Genotyping of a Clinically Important SNP rs1799853 Present in the CYP2C9 Gene Using Tetra-primer ARMS PCR Method

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree ofBachelor of Science in Biotechnology.

Department of Mathematics and Natural Sciences

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August 2020.

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It is hereby declared that

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3. The thesis does not contain material that has been accepted or submitted, for any other degree or diploma at a university or other institution.
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DEDICATED TO MY PARENTS

AND TEACHERS

Acknowledgment

First and foremost, I would like to express my thanks to almighty Allah because He has given me the opportunity and strength to finish this research. I am also thankful for His blessings to my daily life, good health, and a healthy mind.

I acknowledge my esteem to Professor **A F M Yusuf Haider**, Chairperson of MNS Department and Assistant Professor **Iftekhar Bin Naser**, Coordinator of

Biotechnology Program of MNS Department of BRAC University for allowing me and encouraging me to complete my undergraduate thesis.

My regards, gratitude, indebtedness, and appreciation goes to my respected supervisors **MD. Mahmudul Hasan Akash**, Lecturer of Department of Genetic Engineering and Biotechnology, Dhaka University and Professor **Dr. MMahboob Hossain**, Coordinator of Microbiology Program of MNS Department, BRAC University for their constant supervision, constructive criticism, expert guidance, enthusiastic encouragement to pursue new ideas and never-ending inspiration throughout the entire period of my research work. I would like to thank and express my deepest gratitude for guiding me in my report writing and providing time to time suggestions regarding the setting of my experimental designs, interpretation of results, and subsequent directions for the whole work without being a bit of impatient. It would have been impossible to submit my report without their cordial help.

I would like to extend my appreciation to the respective Lab officers **Asma Binte Afzal** and **Md. Nazrul Islam** for their suggestions and moral support during my work.

I also appreciate my thesis partners **WasifaAr Rahman**, **Afia AdibaRupoma**, **Rifaquat Ahmed** for their kind cooperation and active support throughout my work.

Finally, I would like to extend my gratitude to the members of my family and friends for their prayerful concerns and supports.

Anika Tahsin

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Abstract

Pharmacogenetics is the study of how similar drugs affect different people differently according to their unique genetic makeup as an individual. Pharmacogenetics includes a wide variety of research and discovery including essential medication disclosure, genetic research of pharmacokinetics as well as pharmacodynamics, new medication improvement and persistent hereditary testing where the final objective is to find out how a person's gene is responding to different medications and find the best possible treatment for that individual or that group of individuals. By foreseeing the medication reaction of an individual, it will be conceivable to build the accomplishment of treatments and decrease the rate of unfavorable symptoms.In this research, our primary intent is to develop a SNP genotyping method for a particular drug, which is a sulfonylureas drug in the case of type 1 diabetes, and how it affects the functionality of CYP2C9 gene in human body. Finally, our goal is to identify whether our desired SNP is present in the particular locus using the Tetra primer ARMS PCR technique and design the drug dose of that patient accordingly.

CHAPTER 1

INTRODUCTION

Introduction:

Background:

Type 2 diabetes mellitus is a chronic metabolic disorder arising from both the insulin secretion and insulin response defects (DeFronzo, 1999). Identifying high-risk individuals and managing lifestyles can help control diabetes; however, most patients require pharmacological intervention (Davis, 2004).

Five classes of oral agents are currently available in the United States, each of which works through a different mechanism of action, to improve glycemic control in patients with type 2 diabetes (DeFonzo, 1999). The sulfonylureas induce insulin release from pancreatic β cells, and for over 50 years have been a staple of Type 2 diabetes pharmacotherapy.

While sulfonylureas are effective antihyperglycemic agents, there is interindividual variability in drug response (i.e., pharmacodynamics), disposition (i.e., pharmacokinetics), and adverse effects (Aquilante,2010).

The sulfonylureas are mainly metabolized by the cytochrome enzyme p450 2C9 (CYP2C9). Two variants of CYP2C9--*2 (Arg144Cys, rs1799853) and *3 (Ile359Leu)—are related to decreased enzyme activity and impaired metabolism of the substrates (Aquilante, 2010).

The importance of SNPs derives from its ability to influence disease risk, medication efficacy and side effects (Ahmadian *et al.*, 2000) SNP genotyping is the analysis of SNP within a species ' members. SNP genotyping reveals if the SNP is present in an individual and analyses the side effects and minimizes the drug doses.

1.1 Pharmacology and Pharmacogenetics

1.1.1 What is Pharmacology

Pharmacology is the study of how a drug affects a biological system and the reaction of the body to the drug (Pharmacology archives). Within the umbrella of pharmacology, for example, the interactions between drug molecules and drug receptors and the effects of those interactions are studied. The Pharmacology umbrella is a wide one. It requires, among other things, the study of the different groups of drugs, their medical applications, their social functions and their modes of action (Ghosh, 2007). The pharmacology sector has two main branches:

- Pharmacokinetics, that refers to drug intake, delivery, metabolism, and excretion.
- Pharmacodynamics, that refers to the molecular, biochemical and physiological effects of drugs, including the drug action mechanism (Pharmacology archives).

1.1.2 What is Pharmacogenetics

Pharmacogenetics is the study of how people react to drug therapy differently, depending on their genetic structure or genes. Eventually, all drugs must exit the body through a mechanism called elimination– but the time they stay active in the bloodstream is also dictated by genetic differences that affect the ways the drug-processing enzyme function. (Drug absorption - Clinical pharmacology - MSD manual professional edition, 2019) In general, pharmacogenetics refers to the results of a single genetic marker. Pharmacogenetics focuses on the Drug Reaction effect of single genes. Pharmacogenetics can affect both the pharmacokinetics and drug pharmacodynamics (Bishop, 2009).

1.1.3 Pharmacogenetics and Adverse Drug Effects

Polymorphisms in the genes that code for drug-metabolizing enzymes, drug transporters, drug receptors, and ion channels that affect the risk of a person experiencing an adverse drug reaction, or may alter the effectiveness of drug treatment in that person. Mutant alleles are the best-studied human risk factors for adverse drug reactions at a single gene locus and contain several genes coding for drug-metabolising enzymes. These genetic polymorphisms of drug metabolism generate the phenotypes of various drugs called "poor metabolisers" or "ultrarapid metabolisers." Potential risk factors for drug inefficacy or toxicity include drug-drug interactions, patient age, the function of the renal and liver, or other disease factors and variables of lifestyle such as smoking and alcohol use. Factors inherited which affect the kinetics and dynamics of numerous drugs are of even greater importance in determining the individual risk. Therefore, genetic variation in genes for drug-metabolising enzymes, drug receptors, and drug transporters was correlated with individual heterogeneity in drug effectiveness and toxicity. If there are mutant or variant genes in the average population at a level of over 1%, they are considered as genetic polymorphisms. Genetic polymorphisms illustrate why a small proportion of the population could be at a higher risk of drug inefficacy or toxicity; the study of these polymorphisms has brought pharmacogenetics to the forefront (Meyer, 2000).

1.2 Diabetes Mellitus Type 2 and Association of Sulfonylureas drug :

1.2.1 Diabetes Mellitus Type 2

Type 2 diabetes is a chronic disease. It's characterized by elevated blood sugar levels. Food is broken down into basic components during digestion. Carbohydrates,

especially glucose, are broken down into basic sugars. Glucose is a crucially important energy source for the cells of the body. Glucose has to exit the blood to get inside the cells to provide energy to the cells. Insulin travels through the blood signals to take up glucose in the cells. Insulin is a pancreatic-producing hormone. The pancreas, in the abdomen, is an organ. When blood glucose levels rise (for example after a meal) more insulin is released by the pancreas. Type 2 diabetes happens when the cells in body become immune to the usual effect of insulin, which is to push glucose within the cells. This is called insulin resistance. Glucose then starts to build up in the blood. The pancreas "sees" an increase in blood glucose levels in people with insulin resistance. The pancreas responds by producing extra insulin to keep blood sugar stable. Over time, insulin resistance gets stronger in the body. The pancreas produces exponentially more insulin in response. The pancreas finally gets "exhausted" It can't keep up with the demand for insulin progressively. It is pooping out (Harvard Health Publishing, 2019).

1.2.2 Sulfonylureas Drug and Management of Type 2 Diabetes Mellitus

1.2.2.1 Sulfonylureas Drug

Sulfonylureas (e.g. glyburide, glipizide, glimepiride) are insulin secretagogues that induce the release of insulin from pancreatic beta cells and are likely to have the greatest glycemic effectiveness of all of the oral agents (What are sulfonylureas, 2020). Since the 1950s, sulfonylureas have been used in the diagnosis and development of tolbutamide for type 2 diabetes. They were classified into agents of the so-called first generation (tolbutamide, chlorpropamide, tolazamide, and acetohexamide), and agents of the second generation (glibenclamide, glipizide, gliclazide, glimepiride, and gliquidone). The major difference between the two

generations is that of potency, with the agents of the second generation being considerably more efficient. The main variations between the sulfonylureas relate to pharmacokinetic properties, with certain long-acting agents (chlorporpamide, glibenclamide) and others having a far shorter duration of action (tolbutamide, gliquidone). There's been a lot of discussion about the role of sulfonylurea extrapancreatic effects in their metabolic actions. Extrapancreatic effects are likely to result indirectly from the improved regulation of blood glucose because chronic hyperglycemia can produce insulin resistance (Furman, 2007). Sulfonylureas can also improve peripheral sensitivity to insulin secondary to an improvement in insulin receptors or changes in post-insulin-receptor binding events (What are sulfonylureas, 2020).

