powder (SeaKem® LE, Rockland, USA) in 1X Tris-Borate-EDTA (TBE) buffer (Sigma-Aldrich, St. Louis, USA). Initially, two different nucleic acid gel stains, SYBR® Safe (Thermofisher Scientific, Massachusetts, USA) and GelRed™ Nucleic Acid Gel Stain (Biotium Inc., California, USA), were investigated and the SYBR® Safe was selected for subsequent use. A volume of 7.5 µl of SYBR® Safe nucleic acid gel stain was added to the agarose gel for DNA visualisation. The gel was poured into a casting tray with a comb in position and allowed to set. Once the gel had set, it was transferred into a gel tank, filled with 1X TBE buffer. An amount of 5 µl of Quick-Load® 50 bp Ladder (New England BioLabs® Inc., Ipswich, USA) was loaded into the first well, followed by 5 µl of the NTC PCR product into the second well. A volume of 5 µl of each PCR product was subsequently loaded into consecutive wells of the gel. The gel tank was then connected to the Bio-Rad PowerPac™ Basic (Bio-Rad Laboratories, Hercules, USA) and subjected to electrophoresis at 100 V for 80 minutes. The gel was visualised under UltraViolet (UV) light on a Syngene Chemi Genius Bio-Imaging System (Syngene, Gurugram, India).

2.4.3 Sanger Sequencing

Before sequencing commenced, a post-PCR clean-up step was performed, using a Nucleofast 96 well PCR plate (Macherey Nagel, Düren, Germany). The manufacturer's protocol was implemented on a Tecan EVO 150 robotic workstation (Tegan Trading, Switzerland). This purifying step achieved removal of unincorporated dNTPs, primers, short failed PCR products and salts in order to minimise interference with results.

DNA sequencing was performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, California, USA). Sequencing was performed on each of the 9 exons within the *CYP2D6* gene (Sanger *et al.,* 1977). PCR products of exon 1 (part 1, 2 and 3) were sequenced with their respective forward and reverse primers in order for the entire exon including overlapping regions to be sequenced and/or to identify if there was a possible deletion of the entire gene. The PCR products of the remaining exons were sequenced with either their

corresponding forward or reverse primer. The primer that is positioned further from the region of interest was chosen for sequencing, to allow for the standard noise at the beginning of the sequencing strand to not overlap with the region of interest. Certain PCR products were sequenced in duplicate to confirm the presence of potentially novel variants. The Sanger sequencing was performed at the Central DNA Sequencing Facility, University of Stellenbosch, Western Cape; according to their in-house protocol.

2.4.4 Amendment to Protocol During Optimisation

During optimisation, the primer sets used were amended as control 4 (and a case sample, as later identified) had three SNPs within the primer binding region, that did not allow primer binding to occur (see Chapter 3 section 3.1.2). During primer design, these variations were noted, but had extremely low frequencies in the background population. However, due to their presence, the forward primer of exon 1 part 2 could not bind; as such, the primer set was amended. The amended primer set (new exon 1 part 1) now consisted of the original exon 1 part 1 forward primer (5'-GCCATCATCAGCTCCCTT-3') but the exon 1 part **2** original **reverse** primer (5'-CCTATTTGAACCTTGGACGA-3'), which covered a region of 954 bp. The new primer set was assessed using bioinformatics, and optimised according to the methods described above (Table 4).

Table 4: **Amended primer set exon 1 part 1 (with original exon 1 part 1 forward primer and exon 1 part 2 reverse primer).**

Primer Set	Primer Direction	Sequence (5' – 3')	Length (bp)	GC content (%)	Predicted T*m* (°C)	Amplicon size (bp)
1. Exon 1 part 1	Forward	GCCATCATCAGCTCCCTT (unchanged)	18	55.6	54.9	954
	Reverse	CCTATTTGAACCTTGGACGA (originally exon 1 part 2 reverse primer)	20	45.0	52.1	

2.4.5 Data Analysis

The sequence data was analysed using two different DNA sequencing software platforms. ChromasLite version 2.4.4 was used to view the resulting electropherograms and identify known or possible novel variants in the sequenced gene (Technelysium, South Brisbane, Australia). The sequencing results from the participants were also compared to the reference *CYP2D6* sequence, by aligning the sequences, using BioEdit version 7.2.6 Sequencing Alignment Editor and Analysis Program (Hall, 1999). ClustalW was used for multiple sequence alignment with a bootstrap of a 1000. SNPs, Indels and other variations were recorded

and compared to the literature and to genome data available on Ensembl to assess their functional significance and/or novelty.

The resulting optimised assay was then written into a standard operating procedure, which is being published in-house and is available upon request.

2.5 Assay Application

A volume of 500 µl of blood was aliquoted from each of the tubes (Grey, Red and Purple top tubes) that were collected at autopsy for toxicological analysis (performed in-house in parallel study). Sample preparation in the form of acetonitrile protein precipitation was performed on the aliquots, before they underwent screening by means of liquid chromatography-tandem mass spectrometry (LCMSMS) analysis using a Shimadzu Prominence (Tokyo, Japan) High Performance Liquid Chromatography system coupled to a AB SCIEX API 3200 Q-TRAP Mass Spectrometer (Massachusetts, USA), that operates on the AB SCIEX MasterView^{TM} software (Massachusetts, USA). The blood from which the samples that underwent a toxicological screen were aliquoted, remained under storage at 4˚C until DNA extraction was performed. The toxicological analysis was performed in-house by the Division of Clinical Pharmacology, University of Cape Town.

The optimised assay was then applied to the DNA extracted from the cases (see Chapter 2 section 2.2.4), using the developed standard operating procedure. Upon analysis of sequencing results, PCR products of exons 4, 5, 6 and 9 were sequenced in duplicate, using their opposite primer, to clarify the variants observed in the sequencing data.

3. Results

3.1 Assay Optimisation

Assay optimisation was performed using DNA from four healthy, control individuals. DNA was extracted and quantified successfully (Table 5). In all instances, the first elution of DNA was used for optimisation. An A_{260}/A_{280} ratio above 1.80, in Table 5, indicates relatively pure samples with little, if any, protein contamination. The A_{260}/A_{230} ratios were more variable, however, the samples used were considered adequate for further use.

Table 5: DNA quantification results of the four control's blood samples, using a NanoDrop 2000 spectrophotometer.

Controls	DNA concentration (ng/µl)	A_{260}/A_{280} absorbance ratio	A_{260}/A_{230} absorbance ratio
Control 1 – Sample A	16.4	1.73	1.44
Sample B	12.5	1.86	1.00
Control 2 – Sample A	25.0	1.80	1.66
Sample B	10.4	1.86	0.94
Control 3 – Sample A	33.7	1.80	1.49
Sample B	21.6	1.98	0.81
Control 4 – Sample A	17.4	1.71	1.38
Sample B	16.8	1.81	1.17

*Sample A – First elution (used for optimisation); Sample B – Second elution.

3.1.1 PCR Optimisation

Following the selection of the CYP2D6 enzyme, nine sets of primers were designed to amplify and sequence the exons within the *CYP2D6* gene. To this end, temperature gradients were performed for each region of the *CYP2D6* gene. Figure 2 shows the temperature gradient for exon 1 part 1 as visualised on a 1% agarose gel, and is a representation of the typical results obtained for all nine regions (which can be found in Appendix 4). The final annealing temperature selected for each primer set was based on yield and specificity of the PCR products at each of the temperatures within the gradient.

During the first temperature gradient (exon 1 part 1), three different *Taq* polymerase ready mixes and two different visualisation dyes were investigated (Figure 2). The GoTaq polymerase ready mix produced PCR products with the best yields and with the least non-specificity over the range of annealing temperatures (Figure 2C) and was thus selected for all the subsequent PCR reactions.

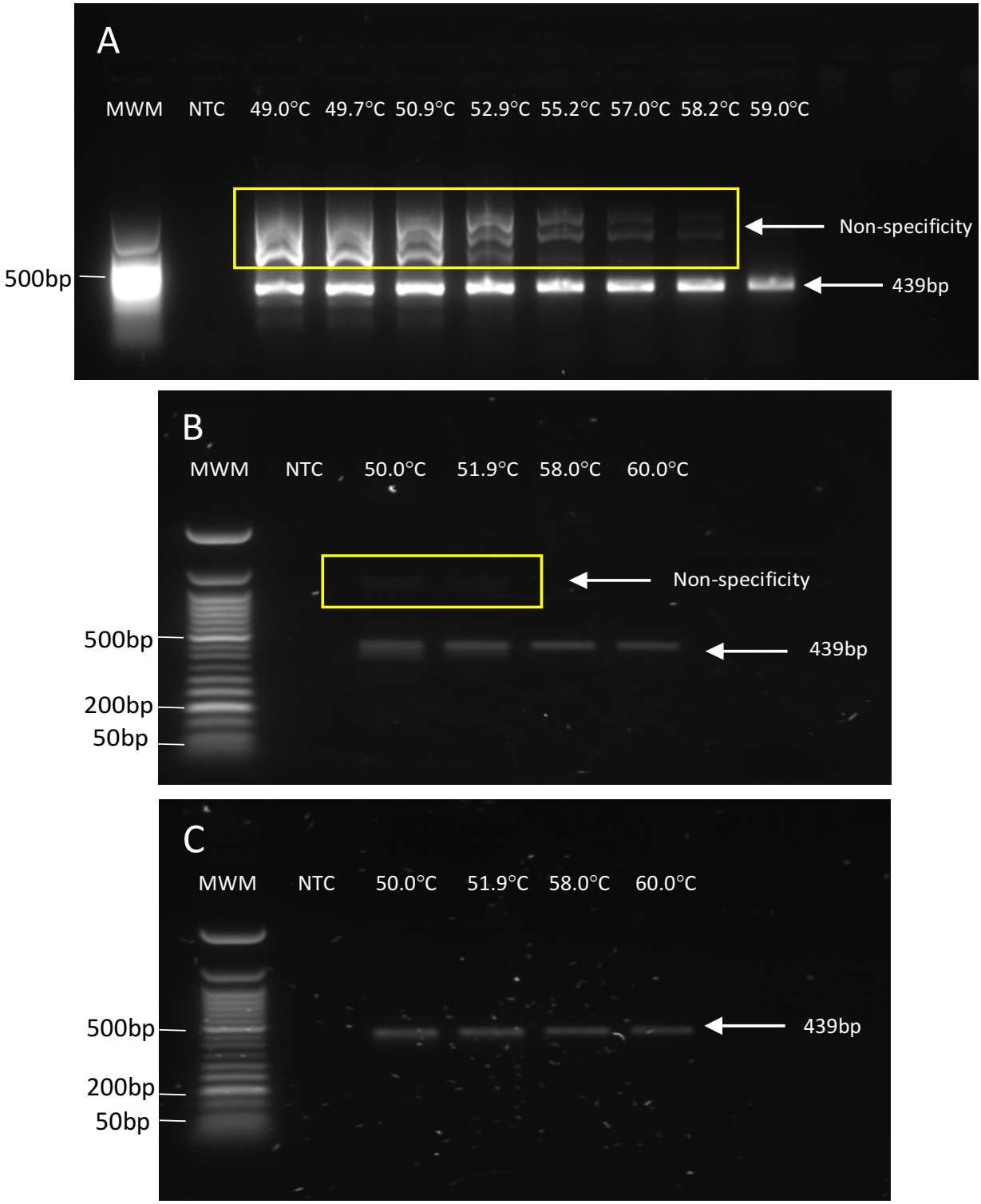

Figure 2: Gel electrophoresis showing PCR products of Exon 1 part 1 using three different *Taq* polymerases. A) *Taq* DNA Polymerase Master Mix Red (AMPLIQON, Odense, Denmark); B) EmeraldAmp® MAX HS PCR Master Mix (Takara Biotechnology, Mountain View, USA); and C) GoTaq® Green Master Mix (Promega, Madison, USA). The gel was visualised under UltraViolet (UV) light on a Syngene Chemi Genius Bio-Imaging System (Syngene, Gurugram, India). MWM: 50bp molecular weight marker; NTC: No Template Control.

The agarose gels prepared using SYBR® Safe nucleic acid gel stain (Figure 2B and 2C) were preferred to the gel prepared using GelRed™ nucleic acid gel stain (Figure 2A), as clearer staining of the MWM and a decreased background fluorescence excitation was observed in the gels prepared with SYBR® Safe. SYBR® Safe was used in subsequent agarose gels.

Overall, for exon 1 part 3 and exon 9, a final annealing temperature of 62.9°C was used for PCR, while the rest of the exon regions incorporated an annealing temperature of 55.3°C. In order to test the assay further, PCR of all exon regions and subsequent agarose gel electrophoresis was performed on DNA from the four controls. The presence of bands at the correct position on the gel based on the known amplicon size was representative of positive amplification. Figures 3 and 4 represent PCR amplification of exon 8 and exon 1 part 2, respectively. Agarose gel images of the remaining exon regions can be observed in Appendix 5.

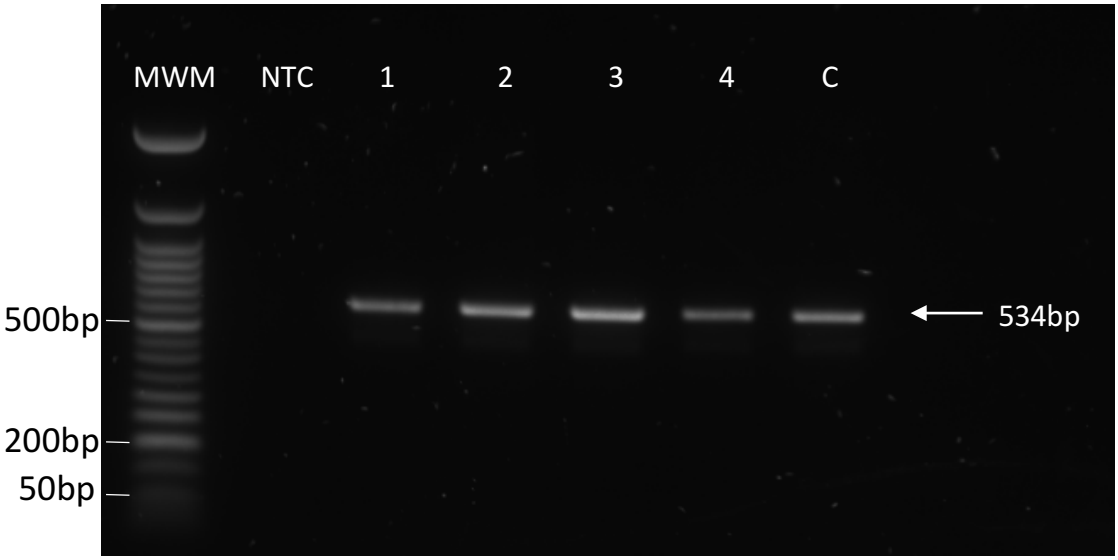

Figure 3: Gel electrophoresis showing amplification of Exon 8 in the four different controls and case 1. The gel was visualised under UltraViolet (UV) light on a Syngene Chemi Genius Bio-Imaging System (Syngene, Gurugram, India). MWM: 50bp molecular weight marker; NTC: No Template Control; 1: Control 1; 2: Control 2; 3: Control 3; 4: Control 4 and C: Case 1.

Amplification of exon 1 part 2 was successful in all the controls except for control 4 (Figure 4). Following repetition of PCR and agarose gel electrophoresis, the same result was observed, which eliminated pipetting error as the cause for lack of amplification. It was thus hypothesised that there may be genetic variation in the primer binding region, which was then investigated through Sanger sequencing.

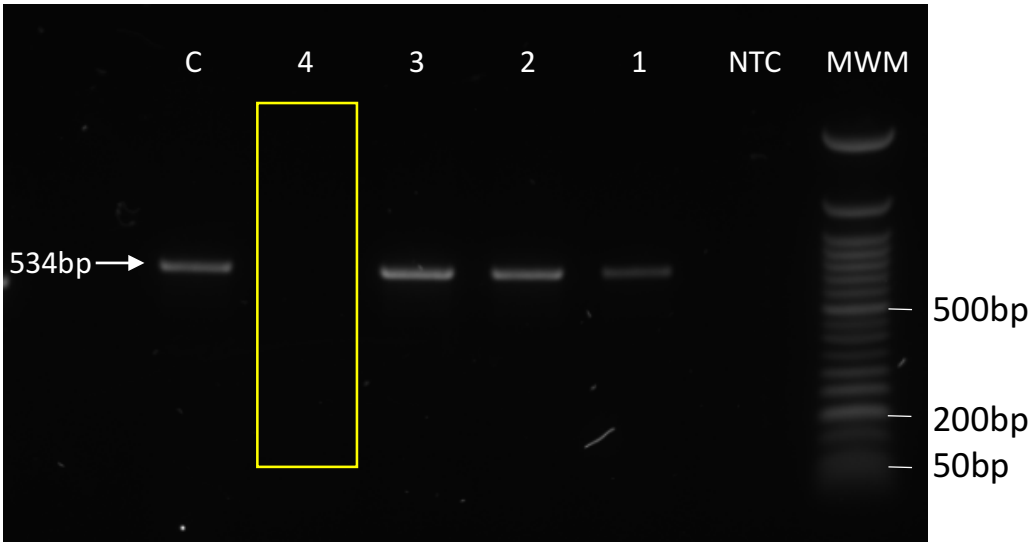

Figure 4: Gel electrophoresis showing amplification of Exon 1 part 2 in the four different controls and case 1. The gel was visualised under UltraViolet (UV) light on a Syngene Chemi Genius Bio-Imaging System (Syngene, Gurugram, India). MWM: 50bp molecular weight marker; NTC: No Template Control; 1: Control 1; 2: Control 2; 3: Control 3; 4: Control 4 and C: Case 1.

3.1.2 Sequencing Optimisation

The PCR products from the four control participants were sequenced and analysed in order to (i) confirm whether the amplified PCR products were indeed the regions of interest; (ii) to confirm which primer was optimal for sequencing (Figure 5); and (iii) to identify the reason for the lack of amplification of the region exon 1 part 2 in control 4.

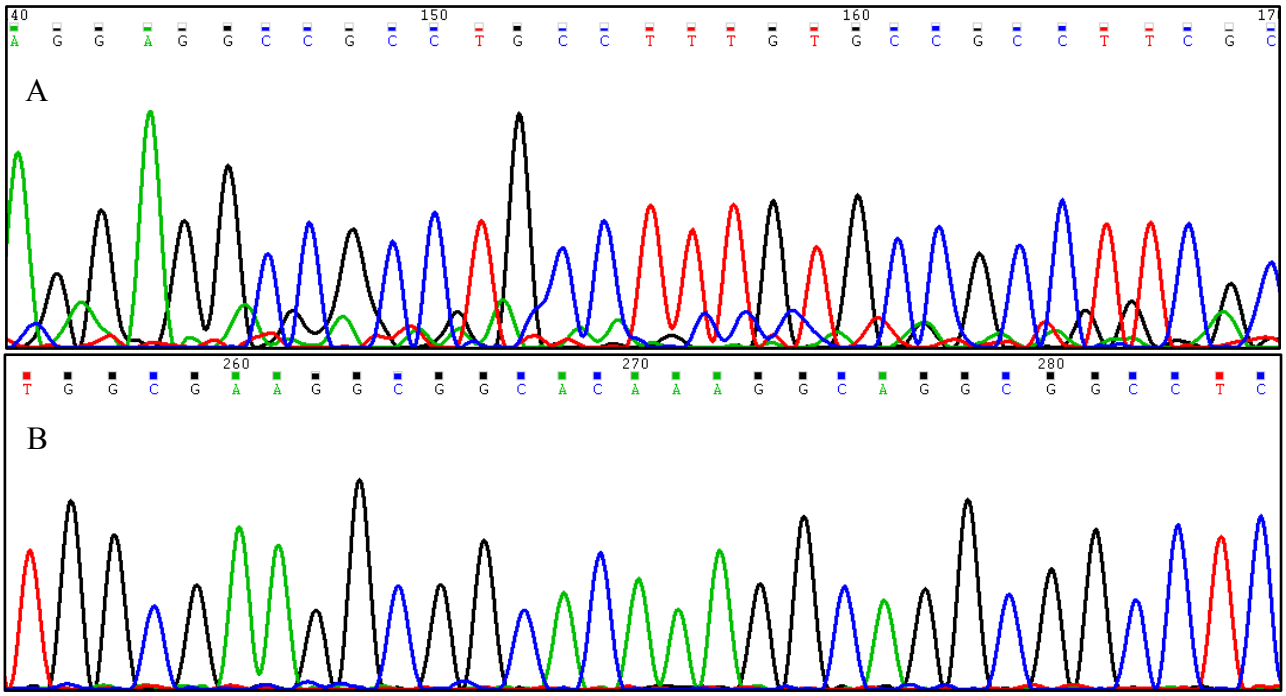

Figure 5: Two electropherograms representing a portion of Exon 2&3. (A) Sequence using the forward primer vs. (B) using the reverse primer.

In Figure 5A, the sequenced region shows background noise, which is not ideal; at worst, it may contribute to the incorrect calling of SNPs. However, Figure 5B, which represents the same region but sequenced with the reverse primer, shows a clean electropherogram. Therefore, the primers in Table 6, were the primers chosen for subsequent sequencing. However, for five of the regions, two sequencing reactions were preformed, one with the forward primer and one with the reverse primer, as these regions either contained an insertion/deletion; or they were overlapping regions of the exon (i.e. exon 1).

Table 6: The selected primers for subsequent sequencing of each exon region.

Primer set	Forward	Reverse
1. Exon 1 part 1	X	X
2. Exon 1 part 2	X	X
3. Exon 1 part 3	X	X
4. Exon 2&3		X
5. Exon 4	X	X
6. Exon 5&6	X	
7. Exon 7	X	
8. Exon 8		X
9. Exon 9	X	X

*X – Represents which primer was selected for sequencing.

It was hypothesised that the lack of amplification seen in the region of exon 1 part 2 in control 4 was due to a null allele. To confirm this, exon 1 parts 1 and part 3 were sequenced in both directions to observe and further analyse the primer binding regions of exon 1 part 2.

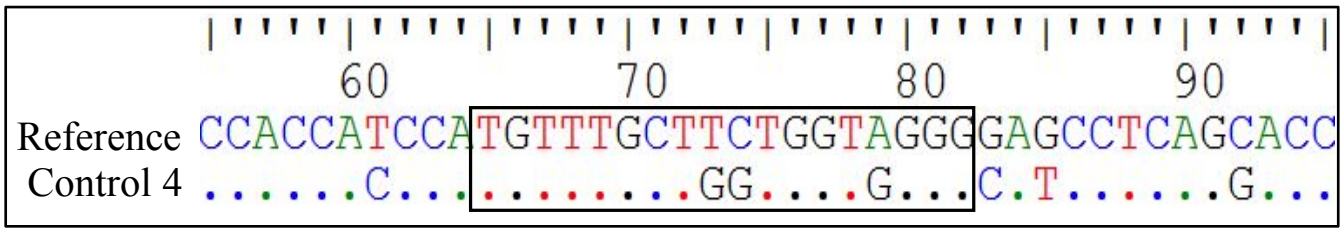

Figure 6: A sequence alignment of a portion of exon 1 part 1 in control 4 in the reverse direction. Illustrating variations within the primer binding region of exon 1 part 2.

In Figure 6, the sequence of DNA in control 4 has been compared to the reference sequence and the primer region of exon 1 part 2 has been highlighted; it shows that there was indeed variation present within the primer binding region of exon 1 part 2. There were three nucleotide changes: at positions 73 and 74 (c.180+52_180+53delGAinsCC) and at position 79 (c.180+47T>C). Therefore the presence of these three homozygous G/G variations likely prevented the forward primer of exon 1 part 2 binding to this region during PCR, thus explaining the null allele. As a result, the primer sets used were amended.

It should be noted that these variations were extremely rare, which was why the site was initially deemed to be suitable for primer binding. The amended primer set (new exon 1 part 1) was amended to consist of exon 1 part 1 forward primer (5'-GCCATCATCAGCTCCCTT-3') and exon 1 part 2 reverse primer (5'-CCTATTTGAACCTTGGACGA-3'), which covered a region of 954 bp.

3.2 Assay Application

3.2.1 Toxicological Screening Results

Blood collected from the two decedents was sent for a toxicological screening and the Table 7 summarised the substances found (Table 7). Full reports are in Appendix 6.

Table 7: Results of the toxicological screening of the two cases, using an LCMSMS.

Cases	Screening Method	Observations
Case 1	LCMSMS	Amitriptyline, Nortriptyline, Protriptyline, Citalopram and Paracetamol
Case 2	LCMSMS	Fluoxetine, Ephedrine, Doxylamine and Diphenhydramine

*LCMSMS – Liquid Chromatography Mass Spectrophotometry Mass Spectrophotometry.

3.2.2 DNA Quality Check

Subsequent to DNA extraction from blood samples of the two decedents, DNA quantification was performed using Nanodrop spectrophotometry. Quantitative PCR (qPCR) was also performed in order to establish whether the samples were degraded; whereby the higher the degradation index (>1), the more degraded the sample. Values close to 1, which was the observed in both cases, indicate relatively little degradation. The results of DNA quantification and the degradation index of each case are summarised in Table 8.

Table 8: DNA quantification results and degradation indices of the two cases, using a NanoDrop 2000 spectrophotometer and Applied Biosystems 7500 qPCR.

Cases	DNA concentration (ng/µl)	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio	Degradation Index
Case 1 – Sample 1	51.2	1.93	2.10	0.96
Case 2 – Sample 1	58.7	1.83	1.86	0.82

The optimised assay was then applied to the two cases. Figure 7 is a representation of an agarose gel showing amplification of each region in case 2. Initially, the original primer sets

(n=9) were used to amplify the DNA from the two case examples; however, exon 1 part 2 failed to amplify in case 2 (Figure 7).

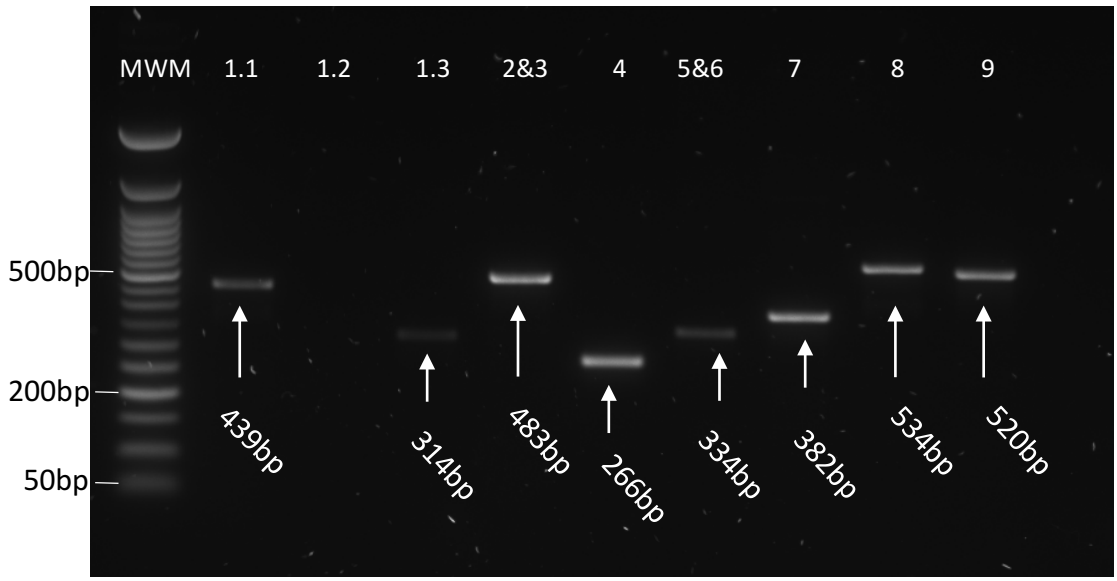

Figure 7: Gel electrophoresis showing amplification of eight regions within the *CYP2D6* gene, in case 2. MWM: 50bp molecular weight marker; 1.1: Exon 1 part 1; 1.2: Exon 1 part 2; 1.3: Exon 1 part 3; 2&3: Exon 2&3 4: Exon 4; 5&6: Exon 5&6; 7: Exon 7; 8: Exon 8 and 9: Exon 9.

In case 2 only, the new primer combination for exon 1 part 1 was then used for amplification (Figure 8).

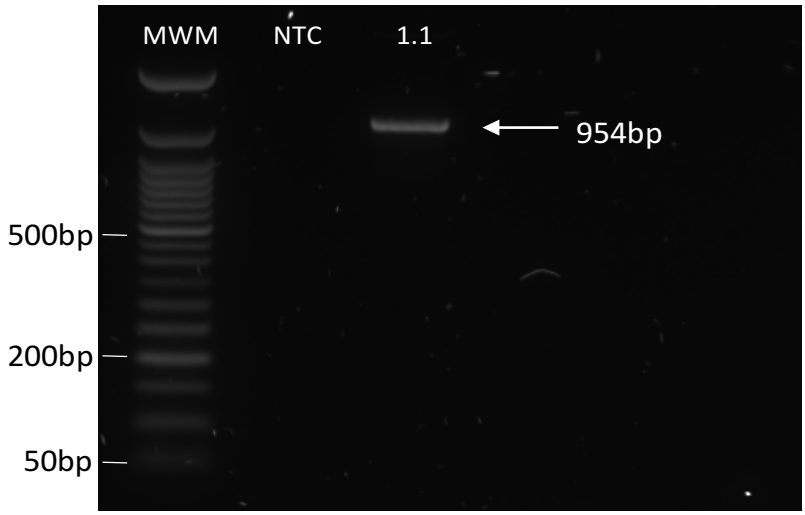

Figure 8: Gel electrophoresis showing amplification of the new Exon 1 part 1 region. Region in Exon 1 part 1 in *CYP2D6* gene in case 2, using the amended primer pair. 1.1: Exon 1 part 1.