1.2.2.2 Mode of Action of Sulfonylureas Drug in Type 2 Diabetes Mellitus

Sulfonylureas are the first OADs to be used but following modern pharmacological principles. Sulfonylureas function on the pancreatic β-cell membrane by closing ATP-sensitive potassium channels, leading to an improved, glucoseindependent insulin secretion. Sulfonylureas will significantly boost glycemic control and should be used as the primary treatment for patients with impaired glycemic control on an acceptable diet.

Sulphonylureas bind to an ATP-dependent K+ (KATP) channel on the pancreatic β-cell membrane which inhibits the potassium tonic, hyperpolarizing efflux. Which causes the electrical potential to become more positive over the membrane. This depolarization opens Ca2 + channels with a voltage gate. The rise in intracellular calcium contributes to an increased fusion of insulin granule with the cell membrane, and hence increased (pro)insulin secretion (Furman, 2007).

Tolbutamide was the very first sulfonylureas. It was very effective in lowering the HbA1c level to 1 to 2%.

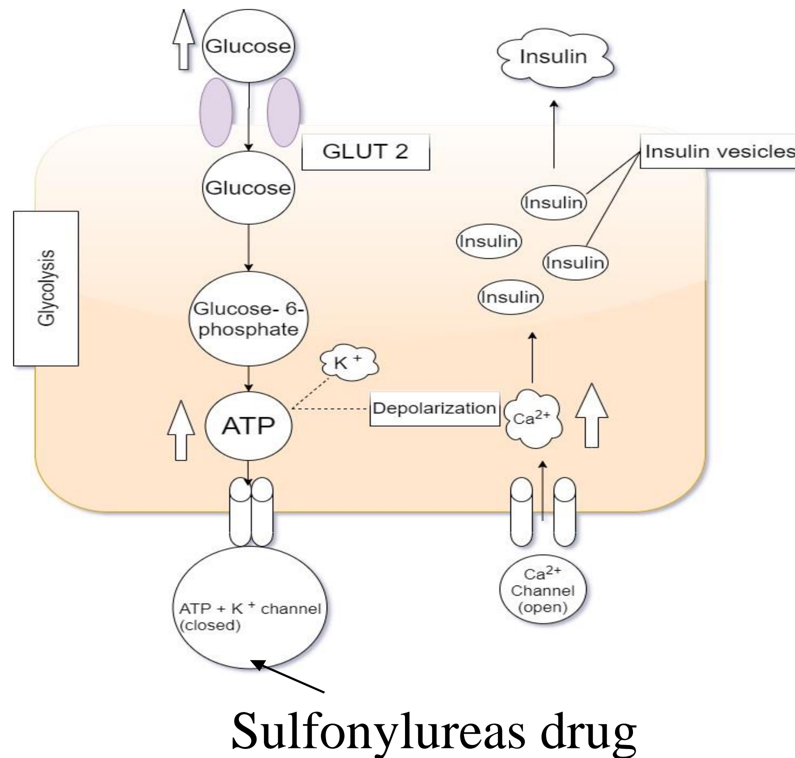


Figure 1.1:Mode of action of sulfonylureas drugs in type 1 diabetes mellitus. Glucose enters the β cell through the GLUT 2 channel which leads to a cascade of reactions resulting in the closing of ATP sensitive potassium channel. The later step leads to depolarization of the cell. This depolarization opens up voltage-gated Calcium channels. The rise in intracellular Calcium eventually leads to the secretion of insulin. Sulfonylureas drugs close the ATP sensitive potassium channel.

1.2.3 Adverse Effect of Sulfonylureas Drug

For approximately 50 years, sulfonylureas have been commonly used in clinical practice in the treatment of type 2 diabetes. Despite several new medications now

available, sulfonylureas remain the most popular anti-diabetic medications doctors are willing to prescribe with first-line therapy medication metformin, in addition to or after failure. The simplicity and higher effectiveness of glucose-lowering are potential benefits that explain why this type of drug is still used in practice. Sulfonylureas are divided into two generations: First and Second. The second-generation sulfonylureas (Glyburide, Glipizide, Glimepiride, Gliclazide) are commonly used, while the first-generation sulfonylureas (Tolbutamide, Chlorpropamide, Tolazamide) are no longer used due to extreme episodes of hypoglycemia. Hypoglycemia, weight gain, and the risk for cardiovascular problems are the most common adverse effects of sulfonylureas (Simmons&Joffe,2019). Sulfonylureas's most severe adverse effect is long-lasting hypoglycemia, which may result in irreversible brain damage and even death, which is usually misdiagnosed as a cerebrovascular insult. In a study of 473 cases of hypoglycemia, 200 of which were serious, Seltzer (Seltzer, 1982)found chlorpropamide had been obtained in most of these patients. 10% of all hypoglycemic patients referred to in hospitals died, and 3% suffered neurological damage. A possible reason why chlorpropamide is more vulnerable to long-lasting hypoglycemia than tolbutamide is that chlorpropamide removes very slowly and thus easily accumulates; its removal half-life is 36 hours or more compared to about eight hours for tolbutamide. The very long halflife in conjunction with slow renal excretion is a result of gradual and only partial metabolic degradation. Glyburide (glibenclamide), the most frequently associated sulfonylurea with long-lasting hypoglycemia, has been in Europe. This is partially due to the widespread use of glyburide; however, other features can also render glyburide more likely to cause long-lasting hypoglycemia. Glipizide is quickly absorbed and removed during long-term treatment, as seen in Fig. 1.4, while glyburide can be absorbed very gradually, allowing for prolonged exposure (Furman, 2007).

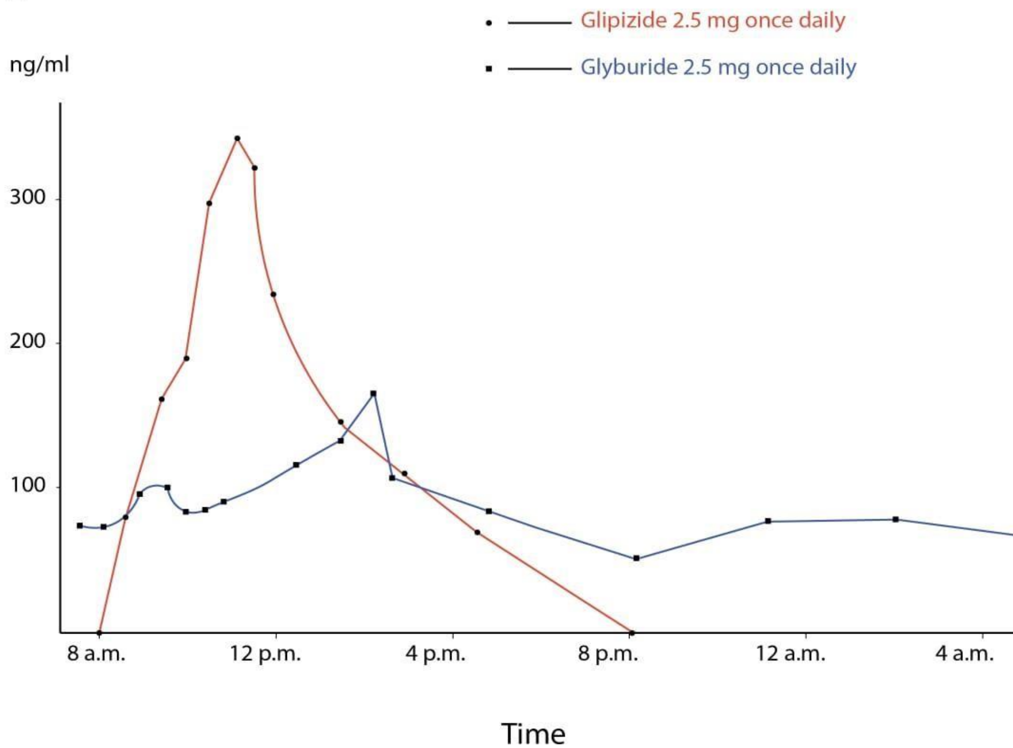


Figure 1.2: Adverse effect of sulfonylureas drugs. Twenty-four-hour plasma concentration profiles of glipizide and glyburide in the same patient during 6 months of treatment with 2.5 mg/d of either drug. Here is the rapid absorption and disappearance of glipizide and the slow and prolonged absorption of glyburide.