After successful amplification, each region was sequenced and aligned with the reference sequence (Ensembl release 90, August 2017) and several variations were observed. For example, in Figure 9, the reference sequence of a region of exon 1 part 1 has been aligned with the same region in both case 1 and case 2; in both the forward and reverse direction. A variation,

more specifically an insertion (c.137_138insT) was observed, disrupting the sequence and causing misalignment thereafter.

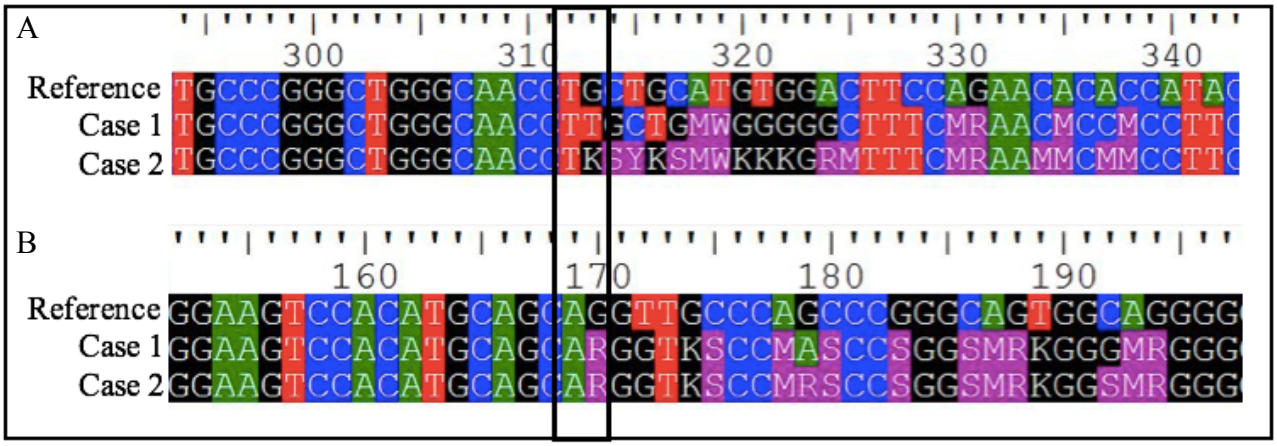

Figure 9: Two sequence alignments of a portion of Exon 1 part 1 in case 1 and 2. (A) forward direction and (B) reverse direction, illustrating a variation identified as an insertion (137_138insT).

3.2.3 Summary and Analysis of Variants

All variations observed during the analysis of the sequence were further investigated using Ensembl (Release 90, August 2017) and literature, to assess their functional significance. The contribution of the variations to haplotypes was also investigated and a summary of all variants observed for case 1 and 2 can be observed in Table 9 and 10, respectively. The reported minor allele frequencies are those of the variations observed in a combined world population as there is no data for South African 'Mixed Ancestry' populations. The frequencies were obtained from Ensembl, representative of data from 1000 Genomes Project Phase 3 (The 1000 Genomes Project Consortium, 2015) and gnomAD (version 2.0.2, October 2017). Enzyme activity of the associated haplotype and phenotype was also recorded.

Table 9: Frequencies and positions of *CYP2D6* genetic variants, their associated amino acid changes, haplotypes and effects on enzyme activity in Case 1.

RefSNP (rs) Number	Haplotype	Nucleotide Change M33388.1 ATG=1 (HGVS Nomenclature)	Genotype Observed	Amino Acid Change	Worldwide Reported Minor Allele Frequency	Enzyme Activity
rs28371696	CYP2D6*43	77G>A	G/A	R26H	1%	Normal
rs774671100	CYP2D6*13	137_138insT	-/T	-	<0.1%	Decreased
rs28371718	CYP2D6*1D	2575C>A (801C>A)	C/A	-	2.3%	Normal
rs77913725	CYP2D6*86	2606G>A (832G>A)	G/A	E278K	0.4%	Unknown
rs1135828		2610T>A (836T>A)	T/A	M279K	0.4%	
rs769157652	CYP2D6*27	3853G>A (1228G>A)	G/A	E410K	0.1%	Normal
Additional SNPs						
rs769258	-	31G>A	G/A	V11M	1.7%	Unknown
rs151226748	-	102A>G	A/G	-	<0.1%	Unknown
rs1080995	-	214G>C (180+34G>C)	G/C	-	30.7%	Unknown
rs1080996	-	221C>A (180+41C>A)	C/A	-	30.7%	Unknown
rs1080997	-	223C>G (180+43C>G)	C/G	-	30.7%	Unknown
rs1080998	-	227T>C (180+47T>C)	T/C	-	30.7%	Unknown
rs29001518	-	232G>C (180+52G>C)	G/C	-	30.7%	Unknown
rs28695233	-	233A>C (180+53A>C)	A/C	-	30.7%	Unknown
rs17002853	-	2466T>C (692T>C)	T/C	L231P	<0.1%	Unknown
rs17002852	-	2470T>C (696T>C)	T/C	-	0.7%	Unknown
rs28371721	-	2661G>A (843+44G>A)	G/A	-	1.8%	Unknown
rs59421388	-	3183G>A (1012G>A)	G/A	-	2.9%	Unknown
rs1135835	-	4131A>G (1408A>G)	A/G	A470T	0.1%	Unknown
rs1135839	-	4166T>C (1443T>C)	T/C	-	-	Unknown
rs28371736	-	4172C>T (1449C>T)	C/T	-	<0.1%	Unknown
rs1135840	-	4180G>C (1457G>C)	C/C	S486T	59.9%	Increased
rs61731577	-	4193T>C (1470T>C)	T/C	-	<0.1%	Unknown

***HGVS** – Human Genome Variation Society Nomenclature.

The variation 137_138insT was heterozygous, therefore causes decreased functionality of one allele. The remaining heterozygous SNPs that have also contributed to haplotypes, namely; *CYP2D6*1D, *27, *43* and **86,* all possess normal or unknown functionality. The combination of one allele with a known decreased activity (*13) and an allele with either a normal or unknown activity, infers either an EM or IM phenotype (Figure 10), however, without parental samples for segregation analysis, the phenotype cannot be classified further.

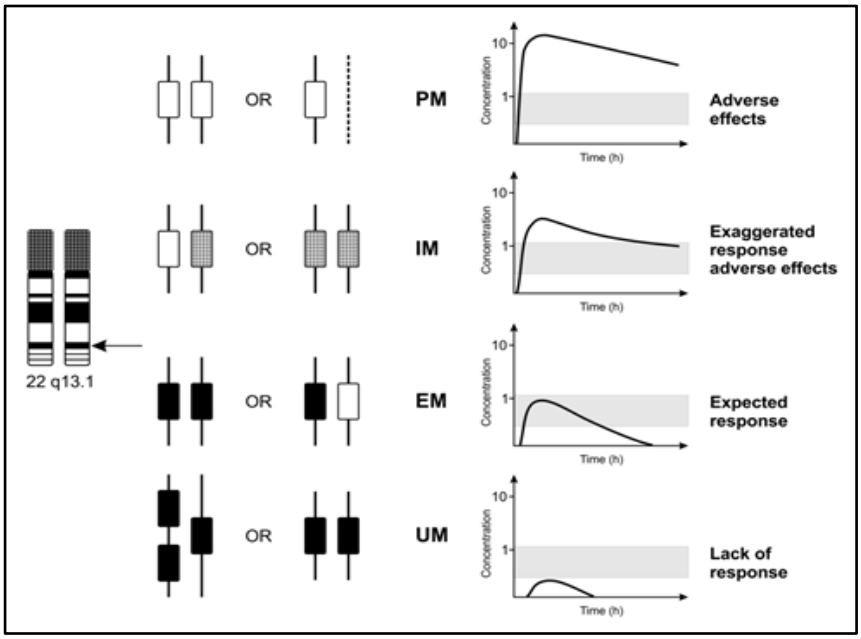

Figure 10: Genotype/Phenotype association and the effects in *CYP2D6* activity. (Taken from Balogh *et al.*, 2011). *Black box – Wild type alleles (Normal function), White box – Null alleles (No function), Dotted box – Decreased function.

Table 10: Frequencies and positions of *CYP2D6* genetic variants, their associated amino acid changes, haplotypes and effects on enzyme activity in Case 2.

RefSNP (rs) Number	Haplotype	Nucleotide Change M33388.1 ATG=1 (HGVS Nomenclature)	Genotype Observed	Amino Acid Change	Worldwide Reported Minor Allele Frequency	Enzyme Activity
rs28371696	CYP2D6*43	77G>A	G/A	R26H	1%	Normal
rs774671100	CYP2D6*13	137_138insT	-/T	-	<0.1%	Decreased
rs28371706	CYP2D6*17	1023C>T (320C>T)	T/T	T107I	5.9%	Decreased
rs1058164		1661G>C (408G>C)	C/C	-	59.9%	
rs16947		2850C>T (886C>T)	T/T	R296C	35.9%	
rs1135840		4180G>C (1457G>C)	C/C	S486T	59.9%	
rs28371718	CYP2D6*1D	2575C>A (801C>A)	C/A	-	2.3%	Normal
rs77913725	CYP2D6*86	2606G>A (832G>A)	G/A	E278K	0.4%	Unknown
rs1135828		2610T>A (836T>A)	T/A	M279K	0.4%	
rs769157652	CYP2D6*27	3853G>A (1228G>A)	G/A	E410K	0.1%	Normal
Additional SNPs						
rs769258	-	31G>A	G/A	V11M	1.7%	Unknown
rs151226748	-	102A>G	A/G	-	<0.1%	Unknown
rs1080995	-	214G>C (180+34G>C)	C/C	-	30.7%	Unknown
rs1080996	-	221C>A (180+41C>A)	A/A	-	30.7%	Unknown
rs1080997	-	223C>G (180+43C>G)	G/G	-	30.7%	Unknown
rs1080998	-	227T>C (180+47T>C)	C/C	-	30.7%	Unknown
rs796532118	-	180+52_180+53delGAinsCC	G/G and G/G	-	-	Unknown
rs1081000	-	245G>A (180+65G>A)	A/A	-	30.7%	Unknown
rs28371699	-	310G>T (180+130G>T)	T/T	-	63.2%	Unknown
rs28371702	-	843T>G (181-41T>G)	G/G	-	63.0%	Unknown
rs17002852	-	2470T>C (696T>C)	T/C	-	0.7%	Unknown
rs28371721	-	2661G>A (843+44G>A)	G/A	-	1.8%	Unknown
rs59421388	-	3183G>A (1012G>A)	G/A	-	2.9%	Unknown
rs1985842	-	3384A>C (1173+40A>C)	C/C	-	-	Unknown
rs4987144	-	3790C>T (1174-9C>T)	C/T	-	-	Unknown
rs1135835	-	4131A>G (1408A>G)	A/G	A470T	0.1%	Unknown
rs1135837	-	4158G>C (1435G>C)	G/C	G479R	<0.1%	Unknown
rs1135838	-	4164T>G (1441T>G)	T/G	F481V	-	Unknown
rs1135839	-	4166T>C (1443T>C)	T/C	-	-	Unknown
rs28371736	-	4172C>T (1449C>T)	C/T	-	<0.1%	Unknown
rs61731577	-	4193T>C (1470T>C)	T/C	-	<0.1%	Unknown

***HGVS** – Human Genome Variation Society Nomenclature.

The same variation 137_138insT observed in case 1 was also observed in case 2; this renders one allele non-functional. Haplotypes with normal or unknown functionality were also observed in case 2 (Table 10). However, the presence of homozygous SNPs forming the *CYP2D6*17* haplotype, meant that both alleles had decreased activity. In this case, it was inferred that the individual possessed an IM phenotype (Figure 10), as both alleles were identified as having decreased activity.

4. Discussion

The main purpose of this research project was to design and optimise a molecular-based assay to aid the procedures surrounding molecular autopsies for specific toxicity cases. In addition to this, the assay was applied to DNA obtained from blood samples of two decedents admitted to Salt River Mortuary, in order to demonstrate a proof of concept. Apart from the assay being applied to DNA from the two cases, blood samples were also sent for toxicological screening, in order for interpretation to be attempted.

4.1 Case 1

4.1.1 Case History

A 45-year-old Coloured female, was admitted to Groote Schuur Hospital complaining about acute shortness of breath. Upon assessment, she was found to be clinically cyanotic and presented with features of airway obstruction. The deceased, while undergoing medical intervention, went into cardiac arrest. Cardiopulmonary resuscitation was performed; however, she developed asystole and was declared dead 20 minutes thereafter. The manner of death was a suspected suicide-overdose, as she had a history of over-the-counter medication abuse. It was known that she had hypertension, diabetes, major depression and anxiety. A post-mortem was performed by a Forensic Pathologist and the following findings were deduced: (i) History of diabetes and hypertension with evidence of target organ damage and (ii) Critically stenosed left anterior descending coronary artery with superadded fresh occlusive thrombus. Given the significance of the coronary artery stenosis, it was concluded that the cause of death was myocardial infarction.

Consent was obtained from the next-of-kin to collect blood for DNA analysis and toxicological screening. The following drugs and/or metabolites were detected: amitriptyline, nortriptyline, protriptyline, citalopram and paracetamol (Table 6 and Appendix 6). Several genetic variants were also observed in the individual, rendering her an IM or EM.

4.1.2 Relationship between an Identified Drug and the Predicted Phenotype

CYP2D6 is reported to play a role in metabolism of amitriptyline and its active metabolite nortriptyline, as well as protriptyline and citalopram. The metabolic pathway of amitriptyline, which is a commonly abused tricyclic antidepressant (TCA), is presented in Figure 11 (Shenouda and Desan, 2013). It has been used in the treatment of depression for many years and is still widely administered (Jornil and Linnet, 2009). Amitriptyline, known to be one of the most studied TCAs, is metabolised by several CYP450 enzymes, most importantly CYP2C19 and CYP2D6 (Jornil and Linnet, 2009; Smith and Curry, 2011) (Figure 11).