1.3 Cytochrome P450 Enzymes Associated with Drug Metabolism and DrugDrug Interaction

1.3.1 Genetic Polymorphism Affecting Gene Expression and Function

Genetic mutations or polymorphisms (genetic variants) of CYP are known to occur among patients. The metabolism of such drugs can be variable based on the phenotype produced by certain genes. The capacity of each person to metabolize drugs is determined by the pairing of individual alleles which he or she inherited from his or her parents. Each allele can be categorized as a wild-type allele

(functional) or variant (defective). Wild-type alleles are considered "normal" and generally occur in the general population, while variant alleles may result in diminished activity or no activity. Persons carrying two wild-type alleles will generally have "normal" metabolism levels (extensive metabolizers), while typically an individual carrying two (defective) alleles would have little to no enzyme activity (poor metabolizers). Those who inherited one of each allele would have reduced enzymatic (intermediate metabolizers) function. In some cases, where gene duplication or amplification leads to more than two gene copies of wild-type alleles, enzyme activity may be greater than average (McDonnell& Dang, 2013).Genetic polymorphisms can have a major effect on drug therapy and should be taken into account in clinical practice, especially when unexpected outcomes occur. For example, due to the accumulation of medications, intermediate and weak metabolisers are at increased risk of toxicity and adverse effects. These patients display hypersensitivity or poor tolerance to certain medications and may need decreased doses or avoidance of the medication entirely afterward. Ultrarapid metabolizers reflect the opposite end of the spectrum, but when the metabolite is more active than the parent compound, they may also be prone to opioid toxicity (McDonnell& Dang, 2013). Variants of loss of function will result in decreased clearance and increased plasma concentrations while variants of gain of function will result in increased clearance and lower concentrations of drugs. If the medication is pharmacologically active, this results in increased and decreased drug effect due to overdose, respectively, and possibly drug-related toxicity. If the drug is activated metabolically (prodrug), the reverse is to be expected and the pharmacological activity or toxicity of the metabolite(s) must be considered, as in the case of CYP2D6-dependent morphine production from codeine, for example (Zanger&Schwab, 2013).

1.3.1.1 Genetic Polymorphism of CYP2C9 Gene in Sulfonylureas Drug Metabolism

The hypoglycemic sulfonylurea is primarily metabolized by the enzyme cytochrome P450 2C9 (CYP2C9). Sulfonylurea is metabolized primarily by the enzyme

cytochromeP4502C9 (CYP2C9). Allelic variants of CYP2C9gene, CYP2C9*2 (Arg144Cys, rs1799853), and CYP2C9*3 (Ile359Leu, rs1057910), encode proteins with less enzymatic activity for multi-substrate metabolism than CYP2C9*1 (Arg144/Ile359) wild-type alleles. Both in vitro and in vivo studies reported a moderate decrease in enzyme activity in humans with polymorphism CYP2C9*2 and a significant decrease in people with polymorphism CYP2C9*3 (Becker *et al.*, 2008).Compared with the genotype CYP2C9 * 1/*1, tolbutamide clearance was decreased by 25% in people with the genotype CYP2C9 * 2/*2 and by 84% in people with the genotype CYP2C9 * 3/*3 (Kirchheiner 2002). For glibenclamide, the clearance reductions were 25% and 57% respectively (Kirchheiner 2002).

1.4 Single Nucleotide Polymorphism (SNP) and SNP Genotyping

1.4.1 What is single nucleotide polymorphism (SNP)

A single nucleotide polymorphism, or SNP (pronounced "snip"), is a difference between individuals at a single location within a DNA sequence (Aylor *et al.*, 2011).The most common form of genetic variation in humans is single nucleotide polymorphisms, also called SNPs. Increasing SNP in a single building block of DNA, called a nucleotide, reflects a difference. For example, in a given stretch of DNA, an SNP can replace nucleotide cytosine (C) with nucleotide thymine (T). SNPs usually occur over the entire DNA of an individual. On average, they occur about once in every 1,000 nucleotides, indicating that a person's genome contains between 4 to 5 million SNPs.

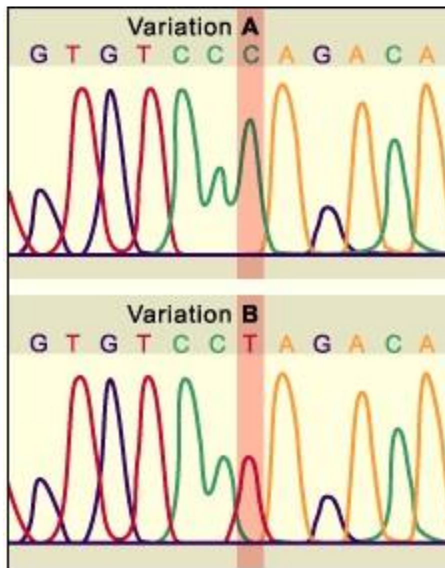


Figure 1.3: Single Nucleotide Polymorphism. This compares two similar segments of the chromosomes of two individuals (A and B). One SNP is shown in a base pair as a variation. In individual B, Cytosine (C) has been replaced by Thymine (T) (Miller *et al.*, 2010).Such variants may be unique or occur in several individuals; in populations around the world, scientists have detected more than 100 million SNPs.

1.4.2 Role of SNPs in Drug Response

DNA is simply a long list of nucleotides arranged in a specific order. Nucleotides that make up the DNA sequence contain purines (adenine (A), guanine (G)) and pyrimidines (cytosine (C), thymine (T)) and are paired inside the double helix such that G is paired with C and A is paired with T by hydrogen bonding. These base pairs also form codons composed of a sequence of three individual nucleotides. For a variety of functions, the combination of these 3 nucleotide sequences is important. One function is to influence the behavior of certainregulatory proteins, such as those involved in the gene transcription process and a protein translation process. During

the process of producing a new protein (such as an enzyme or transporter), another important task is to decide which amino acid to position next in the chain. Throughout the gene translation process, the correct sequence of amino acids must be placed together to ensure that proteins are produced and function properly. Therefore the sequence of individual nucleotides in the DNA determines all of these cellular functions. The ability of genes to be transcribed from the DNA or functional proteins to be generated during gene translation may be significantly affected if any of the individual nucleotides were replaced by a different nucleotide. This transition is SNP in a single nucleotide. The position of the SNP determines a patient's speech or "phenotype." An SNP in the DNA coding region (cSNP) may or may not allow amino acid substitutions to form in the protein. When there is an amino acid substitution, the protein produced can have a different shape or tertiary structure and may have a major impact on the ability of that protein to exert its biological effect. If the SNP occurs in the DNA promoter or enhancer region, gene regulation can be altered resulting in a shift in the amount of protein produced and/or the biological effect it predicts. Unfortunately, SNPs can occur with several drug-transporting proteins, metabolism, and receptors that eventually affect both the pharmacokinetic and pharmacodynamic properties of a variety of drugs (Parvez *et al.*, 2012).

1.4.3 What is Allele Frequency

Allele frequency is a measure of the relative occurrence of an allele in a population at the genetic locus. This is generally expressed as a percentage or a proportion. For population genetics, allele frequencies demonstrate the genetic diversity of a species population or the abundance of its pool of genes equivalently (Luikart *et al.*, 1998). For example, let's consider a population of 100 diploid people. Each human carries two copies of each gene, so a total of 200 gene copies are in the 100-person population. Now let's assume that in this group, 20 individuals are heterozygous for allele A (with the second allele of some other type), and 5 individuals are homozygous for allele A. Each homozygote would contribute two copies of the allele to the overall fraction, while each heterozygote would contribute only one

copy to the overall fraction. And in the population, the total number of A alleles will be 20 + 10, for a total of 30. The frequency of the allele will be that number divided by the total number of gene copies (30/200) to yield 0.15 which is the frequency of the allele. This way, when the numbers of homozygotes and heterozygotes in a population are known, all the frequencies can also be calculated. If heterozygotes can not be differentiated when an allele expresses a recessive trait, Hardy – Weinberg statistics can still be used to estimate the frequency of the allele when other assumptions are made regarding breeding practices (McCarroll *et al.*, 2008).

1.4.4 CYP2C9*2 allele (rs1799853)

1.4.4.1 rs1799853 Associated with Sulfonylureas Drug Metabolism

CYP2C9 is a member of the cytochrome p450 gene subfamily IIC, which is responsible for metabolizing various medications such as phenytoin, tamoxifen, warfarin, fluvastatin and other non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, and naproxen. Individuals who carry variants of CYP2C9 can process these drugs differently. For CYP2C9 the SNPs include:

- rs1057910, CYP2C9* 1, and CYP2C9 * 3 alleles are encoded in two variants.
- rs1799853, whose versions encode CYP2C9 * 2 alleles (Lazar *et al.*, 2004).

Many sulfonylureas are metabolized mainly in the liver by the enzyme cytochrome P450 (CYP) 2C9. Hence, CYP2C9 is a plausible candidate gene to be challenged in relation to clinical pharmacology of sulfonylurea. Studies have shown that the polymorphisms of CYP2C9*3 (Ile359Leu, I359L) and, to a lesser degree, CYP2C9*2 (Arg144Cys, R144C) are correlated with decreased oral clearance and increased tolbutamide, glyburide, glipizide, and glimepiride plasma exposure (Aquilante& Lam, 2013). SUs were mainly inactivated by the cytochrome P450

2C9. According to the GoDARTS report, approximately 6% of the population carries two variants of reduced activity in the CYP2C9 gene (rs1799853 C>T, rs1057910 A>C) correlated with increased response to SUs due to increased concentration of drugs. To avoid hypoglycemia, these patients will need lower starting doses of SUs (Fodor *et al.,* 2019).