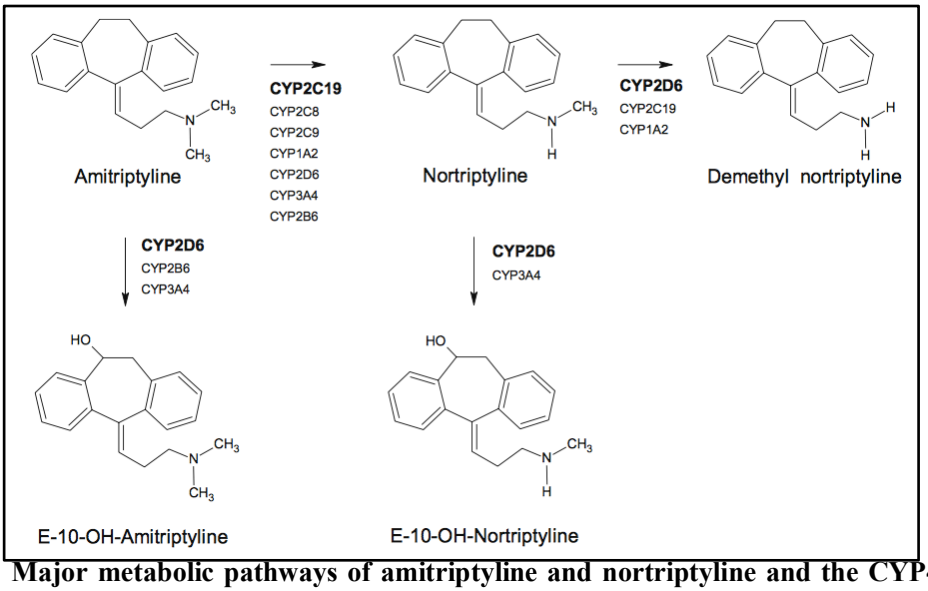

Figure 11: Major metabolic pathways of amitriptyline and nortriptyline and the CYP450 enzymes reported to mediate their metabolism. (Taken from Jornil and Linnet, 2009).

In the metabolic pathway of amitriptyline, CYP2C19 aids its metabolism to its active metabolite nortriptyline, while CYP2D6 is responsible for more than 80% of the metabolism of nortriptyline to demethyl nortriptyline and E-10-OH-nortriptyline (Jornil and Linnet, 2009) (Figure 11). Recently, there has been a major focus on genetic variation within the CYP450 system for the metabolism of TCAs (Jornil and Linnet, 2009). This is attributed to the considerable inter-individual variation of drug metabolism; and the risk of intoxication even from a standard dose. This resulting from the narrow therapeutic index associated with such central nervous system (CNS) drugs (Jornil and Linnet, 2009; Zhou, 2009). Jornil and Linnet (2009), also noted the importance of investigating the metaboliser phenotype with respect to *CYP2D6*, as an association between an altered metaboliser phenotype and an increased plasma concentration of antidepressants, such as amitriptyline, was observed. The importance of a

molecular analysis has been identified, and whether to combine therapeutic drug monitoring with a genetic analysis, is a question that is frequently debated (Jornil and Linnet, 2009).

In case 1, the decedent possessed several variants, which contributed to a number of different haplotypes, namely: *CYP2D6*13*, **1D*, **27*, **43*, and **86*. One of the most significant variants observed in the deceased was an insertion (137_138insT). The 137_138insT insertion causes a frameshift in the open reading frame, which inherently abolishes enzyme activity (Panserat *et al.,* 1995). This variant results from the hybridisation of the *CYP2D6* and *CYP2D7* genes. The hybrid gene shares a *CYP2D7*-derived exon 1, which differs from *CYP2D6* gene by a single nucleotide insertion (T-insertion), thereby differing in respect to the region in which *CYP2D7* switches to *CYP2D6* (Panserat *et al.,* 1995). This variant is associated with both the *CYP2D6*13* and *CYP2D6*15* haplotypes. However, the presence of the additional variants c.696T>C, c.801C>A, c.832G>A and c.836T>A in this case, are associated with a *CYP2D7* gene conversion in exon 5, which suggests the haplotype *CYP2D6*13E* (Gaedigk *et al.,* 2010).

The *CYP2D6*13* haplotype is of clinical significance as it often causes altered drug clearance and drug response (Zhou, 2009). This functionally inactive allele, according to gnomAD (version 2.0.2 – October 2017), occurs in four out of every 14,892 individuals (0.03%) within the African population, which is higher than that seen in any other population group (gnomAD, version 2.0.2 – October 2017). The other haplotypes present, *CYP2D6*1D* (7.3%), *CYP2D6*27* (0.1%), *CYP2D6*43* (2.3%) and *CYP2D6*86* (0%), which are associated with a normal or unknown enzyme activity are, found in African populations in varying frequencies (gnomAD, version 2.0.2 – October 2017).

The deceased was characterised as either an IM or EM phenotype after considering all the possible options/combinations of haplotypes on the two alleles (Zhou, 2009) (Figure 12). In any given situation, the presence of the *CYP2D6*13* haplotype would automatically render one allele inactive (Panserat *et al.,* 1995) (Figure 12). Therefore, for an EM phenotype to result, the alternate allele would need normal or increased functionality (Figure 12). Equally, if the alternate allele presented with a decreased functionality an IM phenotype would result. However, our prediction of the phenotype in this case was limited, as without segregation analysis being performed, or unknown significance of identified SNPs, these are considered the two 'conceivable' phenotypic situations.

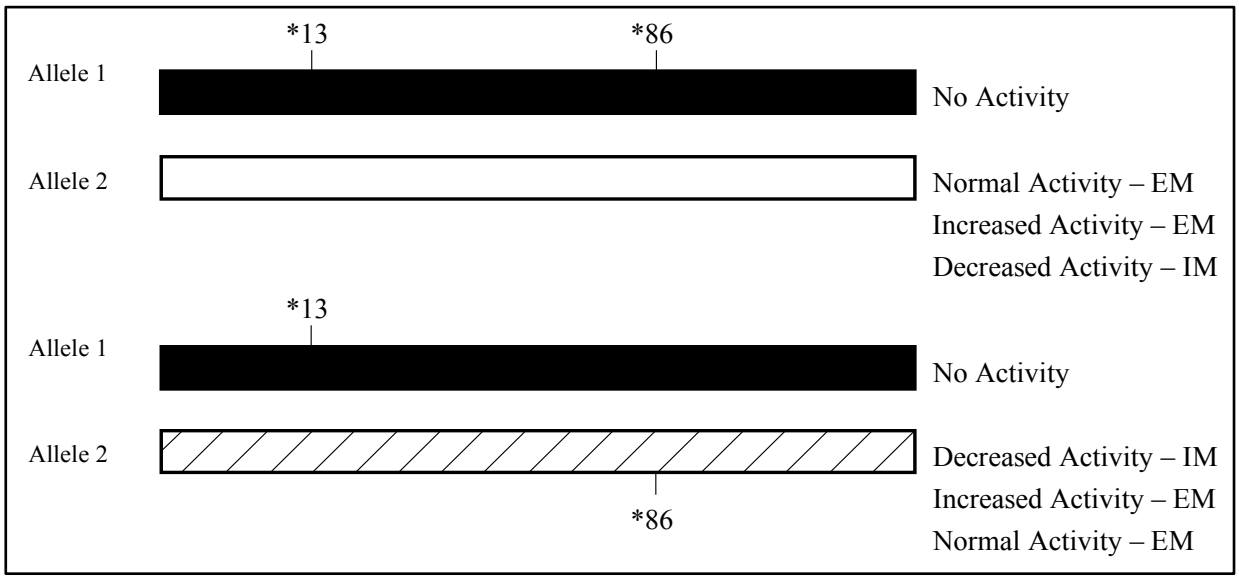

Figure 12: Illustration of the possible phenotypes based on combinations of the different decreased or unknown activity haplotypes observed in case 1.

Typically, these predicted phenotypes suggest that a drug found within the blood of the deceased, such as amitriptyline and its active metabolite nortriptyline would be metabolised successfully, or perhaps, at a slightly decreased rate. However, in this case, this would simply be an assumption, without knowledge of all the contributing factors, including the dose, the unknown significance of genetic variants observed and the functionality of CYP2C19 metabolism.

If it were to be found that there were toxic levels of amitriptyline and/or nortriptyline in the blood of the deceased and that the deceased had reportedly taken a therapeutic dose of the drug, then the presence of both may be associated with a deficient *CYP2D6* activity, which is often observed in an IM phenotype (Smith and Curry, 2011). This may have contributed to a prolonged elevation in antidepressant levels in the deceased, as a result of the inability to clear the drug efficiently (Smith and Curry, 2011). For this to be said with more confidence the metabolic function of CYP2C19 would have to be investigated. A similar mechanism is described in a case report investigated by Smith and Curry (2011), whereby an individual who was suspected to have ingested 99 tablets of 25 mg amitriptyline, had experienced a delayed rise in serum TCA levels and prolonged intoxication as a result of *CYP2D6* deficiency (Smith and Curry, 2011).

Dalén *et al.* (1998) performed a study in which a dose of nortriptyline was administered to several individuals with varying numbers of active *CYP2D6* alleles. As expected, the individuals with inactive alleles possessed the highest concentration of nortriptyline; suggesting that a deficient enzyme activity contributes to high levels of nortriptyline (Dalén *et*

al., 1998). While it is debatable whether it is worthwhile performing genetic analyses on polymorphic CYP450s in suspected amitriptyline overdoses, forensic toxicogenetics may prove constructive in cases where case and drug use history speaks against suicidal intent or where medical-related deaths may have arisen from adverse drug reactions (Koski *et al.,* 2006).

These findings warrant a quantitative toxicological investigation into amitriptyline to distinguish whether there may have been any contribution to death. Given that amitriptyline toxicity has been purported to be associated with myocardial infarction as a result of myocardial cell damage (Kiyan *et al.,* 2006), the genetic results here may assist in distinguishing contributors to death. In this regard, an individual with a decreased CYP2D6 activity, which results in amitriptyline toxicity, may experience abnormal cardiac function (Kiyan *et al.,* 2006). However, the current study was not able to determine whether the case in question was that of amitriptyline toxicity, therefore, such an association cannot be made here. Although, further investigation into cases with similar circumstances should be pursued.

4.2 Case 2

4.2.1 Case History

A 25-year-old Coloured male was found by his mother, unresponsive with creamy white vomitus on his anterior chest and neck area. According to his mother, he had been complaining of headaches for months. The deceased was a known schizophrenic on prescribed medication. He received a set monthly injection, and depending on his wellbeing received additional prescribed medication. His mother claimed that he may have gotten hold of hidden medication. A post-mortem was performed and the following findings were deduced: (i) Low-dose full-body X-ray (LODOX) showed an enlarged heart with widened mediastinum, straightened left border of heart and bilateral lung infiltrates especially on the right; (ii) Vomitus on anterior chest and neck area; (iii) Brain was swollen and oedematous and (iv) Arteries of the heart showed arteriosclerotic occlusion. As a result, it was concluded by the forensic pathologist that the cause of death was associated with acute cardiac disease.

Considering the possibility of contributory intoxication given the history, following next-of-kin consent, blood was taken for DNA analysis and toxicological screening. The following drugs were detected: fluoxetine, ephedrine, doxylamine and diphenhydramine (Table 6 and Appendix 6). Following a genetic analysis, genetics variants observed in the deceased suggest that he was an IM.

4.2.2 Relationship between an Identified Drug and the Predicted Phenotype

CYP2D6 is reported to play a role in the metabolism of the detected drugs fluoxetine and diphenhydramine. Fluoxetine is one of the most widely used selective serotonin reuptake inhibitor (SSRI) antidepressant prescribed drugs, for several different psychopathological disorders (Llerena *et al.,* 2004). Fluoxetine has been the focus of many CYP450 enzyme-related studies because of the role *CYP2D6* plays in the metabolism of the compound, and because of its known potent inhibitory properties of the enzyme (Llerena *et al.,* 2004) (Figure 13). This emphasises the difficulty in assessing the contribution of a drug towards the death of an individual (Llerena *et al.,* 2004).

Plasma drug concentrations of fluoxetine and its metabolites norfluoxetine have shown to be affected by the genetic variations in *CYP2D6* and therefore the efficacy of the drug is ultimately effected (Charlier *et al.,* 2003). A study performed by Charlier *et al.* (2003), suggested that genotyping of *CYP2D6* in addition to therapeutic drug monitoring to explain side effects is the way forward. However, in a forensic context, the investigation should include comprehensive toxicological analyses and ideally a genetic analysis to determine metabolic phenotype in addition to quantitative data.

The metabolic pathway of fluoxetine is presented in Figure 13.

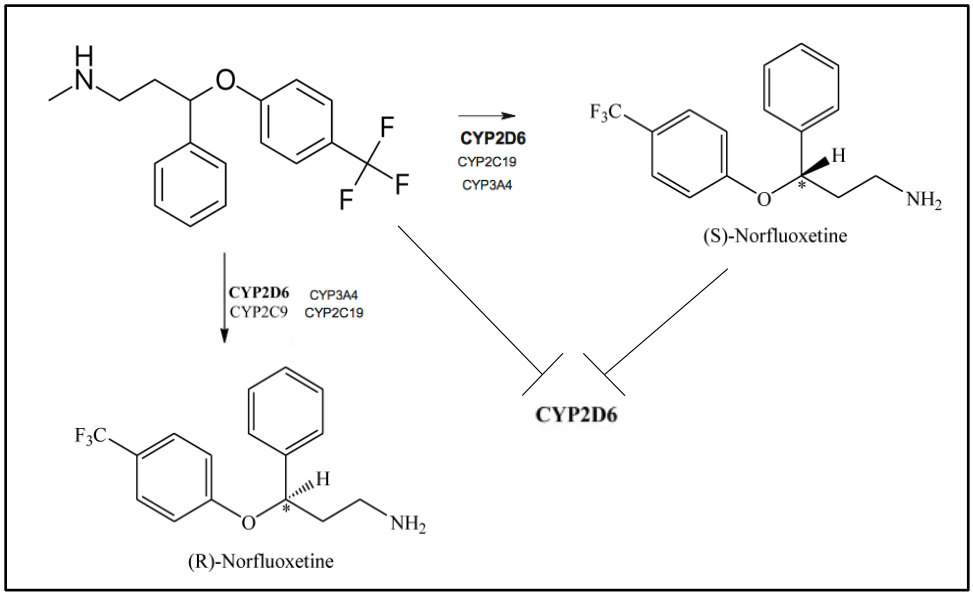

Figure 13: Major metabolic pathways of fluoxetine and CYP450 enzymes reported to mediate the metabolism.

From the variants identified after applying the fully optimised assay to case 2, the deceased was found to have possessed the following haplotypes: *CYP2D6*13, *17, *1D, *27, *43* and *86. Although, the haplotype *13 plays a significant role in the prediction of the phenotype in this case, it is the contribution of another haplotype *17 that reinforces the prediction. The presence of four variants namely; c.320C>T, c.408G>C, c.886C>T and c.1457G>C contribute to the formation of the *CYP2D6*17* haplotype (Masimirembwa *et al.,* 1996).

*CYP2D6*17* has been thoroughly researched and is known to give rise to substrate-dependent decreased activity (Ingelman-Sundberg, 2005; Zhou, 2009). While the *17 haplotype is virtually absent in Caucasians it occurs at a relatively high frequency in African populations (Zhou, 2009). Table 1 in the literature review shows the frequency of *17 in several different African populations, with the highest being that of the Zimbabwean population (34%) (Dandara *et al.,* 2001; Bertilsson *et al.,* 2001). The *CYP2D6*17* haplotype is one of two alleles specific to African populations associated with an IM phenotype and remains one of the most important functional alleles in the metabolism of *CYP2D6* substrate drugs (Alessandrini *et al.,* 2001; Matimba *et al.,* 2008).