1.4.5SNP Genotyping

1.4.5.1 What is SNP Genotyping

Genotyping is the technology that identifies minor genetic variations that can contribute to significant phenotype changes, including both physical differences that make us special, and underlying disease pathological changes. Genotyping identifies genetic complement variations by comparing a DNA sequence with that of another sample or sequence of reference. It detects minor differences in the genetic code within populations, such as polymorphisms of single nucleotides (SNPs) (Kwok & Chen, 2013).SNP genotyping is the analysis of genetic variation between members of a group with single nucleotide polymorphisms (SNPs). It is a type of genotyping, which is measuring genetic variation more broadly (Fan *et al*., 2003) SNP genotyping will speed up the age of personalized medicine by predicting the likelihood of a patient contracting certain diseases, or by designing tailored therapies according to the disease's genetic basis (Kwok & Chen, 2013).

1.4.5.2 SNP Genotyping Methods

1.4.5.2.1 Hybridization

Two allele-specific probes are equipped with the hybridization approach to hybridize to the target sequence only when they match perfectly [Figure 1.6]. Under optimized conditions of the assay, the one-base mismatch sufficiently destabilizes the hybridization to prevent annealing of the allelic probe into the target sequence. Hybridization is the simplest method for genotyping since no enzymes are involved in allelic discrimination.

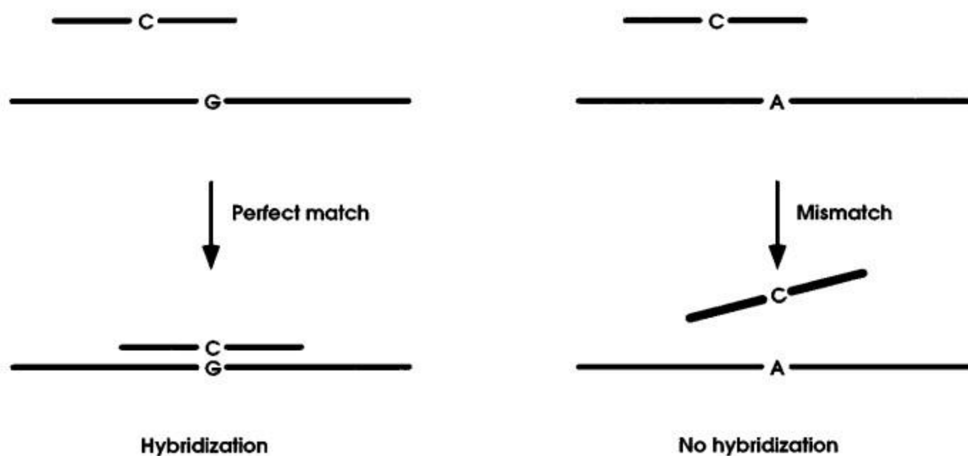


Figure 1.4: Allele-specific hybridization (Kwok, 2001)

When the allele-specific samples are immobilized on a solid support, marked target DNA samples are captured and the hybridization event is visualized by detecting the label after washing away the unbound targets. Knowing the position of the sequences of the probes on the solid support helps one to infer the DNA genotype of the target sample (Kwok, 2001).

1.4.5.2.2 Molecular Beacon Genotyping

Molecular beacons are stem-loop structures that carry a fluorescent reporter in near association with a universal quencher, such that fluorescence is only detected when the stem-loop structure opens. A DNA target with a proper design that is ideally complementary to the sequence of the loop portion of the molecular beacon hybridizes the molecular beacon and causes the stem to open, resulting in fluorescence. The molecular beacon with one base mismatch does not hybridize to the target strongly enough to disrupt the structure of the stem-loop and fluorescence is not observed. Since the presence or absence of fluorescence indicates the open or close status of the stem-loop structure there is no need for purification or separation steps. As long as fluorescence can be tracked one can infer the DNA target genotype.

Advantage of this genotyping technique: This "closedtube" design has many advantages as it minimizes cross-contamination and makes automation simple to achieve. An additional benefit is that the test can be used to measure the amount of DNA present in an unknown sample if real-time fluorescence monitoring is feasible. Multiplex detection is possible to some degree for many fluorescent dyes available in the visible spectrum.

Disadvantage of this genotyping technique: The one downside of this approach is the cost to each SNP marker of the two doubly labeled molecular beacons needed. A fraction of molecular beacons does not work without optimisation until design algorithms are perfected (Kwok, 2001).

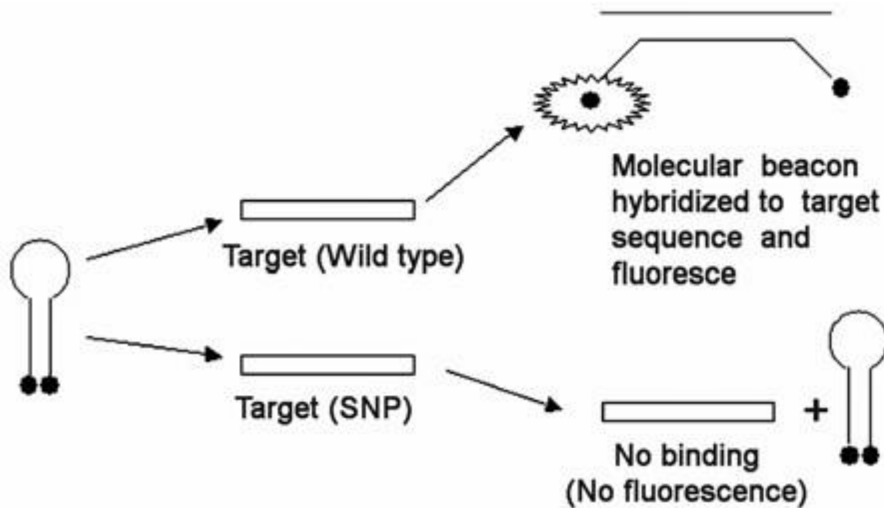


Figure 1.5: Molecular beacon genotyping. Molecular beacon hybridizes target DNA with complementary sequence and opens to isolate reporter from quencher and fluoresce. Therefore, hybridisation does not occur in single base mismatch, no fluorescence signal is obtained (Jehan&Lakhanpaul, 2006).

1.4.5.2.3 PCR Based Genotyping

Allele-Specific PCR: The word suggests that the procedure used in this form of PCR is unique to the particular type of allele. An allele is the gene's alternate form. When one allele has an SNP and the other alternative type is normal, by developing different primers for each allele, we can evaluate all the alleles. To do so, we need to change the single base at the 3 'end of the primer (one primer matches the usual allele, and one primer matches the mutant allele). At the same time, the PCR is done in a single reaction. If there is a mutant allele, then the PCR amplifies the mutant allele, or if there is the usual allele, the usual allele will be amplified (Vannucchi *et al*., 2006).

ARMS PCR: Often known as the (amplification refractory mutation system) ARMS-PCR is the allele-specific PCR because of the use of two different primers for two different alleles. As two sets of primers are constructed, the primer's mutant set is refractory (resistant) to normal PCR, and the primers normal set is refractory to the mutant PCR reaction. The ARMS PCR mechanism is based on the adjustment of the primers for various alleles. Here, the 3 'end of the primers is changed such that the regular allele can be amplified by one set of the primers and the mutant allele can be amplified by others. The single-base mismatch is implemented at the 3 'end of the primer. Such a mutation causes the primer to amplify a single allele. Since the ARMS PCR is mainly designed to detect a mutation or polymorphism, it is also important to be able to determine if the DNA alteration is heterozygous or homozygous. Using ARMS primers for mutant / polymorphic and natural (wild type) alleles differentiates a heterozygote or homozygote. The mutant and standard alleles reactions are normally conducted in separate tubes. But after marking the two primers with different fluorescent dyes, this can be achieved in the same tube (Little, 1995).

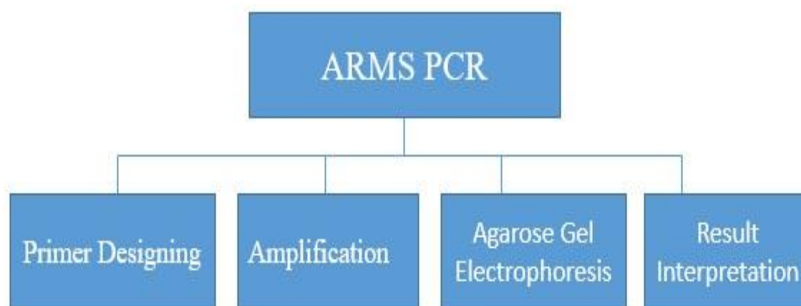


Figure 1.6: ARMS PCR. In this figure, steps of ARMS PCR are shown.