While *CYP2D6**13 renders one allele inactive in this case, the homozygous *CYP2D6*17* was found on both alleles and is also associated with an altered functionality. With both alleles exhibiting decreased function, this individual was classified as an IM. This was the most likely interpretation, but it must be noted that SNPs were found, with unknown significance, and therefore, this interpretation may be different when we learn more about the other SNPs.

In this case, the predicted phenotype was identified as an IM; however, with regards to the presence of fluoxetine, its inhibitory properties on CYP2D6 activity has the ability to make an IM phenotype resemble a PM phenotypes, a phenomenon known as phenocopying (Hemeryck and Belpaire, 2002; Torkamani, 2016). The inhibitory properties of fluoxetine are, according to Kirchheiner *et al.* (2004), a result of multiple doses, which contribute to the decrease in *CYP2D6*-mediated metabolism (Kirchheiner *et al.,* 2004). The ability of fluoxetine and other SSRIs to inhibit their own metabolism, producing increased plasma concentration levels of the drug, constantly highlights the difficulty of assessing the effect of altered *CYP2D6* metabolism on itself. However, the presence of alleles such as *17, found in African populations, that lead to diminished function are very frequent and may, particularly when present in a homozygous form, affect metabolic activity regardless (Steimer *et al.,* 2005).

If a quantitative toxicology analysis was performed on the blood samples retrieved in this case and an inconsistent level of fluoxetine was revealed, the reduced enzyme activity, as a result of *13/*17, and the constant inhibition of the CYP2D6 enzyme could potentially be the contributing factors (Llerena *et al.,* 2004). Since the discovery of the inhibitory properties of SSRIs, it has been noted that when administering other substances, metabolised by CYP2D6, in combination, as seen in this case, caution must be taken (Torkamani, 2016). For example, administering an SSRI such as fluoxetine together with a TCA, would create an antagonistic environment and would make an interpretation complex (Llerena *et al.,* 2004). A significant limitation would be not being able to determine the effect of drug-drug interactions and the interaction with the metabolising enzyme.

4.2.3 Interpretive Challengers

Apart from the drugs reported in the two cases, the polymorphic *CYP2D6* gene is also known for its ability to metabolise drugs from certain illicit drug groups, namely: amphetamine-type stimulants, opioid analgesics and sedative hypnotics (Kupiec *et al.,* 2006; Crews *et al.,* 2012; Koski *et al.,* 2007; Neukamm *et al.,* 2013; Jannetto *et al.,* 2002; Druid *et al.,*1999). *CYP2D6*-mediated metabolism of these drugs is known to be a major source of variation, both in drug effect and pharmacokinetics (Jamei *et al.,* 2009). This makes the interpretation of the results challenging when considering most of the reported substances in case 1 and 2 were not metabolised by CYP2D6, alone. This warrants the development and optimisation of multiple CYP450 assays. As even if an individual were to produce an inactive variant for an enzyme in the major metabolic pathway, the drug may be broken down through an alternate route, assuming all necessary enzymes, are not genetically altered (Wu, 2011). Conversely, individuals may simultaneously possess several reduced function alleles for multiple *CYP450* gene, therefore, exhibiting significant variances in toxicological effects of the drug (Wu, 2011).

Drug-drug interactions also contribute to interpretive challenges according to Hyland *et al.* (2001), who proposed that clinicians should always be aware of the possible drug-drug interactions when prescribing drugs metabolised by CYP450 enzymes, in conjunction with known inhibitors of the enzymes (Hyland *et al.,* 2001). In both cases, several drugs that are metabolised by more than one CYP450 enzyme and are inhibitors of CYP450 enzymes, were reported. For instance, in case 1 Citalopram was reported in the drug screen and is known to be metabolised mostly by CYP2C19, but also by CYP3A4 and CYP2D6 as well (Herrlin *et al.,* 2003). Knowledge of the function each enzyme is responsible for is considered a prerequisite

for predicting variable pharmacokinetics and drug response (Zanger and Schwab, 2013). This allows for further interpretation of possible drug-drug interactions.

4.3 Strengths and Limitations

4.3.1 Post-mortem Toxicogenetics

Even with the substantial improvement in our understanding of the significance, together with vast advances in the application of forensic toxicology, interpretation of post-mortem forensic toxicological results remains a challenge in its own regard (Drummer, 2004; Musshoff *et al.,* 2010). Given the difficulties of interpretation, a full picture of the circumstances surrounding the case needs to be considered (Drummer, 2004).

In a traditional drug testing scheme, two different tiers of testing are employed; a qualitative and quantitative test (Milone, 2012). The first tier comprises of a qualitative presumptive test often in the form of a drug screen, which is used to detect and identify drugs in the blood (Milone, 2012). A quantitative confirmatory test, therefore, forms part of the second tier, which is performed in order to quantify the drugs reported in the initial screen.

In both case 1 and 2, no quantitative analysis was performed. No quantitative analyses were performed as there are currently no relevant standards, in a forensic toxicology context, available in South Africa for the drugs detected. The lack of quantitative toxicological results and case history rendered the interpretation of the drugs reported difficult. The high case load experienced at SRM, often results in only selected cases being sent for toxicological screening, with quantitative drug analyses not being routinely performed.

Essentially the suggested metabolic effects of the phenotypes on the presence of the reported drugs, were merely hypothetical. In addition, determining the exact mechanism of metabolism, becomes an even more difficult task when there is either; (i) a combination of CYP450 enzymes that contribute to the metabolism of drugs present in the blood; (ii) several drugs reported that are metabolised by the same CYP450 enzymes or (iii) the presence of drugs that are known to be metabolised as well as inhibited by CYP450 enzymes.

The value of a quantitative analysis is imperative, when keeping in mind that *CYP2D6* is also prone to inhibition by numerous drugs, as seen in case 2 where fluoxetine was reported (Zanger and Schwab, 2013). The phenomenon known as phenocopying, as previously mentioned, is when an inhibitor is strong enough to change the apparent phenotype of the individual (Zanger

and Schwab, 2013). The inhibition of drug metabolism often results in undesirable elevated plasma drug concentrations of other drugs metabolised by the same enzyme (Hemeryck and Belpaire, 2002). This could be observed in such a case as case 2, whereby the inhibitory effects of fluoxetine, could lead to a decreased metabolism of diphenhydramine, causing an increase in the plasma drug concentration. The importance of the identification of the major CYP450 enzymes involved in the metabolism of a potential drug is paramount in contributing to future predictions for potential drug-drug interactions, which could ultimately help resolve ambiguous cases (Bohets *et al.,* 2000; Hemeryck and Belpaire, 2002).

A co-segregation analysis is used to test the mode of inheritance of a particular genotype (Elston, 1981) and would provide insight into which alleles the different variants were situated on. (Jarvik, 1998). A second limitation was that parents of the deceased were unavailable for the study, which prevented the performing of co-segregation analysis. Without being able to perform a co-segregation analysis, there was an inability to determine the true metabolic phenotype in case 1, based on the alleles that were identified. Not performing co-segregation analysis does not always limit the prediction of a phenotype, as seen in case 2; whereby the homozygosity of variants meant that both alleles had decreased enzyme activity.

Apart from the identified limitations, this assay still needs to be thoroughly researched and validated, before it can be utilised in service.

4.3.2 A Population with Limited Population Frequency Data

During this study, it transpired that there was a paucity of related studies in an African and South African context. This was highlighted during the optimisation phase of this study, whereby a primer set had to be amended as a result of the lack of frequency data on a variant, specifically the c.180+52_180+53delGAinsCC variant. This can be attributed to most pharmacogenetic studies to date have been conducted on a limited number of population groups, namely: Caucasian, Oriental and African American populations (Ikediobi *et al.,* 2011).

A study by Koboldt *et al.* (2006), illustrated how a primer-site variant, such as a SNP, could conceivably have a very rare minor allele frequency and have no effect on the hybridisation of primers, therefore, essentially having no effect on the ability of the primer to contribute to amplification of the target region (Koboldt *et al.,* 2006). However, the variants observed in the primer binding region in this study clearly effected the hybridisation of the primer (5'-CCCTACCAGAAGCAAACA-3') designed by Matimba *et al.* (2009), used in this study, to

the target region.

The pharmacogenetic data categorised in these populations are often extrapolated for the use of inferring information in other populations, despite this introducing numerous problems (Ikediobi *et al.,* 2011). One such problem is the presence of variants, found to have a high frequency in a Caucasian population, are actually very rare in other populations and vice versa (Ikediobi *et al.,* 2011). One such case is that of the *CYP2D6*17* haplotype, which is common in African populations but rarely found in others (Dandara *et al.,* 2001). It is important to appreciate that there are large inter-ethnic differences for *CYP2D6* polymorphisms and their resulting metabolic phenotypes (Allorge and Tournel, 2011; Dandara *et al.,* 2001). African populations are known to be among the most genetically diverse in the world, which highlights the importance that assumptions should not be made for such African populations based on phenotypic predictions from other similar studies and that there is a void of information for these unique African populations (Gaedigk and Coetsee, 2008; Ikediobi *et al.,* 2011).

In South Africa, where *CYP2D6* allele frequency data are sparse and only, recently emerging for geographically defined or indigenous populations, admixed populations, in particular the mixed ancestry 'Coloured' population, should be targeted in order to fill that void (Gaedigk and Coetsee, 2008). Unless an extensive genotyping study is performed, many less frequent, rare SNPs, will go unnoticed, avoiding detection and in turn frequencies of default alleles will be overestimated (Gaedigk *et al.,*2017). Teh and Bertilsson (2012), illustrate how there are marked differences in *CYP2D6* genotypes and phenotypes in populations of different racial origins, whereby certain alleles are found in high frequency in Oriental populations that are either significantly lower or absent in African or Caucasian populations (Teh and Bertilsson, 2012).

4.4 Conclusion

By drawing on similar studies within the literature, a fully optimised molecular-based assay, which was able to amplify and sequence all the exons within the *CYP2D6* gene, was designed. The assay has been optimised from a technical perspective but testing it in a larger cohort would be needed to verify its success rate for sequencing in a larger cohort. A challenge that arose during optimisation, included amending the original nine primer sets to eight, however, this proved to be a minor issue and was successfully dealt with.

To demonstrate a proof of concept, the assay was successfully applied to DNA from two

decedents admitted to Salt River Mortuary and the results were also analysed and interpreted, in context of limited toxicological data. The analysis of the two cases rendered the decedents as (i) either an IM or EM, and (ii) IM respectively. The two chosen cases were initially suspected to have had drugs contribute to death, thus a toxicological screening was performed. Taking into consideration that no quantitative analysis was performed, only a hypothetical reasoning behind the drugs reported in the screening, could be given. Even without a quantitative analysis, knowledge regarding polymorphic CYP450*s* involved in drug metabolism will help anticipate certain observations or guide further investigations.

The genetic variants that contributed to the predicted phenotypes, combined with the presence of the drugs identified in each case, suggests altered drug metabolism could have occurred, which should be investigated further and interpreted within each case context. The findings in this study would be beneficial, not only into resolving ambiguous cases, but also to the decedents' living relatives, who may also carry these variants. Testing for *CYP2D6* variants in the living relatives would identify those at risk for such adverse drug reactions. Overall, this study demonstrates the value of molecular analyses in forensic investigations of toxicological-related fatalities, and lays the foundation for additional future research, particularly since the molecular assay has now been successfully optimised.

The knowledge of South Africa having distinctly diverse populations has made it clear how personalised medicine in a country like South Africa faces many challenges. This largely influences how medical professionals, especially pathologists, are able to analyse and interpret results in toxicological-related deaths. Without a molecular component as a part of routine forensic autopsies, many cases may remain unanswered even after a thorough investigation. It is evident how a genetic analysis attests to its value and could prove instrumental in contributing to a large number of 'would be unresolved' cases actually being successfully resolved. Although, results from a genetic analysis may not always be noteworthy they may always serve as a helpful complement to already identified alterations.

In the little research that has been performed on unique admixed populations in South Africa, the data presented has demonstrated the importance of characterising such populations, in respect to the complement of *CYP2D6* alleles present and their frequencies. It is evident that without an extensive genotyping study being performed, many novel variations, that could ultimately contribute to certain haplotypes, will go unnoticed. Therefore, variations avoiding detection, will result in frequencies of default alleles being overestimated. Without this

knowledge and ultimately the knowledge of the frequencies of the metabolic phenotypes in a local context, one would realise how much still goes undiscovered in routine forensic investigations. It is paramount that forensic pathologists and toxicologists are aware of the biological influences and their possible side effects as this would possibly contribute to the determining a cause and/or manner of death.

Toxicogenetics has been advocated as an important compliment to the investigation of death and if appropriately implemented and utilised in conjunction with routine post-mortem practices can help reduce the number of cases that remain unanswered after thorough investigations.

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Appendix 1

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
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Observatory 7925
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Website: www.health.uct.ac.za/fhs/research/humanethics/forms

28 April 2017

HREC REF: 110/2017

Ms L Heathfield
Pathology
Reception, Forensic & Toxicology
Falmouth Building

Dear Ms Heathfield

PROJECT TITLE: POST-MORTEM MOLECULAR AND TOXICOLOGICAL INVESTIGATIONS: EXPLORING TOXICITY AND GENETIC VARIATION IN DECEASED INDIVIDUALS AT SALT RIVER MORTUARY (MPhil-candidate-D Vincent & L Vuko)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th April 2018.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the following students Mr D Vincent & L Vuko will be involved in this study.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.

HREC 110/2017

Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI),and Declaration of Helsinki (2013) guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

HREC 110/2017

Appendix 2
INFORMATION FORM AND INFORMED CONSENT FORM

INFORMATION FORM

Study title: Exploring Genetic Variation in Drug Metabolising Enzymes in Drug Intoxication Cases at Salt River Mortuary

Researcher: Devin Vincent (VNCDEV001)

Supervisor: Laura Heathfield

Co-Supervisor: Bronwen Davies

Introduction to the study

You are invited to participate in a research study that will take place within the Division of Forensic Medicine and Toxicology at the University of Cape Town. It will be conducted by Devin Vincent who is a researcher in the Division of Forensic Medicine and Toxicology at the University of Cape Town, working towards his Master's degree in Biomedical Forensic Science.

The purpose of the forensic toxicogenetic research study is to explore the genetic variation in drug metabolising enzymes in drug intoxication fatalities by examining the presence of predispositions in *CYP450* gene expression and function. This form explains what is expected of you if you choose to participate in this study. It is important that all information is read carefully and please feel free to ask any questions before you make a decision about participating.

Background

The South African pathology services are often faced with cases in which scene, autopsy and toxicological evidence are all consistent with an overdose and fatal toxicity, however, sometimes the toxicological data in some cases is ambiguous and conclusions concerning the cause and manner of death are limited in scientific validity. In such instances, death may have been caused by issues in drug metabolism – the process by which human bodies process drugs introduced into the body. A test that could be performed to investigate the occurrence of such an event is one that examines the sequence of the specific *CYP450* genes that are responsible for the metabolism of the implicated drug to determine if the decedent is not genetically

predisposed to experience toxicity from the drug even when taken at a therapeutic level. Currently there are no validated molecular-based assays designed for identifying ineffective or genetically altered *CYP450* genes. This makes it difficult to understand the influence genetic polymorphisms have on *CYP450* expression and functionality.