1.4.5.2.4 Sequencing Based Genotyping

Sanger Sequencing: Sanger sequencing is a selective sequencing technique that uses primers of oligonucleotides to search for different regions of DNA. Sanger sequencing starts with Double-stranded DNA denaturation. Using a mixture of deoxynucleotide triphosphates (dNTPs), which provide the required arginine (A), cytosine (C), tyrosine (T), and guanine (G) nucleotides to create the new double-stranded structure, the single-stranded DNA is then annealed to oligonucleotide primers and expanded. In addition, a small amount of chainterminating triphosphates of the dideoxynucleotide (ddNTPs) is used for each nucleotide. With dNTPs, the series will continue to expand until a ddNTP is connected. Since the dNTPs and ddNTPs have equal opportunities to bind to the chain, each chain will end at differing lengths. Often contains a fluorescent marker on each ddNTP (ddATP, ddGTP, ddCTP, ddTTP). Once a ddNTP is added to the elongating chain, the base fluoresces depending on the nucleotide associated with that. By definition, green fluorescence shows A, red T, black G, and blue C. A laser used to read the sequence within the automated system senses a fluorescent intensity that is converted into a "peak." When a heterozygous variant occurs within a sequence, loci will be captured by two fluorescent dyes of equal intensity. When a homozygous variant is present, the expected fluorescent color is replaced completely by the new base pair’s color [Figure 1.10]. (Muotri *et al.*, 2005).

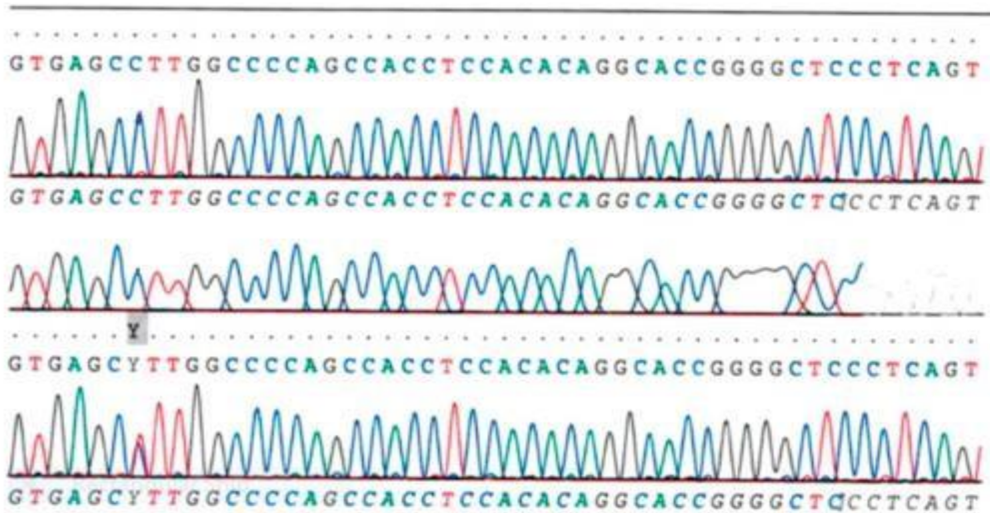


Figure 1.7: Sanger Sequencing. Sanger sequencing electropherogram of a nucleotide shift from C to T (mutation noted with a Y) compared to standard contral sample sequencing. This mutation is a heterozygousmutation because there is a distinct nucleotide in both alleles (Muotri *et al*., 2005).

Advantage of Sanger Sequencing: Sanger sequencing is a reliable research technique that can decide whether there is a point mutation or a slight deletion/duplication.

Limitation of Sanger Sequencing: While one may use individual Sanger sequencing reactions to cover any desired area, compared with other multiplex testing methods, this testing method can be expensive. Hence, most Sanger sequencing tests currently available are gene-specific or examine a small subset of genes. Sanger sequencing is capable of detecting mosaic mutations from as small as 20% of cells, but Sanger sequencing is not precisely quantifiable (Muotri *et al.*, 2005).

1.5 Importance of this Research

Diabetes is one of the four primary forms of non-communicable diseases (NCDs) that make the world's largest contribution to morbidity and death. According to WHO Global Health Days 2016, about 422 million people have diabetes worldwide,

with most living in developed countries, and unfortunately, more than 80% of deaths from diabetes occur in low- and middle-income nations. And 80% of people with diabetes live in countries with low and middle incomes. Diabetes prevalence is growing in urban as well as rural areas of Bangladesh. A recent scoping analysis (1994-2013) showed that type 2 diabetes prevalence in Bangladesh ranged from 4.5% to 35.0%. The International Diabetes Federation in Bangladesh estimated 7.1 million people with diabetes and nearly equal numbers with undetected diabetes. It is predicted the thenumber will double by 2025. It can cause stroke, heart attack, chronic kidney disease, neuropathy, vision impairment, and amputation. Although most of these complications can be avoided primarily through cheap, easy-to-use, and cost-effective therapies (Mohiuddin, 2019). Sulfonylureas are a class of oral antidiabetic drugs used worldwide for the treatment of type 2 diabetes mellitus. Through this study, we have developed a (an) SNP genotyping method which will eventually determine whether a person is homozygous wild type, homozygous mutant, or heterozygous mutant for this particular SNP (rs1799853) and whether he/she is prescribed for this particular drug then how would the dose be. The SNP genotyping approach employed for this work is genotyping based on ARMS PCR.

1.6. Objective of this Research

1. Development of the SNP genotyping method: The gene CYP2C9 has been chosen because it is commonly associated with the metabolism of the drug Sulfonylureas and CYP2C9*2 allele (rs1799853) and CYP2C* 3 allele (rs1057910) are the most studied CYP2C9 gene alleles. If a person is mutant heterozygous or homozygous for this SNP (rs1799853) after genotyping, the drug should be given to him/her at a reduced dose or should not be given the drug.
2. Primer Designing: Primer was designed for this particular SNP (rs1799853, Arg144Cys) at CYP2C9*2 gene locus for the ARMS PCR method. The primers will be 18-25 bp long with a GC percentage ranging from 40% to 60%. There shouldn’t be any formation of primer dimer.

3. Another goal of this research is to find out the frequency of this CYP2C9*2 allele in the Bangladeshi population.

CHAPTER 2
MATERIALS AND METHODS

Materials and Methods

2.1 Study design

As seen in Figure 2.1, this research project comprises three different parts – In silico studies, In vitro experiments, and Data analysis. Recovery of the CYP2C9 gene sequence from the NCBI website includes in the in silico experiment. The bulk of this research project is priming design. In this research, the ARMS PCR technique was used to identify SNPs by tetra primers and a set of four primers, two outer forward primers, and two inner reverse primers were designed for this technique. The outer primers are for individuals of the wild variety and mutant variety and the inner reverse primers are for individuals having the SNP. A mismatch was deliberately inserted in the inner reverse primer. In vitro experiment includes collecting DNA samples from healthy people, optimizing PCR conditions, and analyzing data include analyzing poor metabolizer clusters and Hardy-Weinberg equilibrium testing.

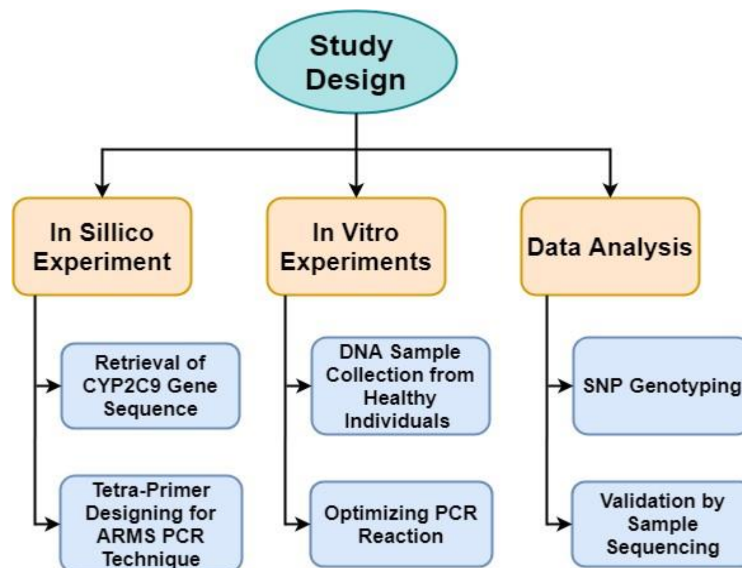


Fig 2.1: An overview of three different parts of the methodology of identifying SNPs

2.1.1 In Silico Experiment

The main focus in this section was to retrieve the sequence of the CYP2C9 genes for priming design. And a set of four primers for the ARMS PCR reaction was designed, and the suitability of these primers for PCR reaction was checked.

2.1.1.1 Retrieval of CYP2C9 Gene Sequence

The accompanying advances were performed to recover the CYP2C9 gene sequence-

Use the URL the NCBI web site was browsed: https:/www.ncbi.nlm.nih.gov. On the search box was typed "Homo sapiens CYP2C9" "All database" was set to "Gene" by pressing the drop-down menu on the left-hand side. Clicking on the "Search" tab activated the question. The first search result entry was "CYP2C9-cytochrome P450 family 2 C member 9." This entry is GeneId 1559. The next page was arrived by clicking on the "Name / Gene" bar to share more detail about the outcome of this article. On this page, "RefSeqGene" was clicked under the "Related Information" section and the FASTA format was checked out. This FASTA format represents the CYP2C9 gene sequence.

2.1.1.2 Tetra-Primer Designing for ARMS PCR Technique

We designed our desired primers for Tetra Primers ARMS PCR technology using **BatchPrimer3 v1.0 A high throughput web application for PCR and sequencing primer design 1.** We used the following parameters stated in table 2.2 to design our primer.

Table 2.1: Parameters used in designing primers for the ARMS PCR method

Parameters	Min.	Opt.	Max.
Primer Size	20	22	30
Primer Tm	50	65	80
Tm Difference			5
Primer GC%	20		80
Inner Product Size	100	250	400
Relative Size Difference Between Inner Product Sizes	.5		1.5
Max#N's	0		
Salt Concentration	50		
DNA Concentration			50
Self Complementarity			8
3′ Self Complementarity			3

Fig 2.2: USDA-ARS, Albany Server 1 Page of BatchPrimer3 Gateway.

2.1.2 In Vitro Experiment

This section concerns the processing of DNA samples and the optimisation of PCR reaction conditions.

2.1.2.1 DNA Sample Collection from Healthy Individual

A good amount of DNA sample was needed to run this project. The PCR reaction required DNA sample. Samples from the university compound were obtained from people of various ages, mainly from the students and the teachers. Samples were

collected via gargling of saline water. Saline water was made from 0.9 g NaCl, and 100 mL DH2O, and samples were obtained in the falcon tubes.

2.1.2.1.1 DNA Extraction from Saline Water

By pipetting with pipettes, 1500 ml of the sample collected was inserted into the eppendorf tubes. To obtain a good pellet, the Eppendorf tubes were then placed in the centrifuge machine for centrifugation at 4500 rpm for 5minutes. A good amount of pellet is needed to increase the cell concentration. The supernatant was discarded after obtaining the pellet from the first centrifugation, and 1500 ml of the sample was taken again, and this process was repeated for 4 to 5 times to achieve a decent pellet.

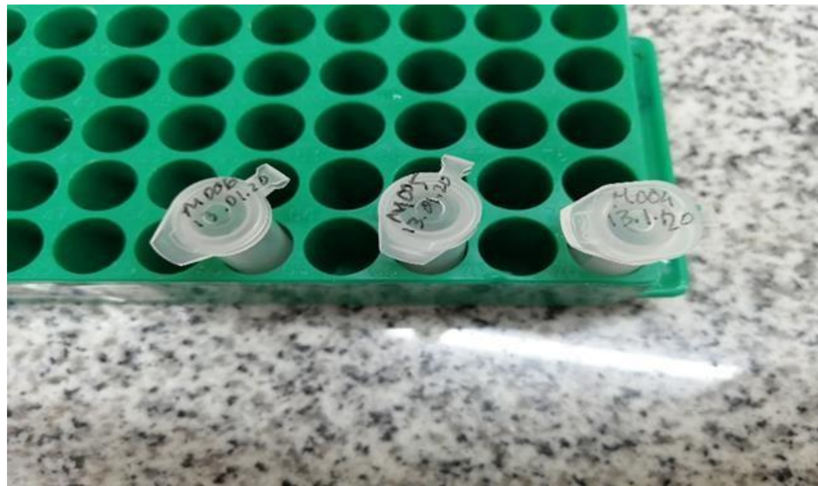


Fig 2.3: Eppendorf tubes for collecting extracted DNA

The pellet collected was then applied to the 500 µl lysis buffer [10 Mm Tris (pH 8.0), 10 mM EDTA, and 2.0% SDS], and 50 µl 10% SDS, followed by 5-10 µl 20 mg/ml K proteinase. Then the samples were incubated at 65ºC for 2 hours in the water bath until the tissue was fully dissolved. From each sample the DNA was then collected with an equivalent amount of phenol : chloroform: isoamyl alcohol

solution (25:24:1) and mixed gently by inverting the tubes for 3 min. The samples were then centrifuged with 10,000 g (4ºC) for 10 min and transferred the upper aqueous layer to a fresh, sterilized microcentrifuge tube. RNase A was applied (10 µl 10mg / ml) and the solution was incubated for 30 min at 37ºC. Chloroform: isoamyl alcohol solution was added in equivalent amounts and centrifuged again for 10 min with 10,000 g (4ºC). Chloroform: isoamyl alcohol solution is used to extract the proteins from nucleic acid preparations. The upper aqueous layer was moved to a sterilized microcentrifuge pipeline, and the chilled isopropanol volume was doubled; along with the one-tenth volume of 3 M sodium acetate, and chilled at -20ºC for 1hour for precipitation. The sample was centrifuged for 10 min at 10,000 g (4ºC) after 1 hour. 250 µl of 70% ethanol was applied after decanting the supernatant, and the pellet was dissolved; the mixture was centrifuged at 10,000 g for 10 min, and the supernatant was gently decanted. The pellet was air-dried and the dried pellet was resuspended in 30µL of nuclease-free water.

2.1.2.1.2 Confirmation of the Presence of DNA through Agarose Gel Electrophoresis

The pellets extracted were then placed in agarose gel of 0.8% to ensure the presence of DNA. 2 µL of the loading dye and 6 µL of the DNA sample and a total of 8 µL of the agarose gel was prepared. The sample was visualized under UV light, after conducting the gel electrophoresis for up to 30 minutes. 2 µL ladder of 100 kb was used.

2.1.2.2 Optimization of PCR Reaction Condition

PCR sometimes requires reaction conditions to be optimized to achieve a successful result. There would always be an outer band for outer primer, from which the PCR

reaction is recognized. Annealing temperature and concentration of the primer was continuously changed until the condition of PCR was optimised.

2.1.2.2.1 First Trial of Polymerase Chain Reaction

The following steps were followed to accomplish the first trial of Polymerase chain reaction-

Five autoclaved PCR tubes were labeled. There were two sets of primers. Each of them was ran at three different temperatures: 60ºC, 61ºC, and 62ºC. These temperatures were chosen based on the optimal temperature of primers during primer designing. Annealing temperature should be 5ºC bellow the T_m of our designed primer. PCR master mixture was prepared according to table 2.1. Each of the components were placed in sequence combinedly in the same order as shown here. Four PCR tubes contained the master mixture along with the DNA template.
Only one tube didn't contain any DNA template. This was our control to check whether the polymerase chain reaction is working.

10X DreamTaq Reaction Buffer	2.5
10 µM dNTP mix	.5
10µM Forward Outer Primer	.5
10 µM Forward Inner Primer	.5
10 µM Reverse Outer Primer	.5
10 µM Reverse Inner Primer	.5
DreamTaq DNA Polymerase	.2
PCR H_2O	16.8
Template DNA	3.0

Total	25

Table 2.2: Recipe for the first trial of PCR reactions. (All the volumes are taken in µL)

The PCR tubes were placed in the middle portion of the thermal cycler where the temperature 60ºC, 61ºC, 62ºC were generated. The polymerase chain reaction was started. The reaction was taken place for #32 cycles. The PCR products were primed for 60 minutes in 2% agarose gel under 90 voltage-current after the completion of the reaction process. Under UV radiation, the DNA bands in the agarose gel are visualized.

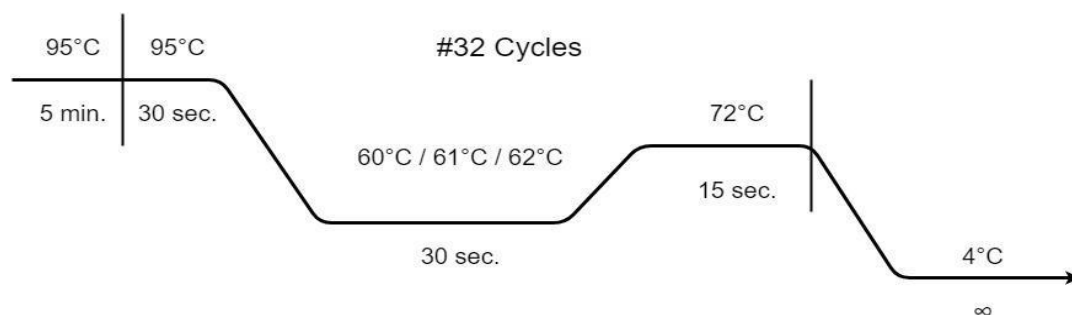


Fig 2.4: Conditions of the thermal cycling for the first trial of PCR reaction condition optimization

2.1.2.2.2 Fixing the Polymerase Chain Reaction Conditions

The following steps were followed to fix the PCR conditions-

Five autoclaved PCR tubes were labelled. Each tube contained the master mixture according to table 2.1 along with the DNA template except one tube. One tube contained all of the components except the DNA template. At first, the tubes were placed in the thermal cycler for #32 cycles at annealing temperature followed by Fig 2.4.

These condition didn't yield good PCR products. The PCR conditions were then fixed again.