However, to be able to design a validated molecular-based assay that could be incorporated into the routine tests done by forensic pathologists, biological samples such as blood needs to be collected from individuals and the DNA extracted and analyzed in order to help with optimization. The optimized assay will help forensic pathologists in their postmortem examinations, which will in turn facilitate and increase the accuracy by which they determine the manner and cause of death.

Procedure

You will be asked to give permission to allow for blood to be collected by a registered doctor/nurse using an invasive blood collection technique:

- 4 – 5 ml of blood will be collected in a purple top tube, which is done using an invasive method which incorporates the use of sterilised needles and blood collection vials.

Once samples are collected, there will be laboratory tests on the samples to extract the DNA, study the DNA more closely and use the DNA to design molecular-based assays. Please note that technology in the laboratories are always getting better so in time there might be newer laboratory techniques invented that may become accessible to the researcher. You will also be asked to allow the biological samples collected to be used in these newer technologically advanced methods if and when they become available.

The biological material collected and the extracted DNA will be stored for a period of 20 years at the Division of Forensic Medicine (authorised institution) at the University of Cape Town. After this time the samples will be appropriately discarded.

Each participant's name will not be made known. Instead the samples, extracted DNA and generated data will be coded which means that any identifiers will be replaced with numbers or symbols. The confidentiality of the samples and data will be maintained in the following ways:

1. Every person involved in the project will be required to fill in a confidentiality agreement.
2. Only the primary researcher (Devin Vincent) will have access to the database.
3. All samples, genetic data and results generated will be stored in locked filing cabinets and password protected files in the Division of Forensic Medicine at the University of Cape Town.
4. Sharing of experimental data will be done on the basis of alphabetical and numerical codes.
5. The results of the study may be published or presented at meetings but the identity of the participant will not be revealed.
6. Your participation will be kept confidential.

The biological samples will not be used for any research unless the research study is reviewed and approved by the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee. This committee is responsible for protecting the rights and welfare of individuals who volunteer for participation in research studies.

Several things that you need to know before providing biological samples:

1. When research is carried out it is not the policy of the University of Cape Town to provide genetic information uncovered about the participant.

2. Participation in this study is completely voluntary. You are free to not participate or to withdraw at any time, for whatever reason, without negative consequences. In such cases no samples will be collected and your choice will not affect the way you will be treated at the Division of Forensic Medicine and Toxicology.

3. If you participate in the study, and you wish to not participate any longer and you do not want the biological sample to be used in this study. Please let us know and we will destroy the samples, immediately. If the sample has already been analysed at the time you change your mind, your results and other data may have already been shared with other researchers. In that case, we will not be able to destroy this data. The data will,

however, be removed from the secured database. That means that no additional/future researchers can get the data.

4. You will not receive feedback of possible genetic variations that are found in the DNA. This study is unlikely to benefit you directly, but it is hoped that it will contribute to knowledge about the presence of predispositions in *CYP450* gene expression and function with regards to drug intoxication fatalities in the future.

5. There will be no cost to you and there will be no compensation for your participation.

Making your choice

Please read each sentence below and think about your choice. After reading each sentence; please tick the Yes or No box. No matter what you decide, it will not negatively affect you in any way.

If you may have any questions please don't hesitate to ask the person taking the consent. If you may have any questions with regards to the rights and welfare of a research subject in the study, please contact the Chairperson of the University Of Cape Town Faculty Of Health Science Human Research Ethics Committee, **Professor Marc Blockman** on (021) 406 6496. If you require any further information about this study please contact Devin Vincent at 082 496 7301 or email at VNCDEV001@myuct.ac.za

If the volunteer agrees then the consent form needs to be read and informed consent will be taken.

- Thank you for your time -

Please find attached the consent form to be signed if you wish to proceed.

Study Case no._____

CONSENT FORM

I, _____ (full name), hereby acknowledge my participation in the research study following the completion of this form.

1. I confirm that I have:	**Yes**	**No**
a) Read and understood contents of this form and agree to be a part of the research study.		
b) Been informed about this study's purpose, procedures, possible benefits, and risks.		

2. I give consent and agree that:	**Yes**	**No**
a) Biological samples in the form of blood can be taken and subjected to laboratory tests at the Division of Forensic Medicine and Toxicology in which DNA will be extracted and analysed to better understand genetic predispositions in *CYP450* gene expression and function.		
b) Agree that the DNA extracted from the sample may be stored in the Division of Forensic Medicine at the University of Cape Town for a period of 20 years after which it will be appropriately discarded.		
c) The stored genetic material may only be used further research studies which have been reviewed and approved by the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee.		

3. I further understand that:	**Yes**	**No**
a) The treatment and management of the biological samples will be in accordance with guidelines of the University Of Cape Town Faculty Of Health Science Human Research Ethics Committee.		
b) The genetic laboratory is **under obligation** to respect medical confidentiality.		
c) This study is unlikely to benefit me directly.		

d) I can at any time withdraw my consent and that I have to notify the primary researcher of my decision to withdraw.		
e) I may be contacted in the future by someone from the University of Cape Town who may ask me to take part in research that may or may not be directly linked to the proposed study.		

I, have explained to _____(full name); the purpose, procedures, possible benefits and discomfort of this research study; and how the samples will be collected and stored in the Division of Forensic Medicine and Toxicology at the University of Cape Town for use in the study and in possible further research by other individuals within the Forensic Medicine at the University of Cape Town.

_____

Full name of person obtaining consent

_____

Signature of person obtaining consent Date

_____

Full name of person authorising consent for collection of samples for use in research study

_____

Signature of person authorising consent for collection of samples for use in research study Date

_____

Full name of witness

_____

Signature of witness Date

PARTICIPANT QUESTIONNAIRE

Participant reference number: _____

1. Sex ☐ Male ☐ Female

2. Age ☐ 18 – 29 ☐ 30 – 50 ☐ 50+

3. Self-reported population group according to South African Census categories

☐African Black ☐Coloured ☐Indian/Asian ☐White

4. Ancestral origin (if known)

☐ European

☐Africa

☐Asian

☐ Middle Eastern

☐ Other

Specify: _____

7. Your self-reported ethnicity (With reference to cultural self-identification)

_____

Appendix 3
INFORMATION FORM AND INFORMED CONSENT FORM

INFORMATION FORM

Study title: Postmortem Toxicogenetics: determining the suitability of blood samples collected for routine toxicological analyses for use in subsequent genetic analyses.

Principal investigator: Loyiso Vuko

Supervisor: Ms. Bronwen Davies

Co-supervisors: Ms. Laura Heathfield

Ms. Katrina Auckloo

Introduction to the study

You are invited to participate in a research study under University of Cape Town's Division of Forensic Medicine and Toxicology, in the Department of Pathology.

It will be conducted by Loyiso Vuko, who is a researcher in the division, and a candidate for an M.Phil degree in Biomedical Forensic Science.

The purpose of this study is to determine whether blood – a biological sample that is routinely collected at autopsy for toxicological analyses (tests that investigate the presence and amount of drugs/chemicals in the body of the deceased) – can be used for a genetic analysis following toxicological analysis. This form explains what you will be asked to do if you decide to participate in this study. Please read it carefully and feel free to ask any questions you may have before you make a decision about participating.

Background

Determining the cause and manner of death at autopsy is one of the core functions of forensic pathology services. This is performed by conducting an anatomical autopsy and incorporating all the evidence that is at the disposal of the responsible Forensic Pathologist. This exercise is not always straightforward in cases of drug-related deaths and those in which the decedent died in a sudden and unexpected manner. This is because while there are cases in which scene, autopsy and toxicological evidence are all consistent with an overdose and fatal toxicity, South African forensic services are often faced with cases in which the toxicological data is ambiguous and conclusions concerning the cause and manner of death are limited in scientific validity. In such instances, death may have been caused by issues in drug metabolism – the process by which human bodies process drugs introduced into the body.

The current postmortem procedure in South Africa does not involve a test by which such phenomenon could be investigated. One test that could be performed to investigate the

occurrence of such an event is one that examines the sequence of the specific genes that are responsible for the metabolism of the implicated drug to determine if the decedent is not genetically predisposed to experience toxicity from the drug even when taken at a therapeutic level.

However, in order for such a test to be incorporated into the routine work of forensic pathology, it should be determined whether the blood that will be used – collected at autopsy by the Forensic Pathologist for toxicological analysis – will have Deoxyribonucleic acid (DNA – genetic material) of sufficient quality to perform the test after it (the blood sample) has undergone the process of toxicological analyses. This is what this study aims to investigate. Information gathered from this study will help the practitioners in postmortem investigation to make an informed decision on whether to use blood samples that are routinely used for toxicological investigations or to collect separate blood samples for the aforementioned genetic analysis, which will in turn facilitate and increase the accuracy by which Forensic Pathologists pronounce on the cause and manner of death.

Procedure

You will be asked to give permission to allow for blood to be collected at autopsy by the Forensic Pathologist assigned the case for your deceased family member. The samples will be collected into three different types of collection tubes, two of which are routinely used to collect blood at autopsy for toxicological analysis, while the other one is custom-made to preserve DNA for the purpose of making comparisons with the former.

Some of these samples will undergo toxicological analysis as is routinely performed in postmortem investigations, following which the DNA will be extracted in preparation for the determination of the quality of the DNA in those blood samples by means of laboratory tests that include spectrophotometry, quantitative Polymerase Chain Reaction and DNA Profiling. These samples will be compared to those that had undergone toxicological analyses. Please note that technologies in the laboratories are constantly being improved, and that it is possible that by the time the samples get processed in this study the laboratory may have upgraded to newer and more advanced technologies. You will also be asked to allow for the samples to be processed using these advanced technologies in the event of such an upgrade occurring.

The biological material collected from your deceased family member and the extracted DNA will be stored for a period of 20 years at the Division of Forensic Medicine and Toxicology (authorised institution) at the University of Cape Town. After this time the samples will be appropriately discarded.

The name and every other personal detail of your family member will not be made known. Each case will be given a unique identification code to maintain the anonymity of the deceased. These identification codes will be used to trace the collected samples throughout the course of the research. The confidentiality of the samples and data will be maintained in the following ways:

7. Every person involved in the project will be required to fill in a confidentiality agreement.
8. Only the primary investigator will have access to the database.
9. All samples, genetic data and results generated will be stored in locked filing cabinets and password protected program in the Division of Forensic Medicine and Toxicology at the University of Cape Town.
10. Sharing of experimental data will be done on the basis of the unique identification codes.
11. The results of the study may be published or presented at meetings but the identity of the deceased and the family will not be revealed.
12. Your participation will be kept confidential.

The biological samples will not be used for any research unless the research study is reviewed and approved by the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee. This committee is responsible for protecting the rights and welfare of individuals who volunteer for participation in research studies.

There are several things you need to know before allowing biological samples to be taken from your deceased family member:

6. When research is carried out, it is not the policy of the University of Cape Town to provide genetic information about the deceased to the family members.

7. Participation in this study is voluntary. You are free not to participate or to withdraw at any time, for whatever reason, without negative consequences. In such cases, no samples will be collected and your choice will not affect the way you will be treated at the Salt River Mortuary.

8. If you participate in the study, you can change your mind later and decide that you don't want to participate anymore and you do not want the tissue to be used in this study. Please let us know and we will destroy the samples. If the sample has already been analysed at the time you change your mind, your results and other data may have already been shared with other investigators. In that case, we will not be able to destroy this data. The data will be removed from the secured database. That means that no additional researchers can get the data.

9. You will not receive feedback of possible genetic variations that are found in the DNA of the deceased. This study is unlikely to benefit you or your family directly, but it is hoped that it will contribute to knowledge about the influence of genes in drug-toxicity in the future.

10. There will be no cost to you and there will no compensation for your participation.

Making your choice

Please read each sentence below and think about your choice. After reading each sentence, please tick the Yes or No box. No matter what you decide, it will not negatively affect you or your deceased family member in any way.

If you may have any questions or require referral to a grief centre or psychological support, please don't hesitate to ask the person taking the consent. If you may have any questions with regards to the rights and welfare of a research subject in the study, please contact the Chairperson of the University Of Cape Town Faculty Of Health Science Human Research Ethics Committee, **Professor Marc Blockman** on (021) 406 6496. If you require any further information about this study, please contact Loyiso Vuko at 083 538 4283 or email at VKXLOY001@myuct.ac.za

If the spouse/partner/major child/parent/guardian/major sibling agrees then the consent form needs to be read and informed consent will be taken. **Please note that the information and consent forms will be translated into the family member's language of choice.**

- Thank you for your time -

Please find attached the consent form to be signed if you wish to proceed

CONSENT FORM

I, _____ (full name), the spouse/partner/major child/parent/guardian/major brother/major sister (circle relationship) of the deceased; fill in the Western Cape death register number: WC11

I confirm that I have:	**Yes**	**No**
c) Read and understood contents of this form and agree to be a part of the research study.		
d) Been informed about this study's purpose, procedures, possible benefits, and risks.		

I give consent and agree that:	**Yes**	**No**
d) Blood sample(s) can be taken from my deceased family member.		
e) The blood sample(s) can be subjected to laboratory tests at the Division of Forensic Medicine and Toxicology in which DNA will be extracted and its quality assessed after toxicological analysis.		
f) Agree that the samples and extracted DNA may be stored in the Division of Forensic Medicine and Toxicology for a period of 20 years after which it will be appropriately discarded.		
g) The stored genetic material may only be used for further research studies which have been reviewed and approved by the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee.		
h) I may be contacted in the future by someone from the University of Cape Town who may ask me to take part in research that may develop from the results of the study.		

I further understand that:	**Yes**	**No**
f) The treatment and management of the biological samples of my deceased family member will be in accordance with the guidelines of the University Of Cape Town Faculty Of Health Science Human Research Ethics Committee.		
g) The forensic laboratory is **under obligation** to respect medical confidentiality.		
h) This study is unlikely to benefit me or my family directly.		
i) I can, at any time, withdraw my consent and that I have to notify the primary investigator of my decision to withdraw.		
j) Research conducted with this DNA may result in publication, but neither the deceased nor the family of the deceased will be identified.		

I, have explained to _____(full name) who is the spouse/partner/major child/parent/guardian/major brother/major sister of the deceased; the purpose, procedures, possible benefits and discomfort of this research study; and how the samples will be collected and stored in the Division of Forensic Medicine and Toxicology at the University of Cape Town for use in the study and in possible further research by other individuals within the Division of Forensic Medicine and Toxicology at the University of Cape Town.

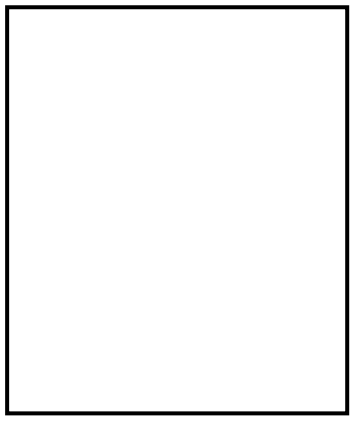
Full name of person obtaining consenting

Signature of person obtaining consenting Date

Full name of person authorising consent for
collection of samples at autopsy for use in research study

Signature of person authorising consent for Date
collection of samples at autopsy for use in research study

Thumb print of the spouse/partner/major child/parent/guardian/major brother/major sister of the deceased:



Full name of witness

Signature of witness Date

Appendix 4

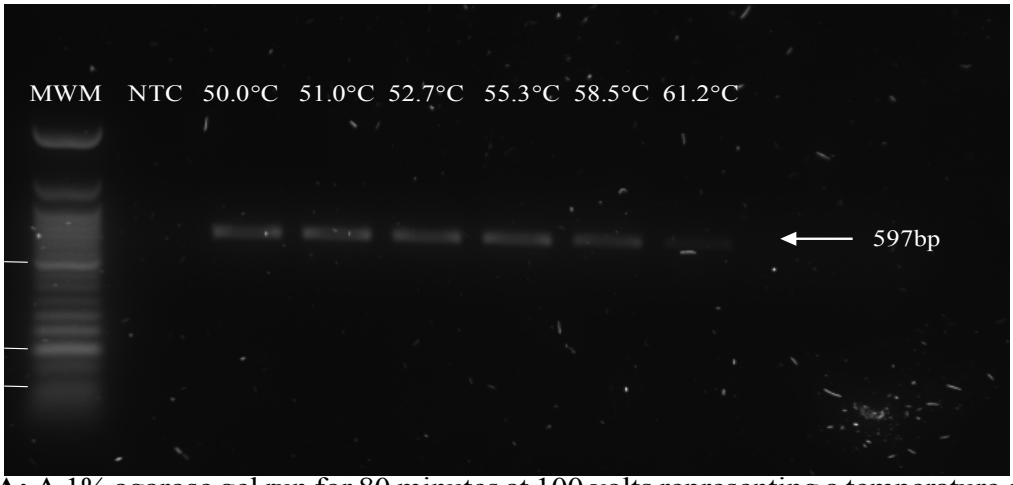


Figure A: A 1% agarose gel run for 80 minutes at 100 volts representing a temperature gradient of exon 1 part 2. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control.

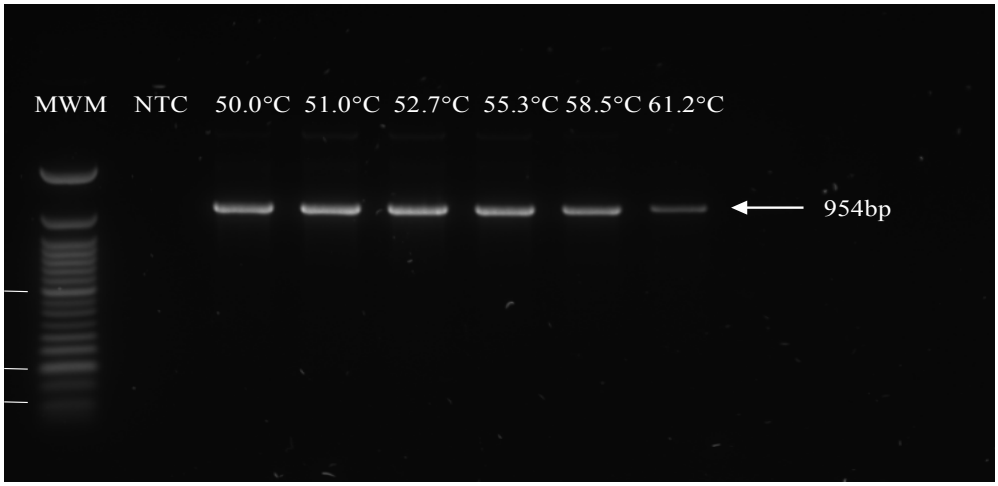


Figure B: A 1% agarose gel run for 80 minutes at 100 volts representing a temperature gradient of exon 1 part 1 **new**. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control.

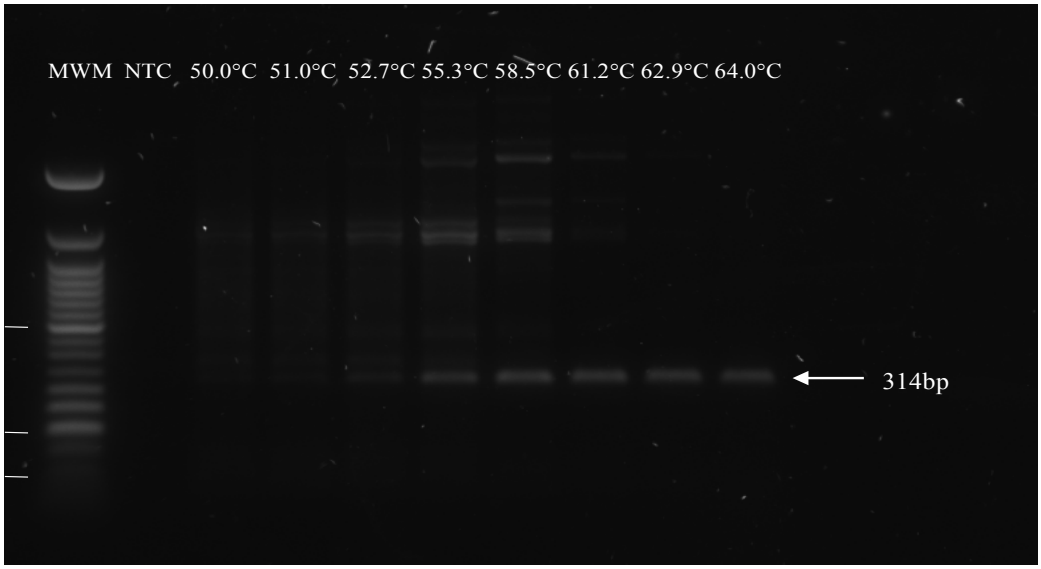


Figure C: A 1% agarose gel run for 80 minutes at 100 volts representing a temperature gradient of exon 1 part 3. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control.

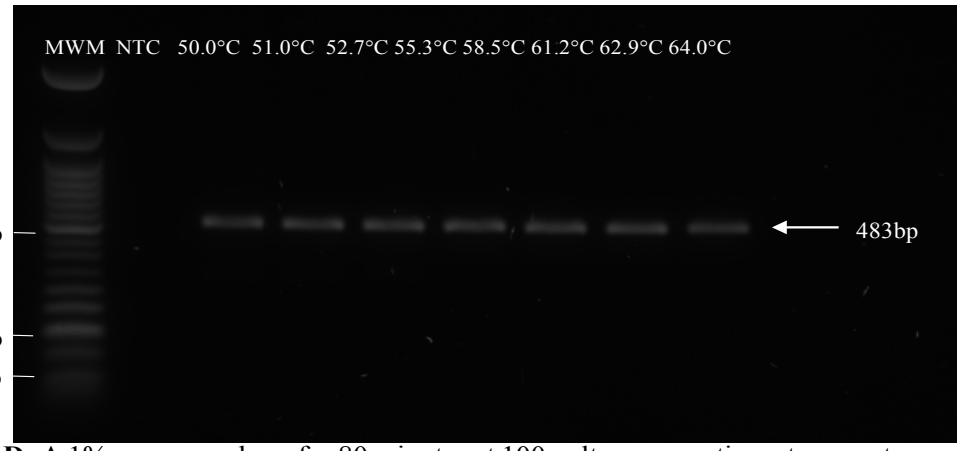


Figure D: A 1% agarose gel run for 80 minutes at 100 volts representing a temperature gradient of exon 2&3. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control.

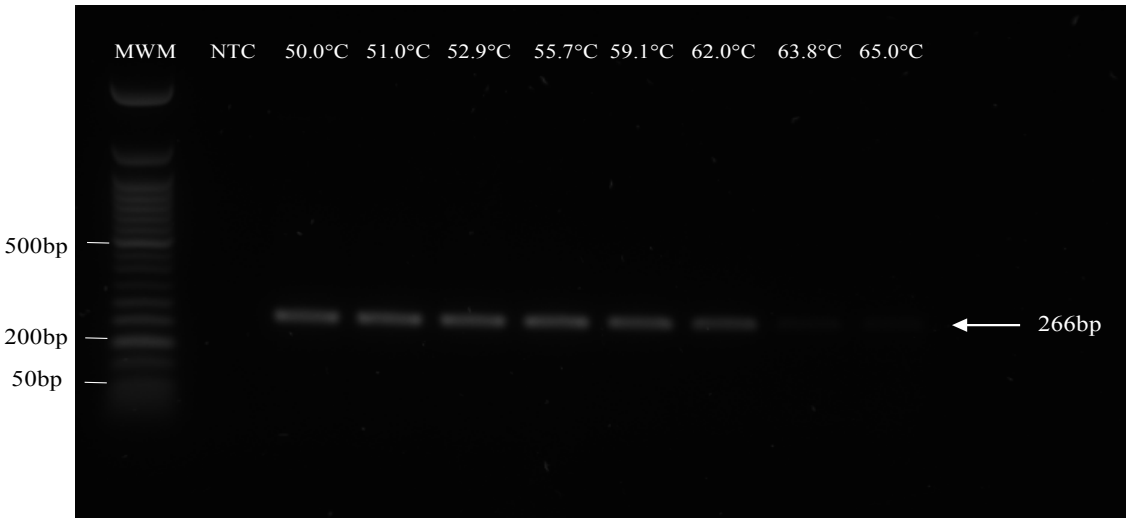


Figure E: A 1% agarose gel run for 80 minutes at 100 volts representing a temperature gradient of exon 4. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control.

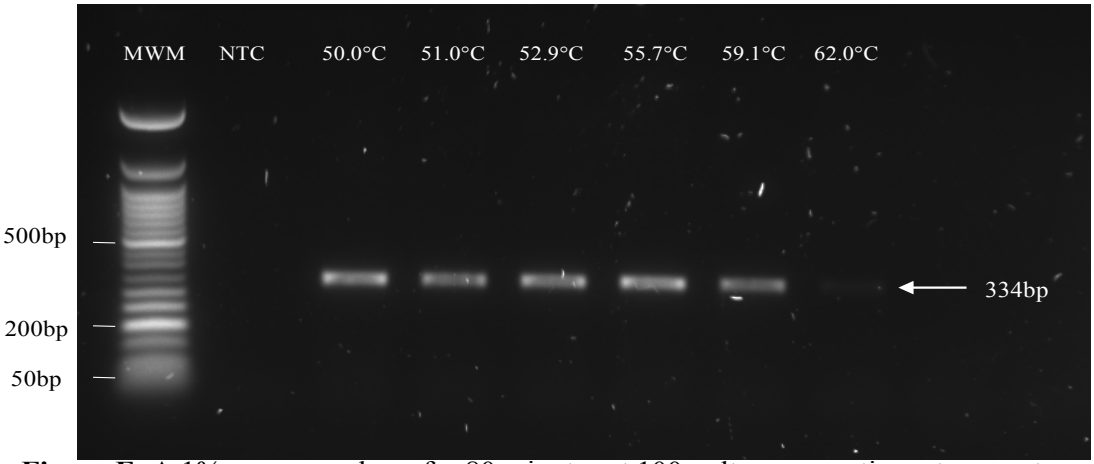


Figure F: A 1% agarose gel run for 80 minutes at 100 volts representing a temperature gradient of exon 5&6. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control.

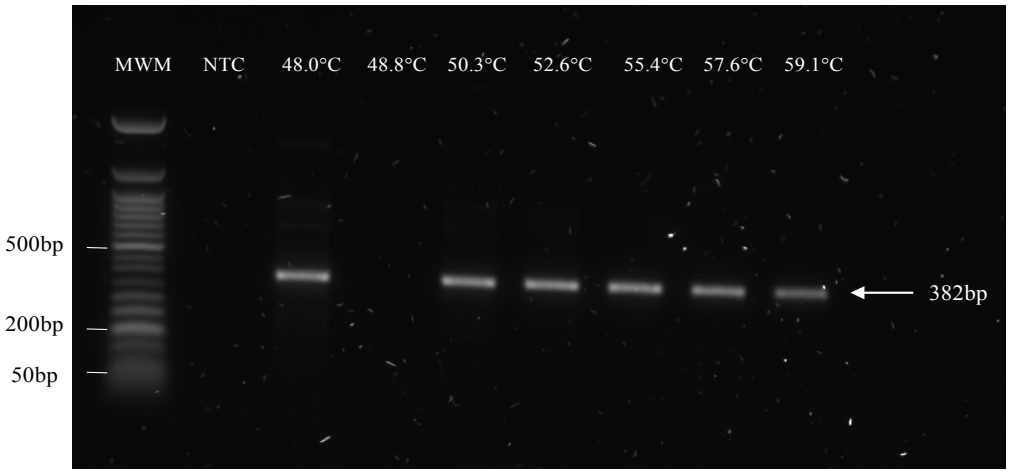


Figure G: A 1% agarose gel run for 80 minutes at 100 volts representing a temperature gradient of exon 7. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control.

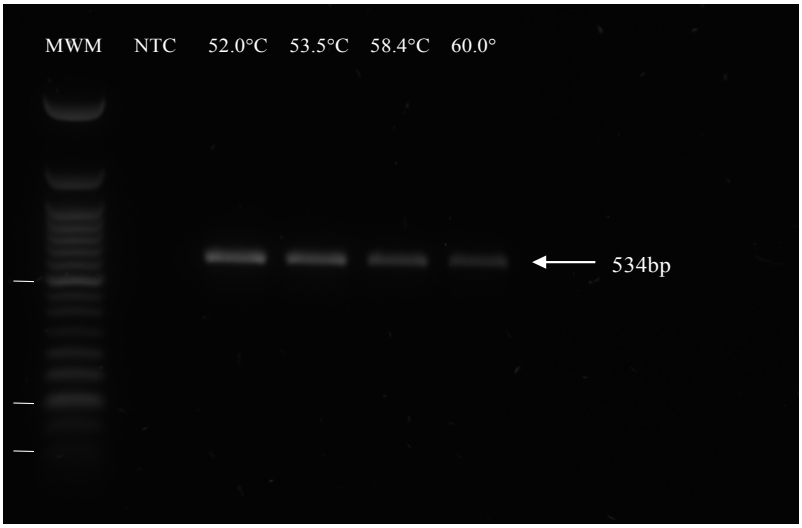


Figure H: A 1% agarose gel run for 80 minutes at 100 volts representing a temperature gradient of exon 8. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control.