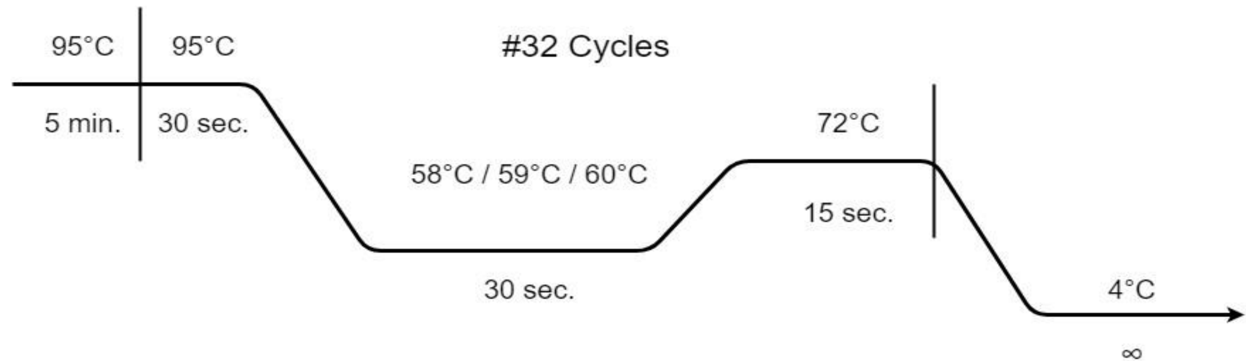


Fig 2.5: The annealing temperature was set at 58ºC, 59ºC, 60ºC the PCR condition

2.1.2.2.3 Final Optimization of the PCR Condition

We all know about the COVID-19 pandemic situation and suddenly our university was shut down because of this pandemic. So we couldn't be able to complete the final optimization of the PCR condition.

2.1.3 Data Analysis

2.1.3.1 SNP Genotyping

SNP genotyping is the calculation of genetic variability between members of a group with single nucleotide polymorphisms (SNPs). It is a type of genotyping, which measures genetic variance more broadly. In this project, Tetra Primer based ARMS PCR was applied to detect SNPs.Two sets of primers were designed for this purpose. The four primers were used to amplify the genotype for SNP rs1799853 (C > T) using Tetra-primer based ARMS PCR. Optimized PCR condition was observed to determine whether an individual is homozygous, homozygous, or heterozygous of

the wild type. Yet owing to this pandemic condition, we could not take our experiment further to this phase of genotyping.

2.1.3.2 Validation by Sample Sequencing

This phase of methodology is the further stage of SNP genotyping. After observing wild type homozygous or mutant homozygous or heterozygous in genotyping method, it is supposed to do sample sequencing to validate the genotyping result.

But again unfortunately we couldn’t make it possible to do sample sequencing due to the pandemic situation.

CHAPTER 3
RESULTS

Result

3.1 DNA Sequence of *Homo sapiens* CYP2C9 gene

From the NCBI GeneBank entry, the key attributes of the *Homo sapiens* CYP2C9 gene have been derived and outlined in **Table 3.1**.

Homo sapiens CYP2C9 Gene	
Official Symbol	CYP2C9
Official Full Name	cytochrome P450 family 2 subfamily C member 9 (provided by HGNC)
Accession code	NC_000010.11
Gene ID	1559
Gene Type	Protein Coding
RefSeq Status	Reviewed
Organism	*Homo sapiens*
Location	10q23.33
Length of gene sequence	51434 bp
Region of the gene sequence	94938658..94990091
Total Exon Count	9
Exon Position	1...193, 194…356, 357…506, 507…667, 668…844, 845…986, 987…1174, 1175…1316, 1317…2561
Total transcript variants	1
Size of the transcript	2561 bp
Length of the protein	490aa
Transcript ID	NM_000771.4

RefSeq Protein ID	NP_000762.2

Table 3.1 : The key attributes of the *Homo sapiens* CYP2C9 gene

3.2 Designing Tetra Primers for ARMS PCR Technique

5′ - AGCATTGAGGACCGTGTTCAAG - 3′ , this is the 5′ flanking sequence of the CYP2C9 gene, which is the search result in dbSNP from NCBI gateway (**Table 3.2**).

AGCATTGAGGAC[C/T]GTGTTCAAG

Rs	1799853
Varient type	SNV
Alleles	C>T
Chromosome	10:94942290 (GRCh38) 10:96702047 (GRCh37)
Gene	CYP2C9
Functional Consequence	coding_sequence_variant,missense_variant

Table 3.2: Search result of CYP2C9 gene in dbSNP from NCBI gateway indicating the flanking sequence

Here is a figure of the word document containing CYP2C9 gene sequence, highlighted with important portions and positions.

TTTTCAGCAATGGAAAGAAATGGAAGGAGATCCGGCGTTTCTCCCTCAT

GACGCTGCGGAATTTTGGGATGGGGAAGAGGAGCATTGAGGACCGTGT

TCAAGAGGAAGCCCGCTGCCTTGTGGAGGAGTTGAGAAAAACCAAGGG

TGGGTGACCCTACTCCATATCACTGACCTTACTGGACTACTATCTTCTCT
ACTGA CATTCTTGGA AACATTTCAG GGGTGGCCAT ATCTTTCATT

Word document containing CYP2C9 gene sequence, highlighted with important portions and positions. 5′ flanking sequence, intermediate position and SNP locus is highlighted with red, yellow and blue respectively.

Two sets of primers were designed, Forward inner, forward outer and Reverse inner, reverse outer primer.

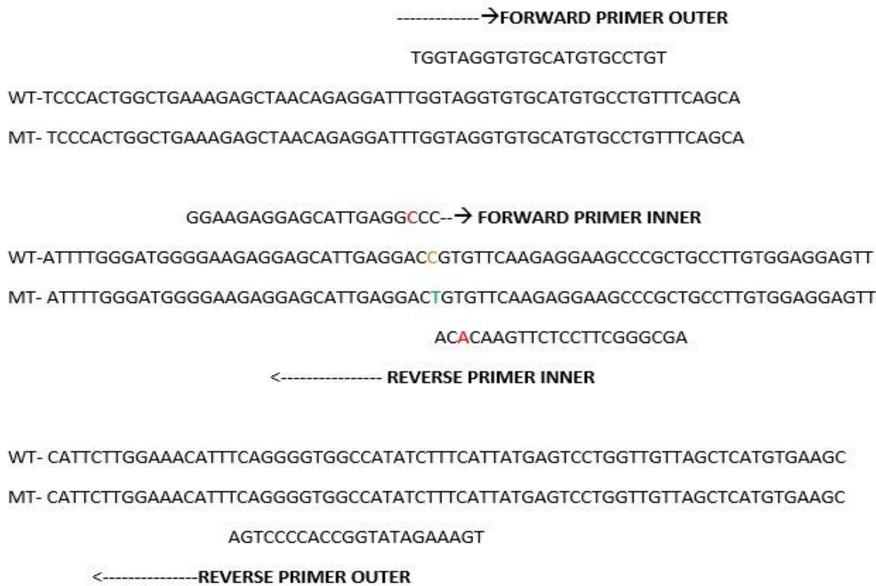


Fig 3.1: Two sets of primers for ARMS PCR technique

Fig 3.2 shows important parameters for these four primers. Forward outer and Reverse outer primer is of 22 bp long and Forward inner primer is 21 bp long and reverse inner primer is 20 bp long.

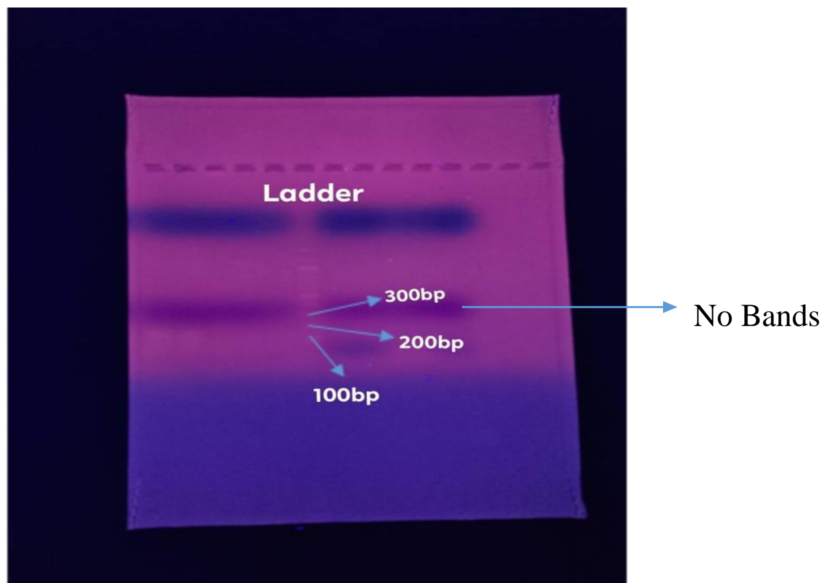
Index	Seq ID	Count	Primer_type	Orientation	Start	Length	Tm	GC%	Any compl	3' compl	Q Score	SNP	Pos
1	gnl|dbSNP	1	Outer primer	FORWARD	422	22	66.01	54.55	4	0			
2	gnl|dbSNP	1	Outer primer	REVERSE	838	22	65.83	50	6	1			
3	gnl|dbSNP	1	Inner primer	FORWARD	673	21	66.59	61.9	4	3	91.82	C	693
4	gnl|dbSNP	2	Inner primer	REVERSE	714	20	66.83	60	4	1	88.83	T	693

Fig 3.2: Important parameters used for designing the primers

3.3 In vitro Optimization Result of PCR Condition

3.3.1 First Trial of Optimization

At the very first step of optimization of the PCR condition, 60ºC temperature was used as annealing temperature. 100 bp length of ladder was used to measure the approximate size of the PCR product in the gel.