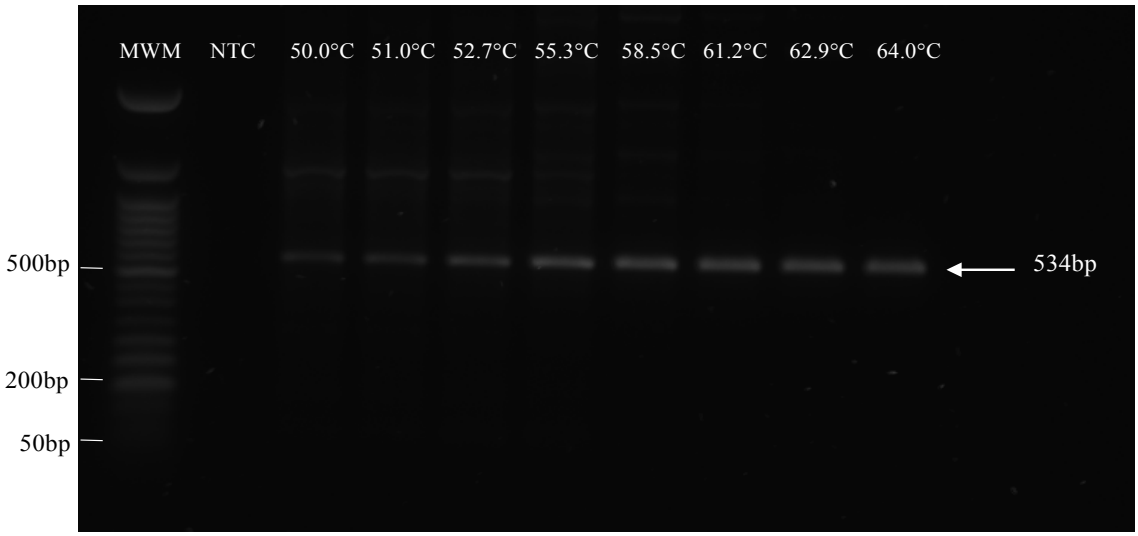


Figure I: A 1% agarose gel run for 80 minutes at 100 volts representing a temperature gradient of exon 9. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control.

Appendix 5

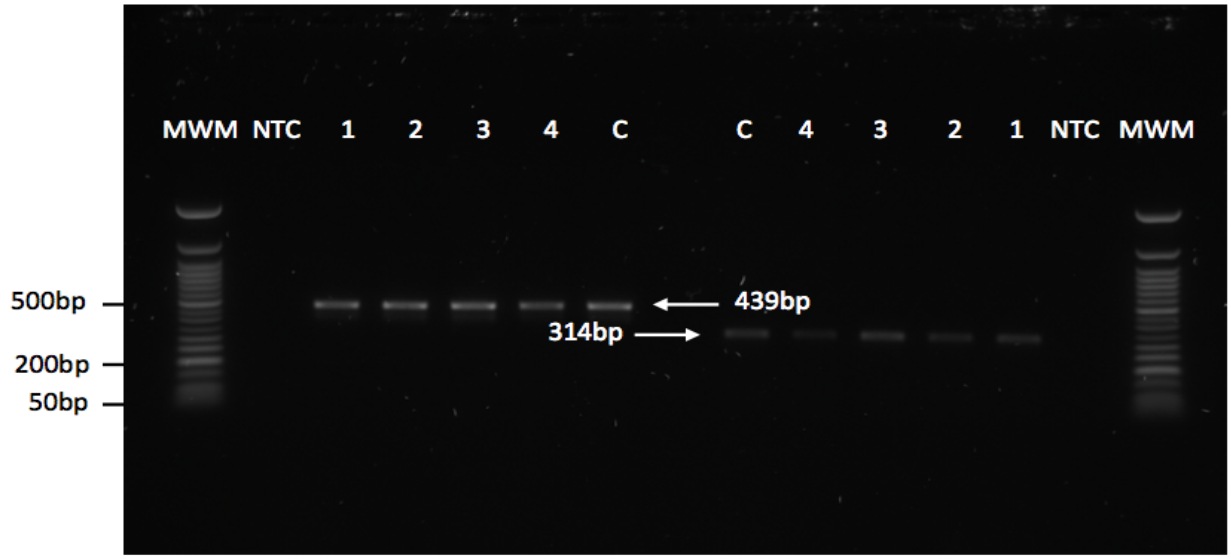


Figure A: A 1% agarose gel run for 80 minutes at 100 volts, representing amplification of exon 1 part 1 and 3 in the four different controls and case 1. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control; **1:** Control 1; **2:** Control 2; **3:** Control 3; **4:** Control 4 and **C:** Case 1.

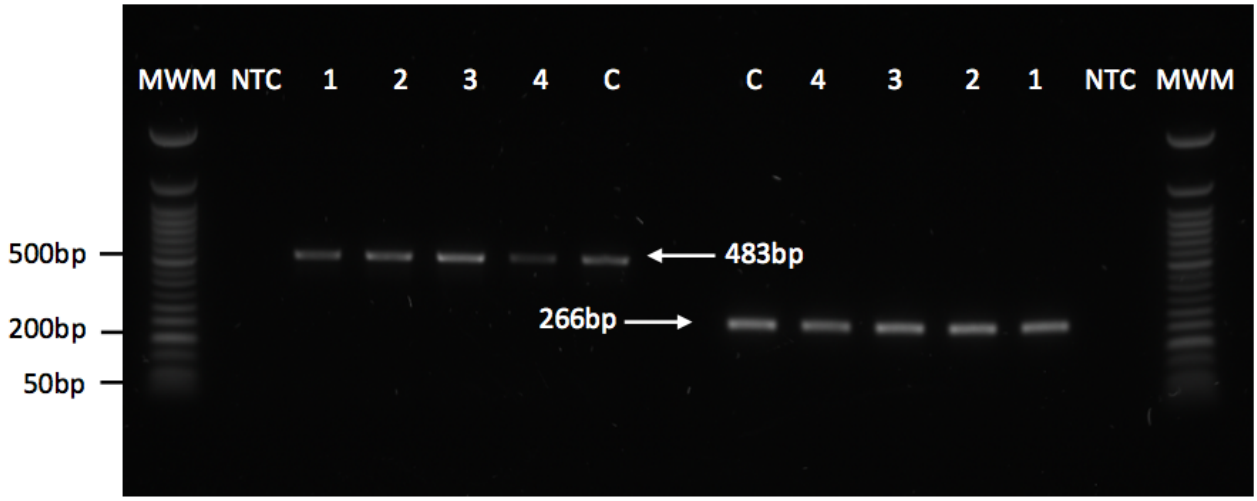


Figure B: A 1% agarose gel run for 80 minutes at 100 volts representing amplification of exon 2/3 and 4 in the four different controls and case 1. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control; **1:** Control 1; **2:** Control 2; **3:** Control 3; **4:** Control 4 and **C:** Case 1.

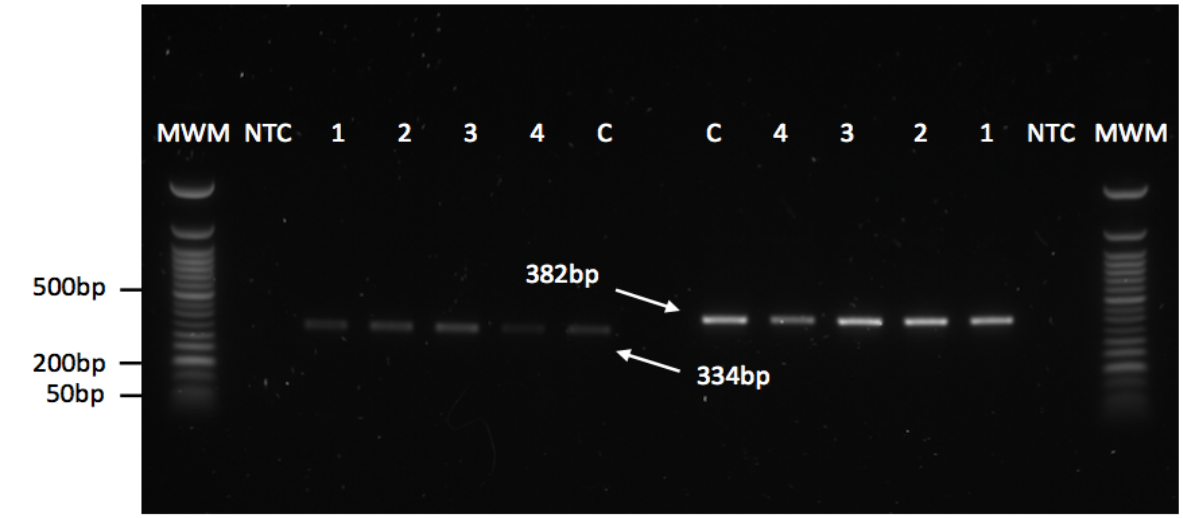


Figure C: A 1% agarose gel run for 80 minutes at 100 volts representing amplification of exon 5/6 and 7 in the four different controls and case 1. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control; **1:** Control 1; **2:** Control 2; **3:** Control 3; **4:** Control 4 and **C:** Case 1.

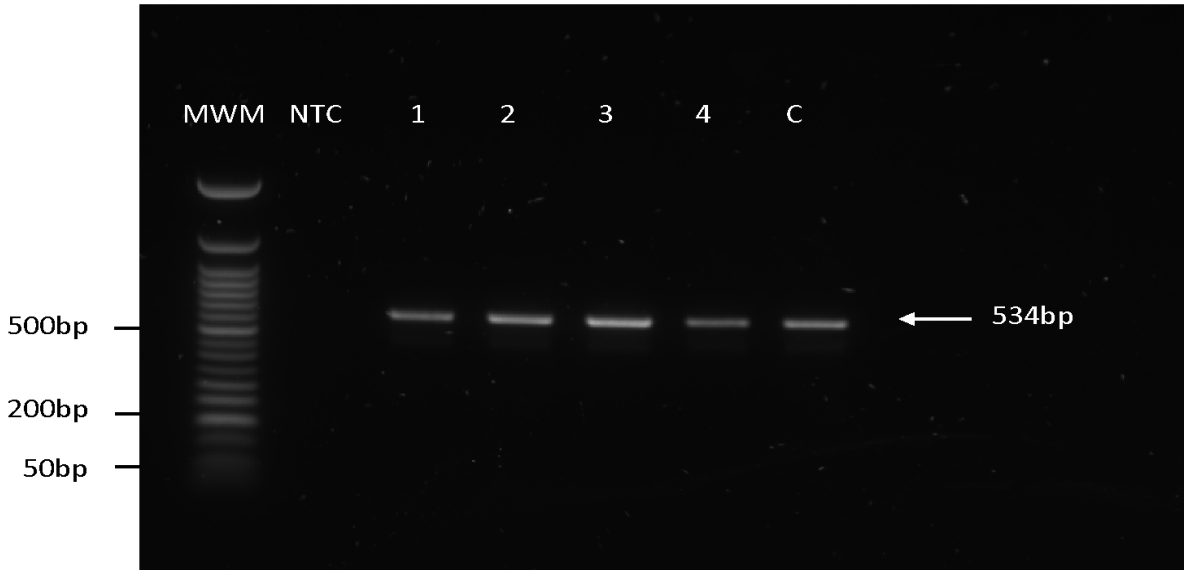


Figure D: A 1% agarose gel run for 80 minutes at 100 volts representing amplification of exon 8 in the four different controls and case 1. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control; **1:** Control 1; **2:** Control 2; **3:** Control 3; **4:** Control 4 and **C:** Case 1.

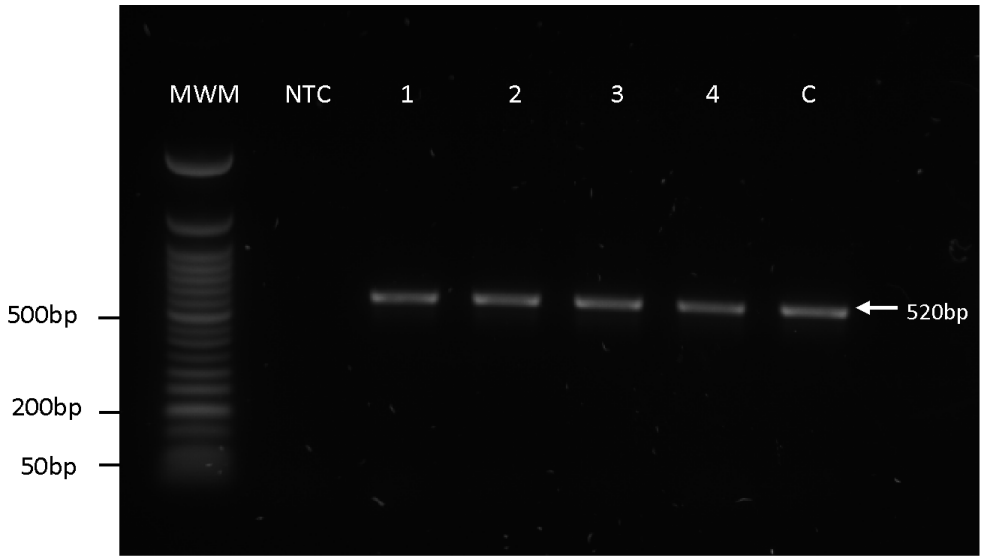


Figure E: A 1% agarose gel run for 80 minutes at 100v representing amplification of exon 9 in the four different controls and case 1. **MWM:** 50bp molecular weight marker; **NTC:** No Template Control; **1:** Control 1; **2:** Control 2; **3:** Control 3; **4:** Control 4 and **C:** Case 1.

Appendix 6

UCT TOXICOLOGY
Division Pharmacology, K50.30 OMB, GSH,
Observatory, 7925. ☎: 021 4066257

Printed: 6/13/2017 10:46:00 AM

REPORT

Attention: FORENSIC PATHOLOGY SERVICES

email:
cc:

Date: 12 June 2017

Patient ID	Case 1
Lab Number	
Date received	06/06/17
Date analysed	12/06/17
Sample Type	☒blood ☐urine ☐stomach contents ☐eye fluid ☐bile ☐tissue ☐other
Assay Method	LCMSMS
Observation	Amitriptyline, nortriptyline, protriptyline, citalopram and paracetamol detected

NOT FOR MEDICAL LEGAL PURPOSES

Analyst:

Lab Director:
Pharmacology Laboratory UCT

Page 1 of 1

UCT TOXICOLOGY
Division Pharmacology, K50.30 OMB, GSH,
Observatory, 7925. ☎: 021 4066257

Printed: 6/28/2017 2:15:00 PM

REPORT

Attention: **FORENSIC PATHOLOGY SERVICES**

email:
cc:

Date: **28 June, 2017**

Patient ID	Case 2
Lab Number	
Date received	22/06/17
Date analysed	28/06/17
Sample Type	☒blood ☐urine ☐stomach contents ☐eye fluid ☐bile ☐tissue ☐other
Assay Method	LCMSMS
Observation	Fluoxetine, ephedrine, doxylamine, diphenhydramine detected

NOT FOR MEDICAL LEGAL PURPOSES

Analyst:

Lab Director:
Pharmacology Laboratory UCT