No Bands

Fig 3.3: First trial of PCR The annealing step was continued for 30 seconds and PCR was run in 2% agarose gel electrophoresis for 60 minutes under 80V of electricity. There was no bands for this annealing temp.

3.3.2 Second Trial of Optimization

As at the first trial of optimization, 60ºC annealing temperature was used, at the second trial of optimization the annealing temperature was decreased to 59º C . If the annealing temperature is too high, there is a chance that primer will not bind to the DNA template.

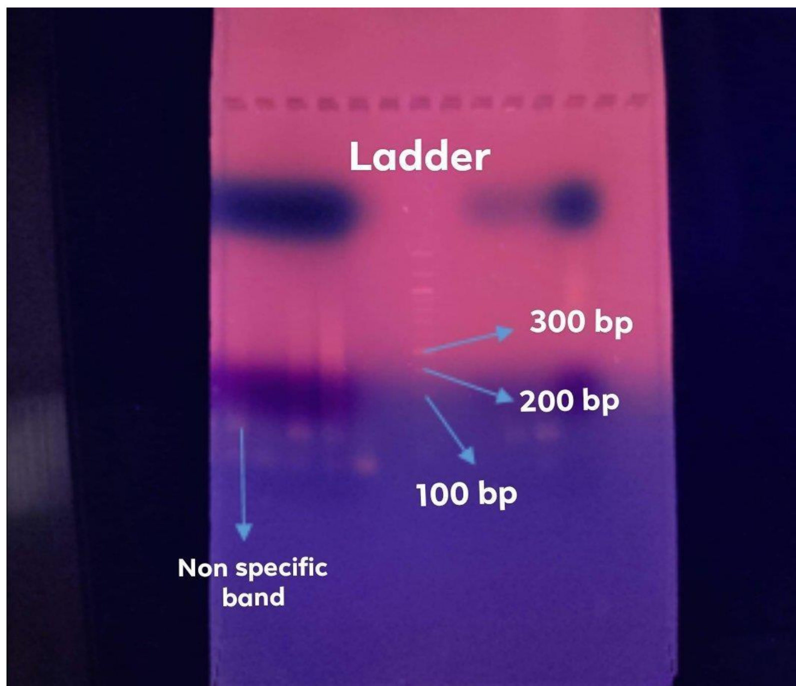


Fig 3.4: Second trial of PCR optimization condition.

This annealing step was also continued for 30 seconds. At this trial step, non-specific bands were found which were wild type homozygous.

3.3.3 Third Trial of Optimization

From the second trial, non specific bands were found. Temperature was again decreased to 58ºC at third trial of optimization.

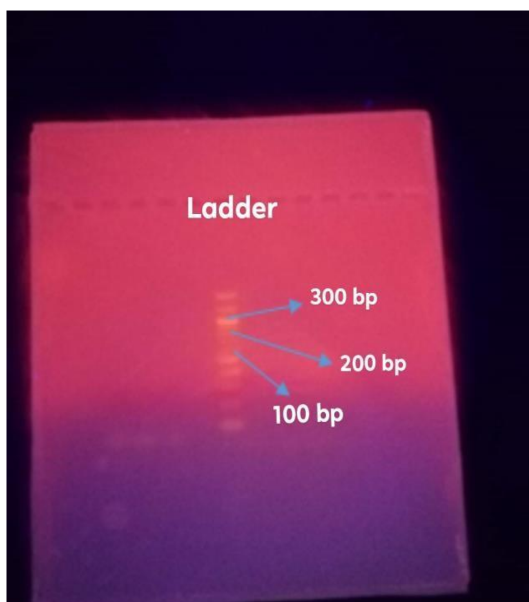


Fig 3.5: Third trial of PCR condition optimization.

Annealing time was 30 seconds and extension time was extended from 15 seconds to 30 seconds. No bands were found at annealing temperature 58ºC.

CHAPTER 4
DISCUSSION

Discussion

4.1 Discussion on Overall of the Project

Pharmacogenetics is the science of understanding how hereditary changeability influences the results of the medicate treatment (Eum *et al.*, 2016). Pharmacogenetics centers on the impact of a single gene on medicate reaction. One of the pharmacogenetics goals is to correlate variation in the response to genetic variation in drugs. In humans, the DNA sequences are 99.9% the same. That is, 0.1% of human DNA sequence varies according to person. Scientists see this genetic variation as leading to discrepancies in how people react to drugs. Scientists have noted that many variations in DNA are single nucleotide replacements, too known as Single Nucleotide Polymorphism (SNP) (Chasman *et al.*, 2004). A SNP is a modification of 1 nucleotide or base-pair within a DNA codon. Depending on its position, an SNP can alter how a gene is transcribed or the sequence of amino acids for the protein being produced, eventually causing a change in the protein 's activity. A SNP in the DNA coding region (cSNP) can or may not allow amino acid substitutions to form in the protein. When there is an amino acid substitution, the protein produced can have a different shape or tertiary structure and may have a major impact on the ability of that protein to exert its biological effect (Lieberman, 2009). So scientists started to focus on identifying clinically significant SNPs to regulate people's variation in drug response. There are various SNP genotyping methods described in **Section 1.4.6.2** to detect clinically significant SNPs. We tried to genotype one of the most important SNPs in the drug-metabolizing enzyme CYP2C9. Because of this pandemic situation with COVID-19, we could not finish our experimental work but at least we developed a method of genotyping using the ARMS PCR technique.

4.2 Importance of CYP2C9*2 (rs1799853)

Cytochrome P450 2C9 is an important drug-metabolizing enzyme. It is involved in the metabolism of many drugs, including non-steroidal anti-inflammatory drugs, losartan, tolbutamide, warfarin, phenytoin, or carbamazepine. Genetic polymorphism has been found in the gene encoding for CYP2C9 protein which gives rise to significant differences in drug disposition interindividual variation. As well as the most common CYP2C9*I allele (wild type), polymorphism of 430 C >T single nucleotide in exon 3 and 1075 A > C polymorphism of a single nucleotide in exon 7 is the most common polymorphisms causing the exchange of amino acids i.e. Cys (CYP2C9*2) allele) and Ile359Leu (allele CYP2C9*3), respectively. Individuals expressing the defect alleles (poor metabolizers) are more sensitive to adverse events upon CYP2C9 metabolized drug administration. Wild type is found in about two-thirds of individuals in the Caucasian population, whereas one-third express heterozygous genotype CYP2C9*1/2 or CYP2C9*1/3. Prevalence of subjects having two alleles with low activity i.e. in these populations, CYP2C9*2/2, CYP2C9*2/3, and CYP2C9*3/3 accounted for less than 2.5%. In contrast, genetic polymorphism CYP2C9 is unusual in African-American and Asian populations. Individuals with at least one CYP2C9*2 or CYP2C9*3 defect allele exhibit reduced biotransformation of CYP2C9-metabolized drug (Lazar *et al.*, 2004) The CYP2C9 * 2 allele is the result of a C > T transition to position 430 of the CYP2C9 gene, resulting in an Argto-Cys substitution at the CYP2C9 molecule residue 144 (Lazar *et al.*, 2004). The two most characterized variant alleles are CYP2C9 * 2 (C > T, Arg144Cys, rs1799853) and CYP2C9 * 3, both associated with reduced enzyme activity and impaired phenotypes of the drug metabolism (Pratt *et al.*, 2018) That is why we attempted to genotype the most important SNP of CYP2C9 enzyme, CYP2C9*2 (rs1799853). For this purpose, we choose the Tetra primer ARMS PCR technique which was easy to perform and cost-effective.

4.3 Attempting DNA Extraction from Saline Water

At first, we tried to collect DNA samples from individuals with buccal swabs and we were supposed to extract DNA from buccal swabs. But there were problems regarding extracting DNA from buccal swabs. Achieving pellets from buccal swabs was quite difficult and also time-consuming as the swabs had to kept in lysis buffer in a small beaker within a shaker for minimum of 24 hours to extract the pellet. That's why we decided to collect DNA from individuals with saline water which was less time-consuming in extracting DNA. We extracted DNA by Phenol chloroform extraction method. The first step of this extraction method includes proteinase K and lysis buffer which is composed of Tris, EDTA, and SDS. Lysis buffer for fast retrieval of DNA is used to separate the cells. Tris is used for regulating the pH and osmolarity of lysate in the lysis buffer. EDTA precludes DNA loss in the lysis buffer. Its primary function is to round up free zinc, magnesium, and calcium in the buffer, thereby avoiding the oxidation of DNA by other mechanisms involving certain metals. The cell membranes shall be solubilized by SDS in the lysis buffer. SDS also denatures the bulk of cell proteins. Proteinase K is widely used for digesting protein and extracting contaminants from nucleic acid preparations. The DNA samples were incubated in a water bath at 65ºC for 2 hours to dissolve them completely. Phenol: chloroform: isoamyl alcohol is often used to remove the proteins from nucleic acid preparations. In the case of genomic DNA, the sample should be handled gently. RNase A is used to remove RNA from the samples. Chloroform: isoamyl alcohol solution is used to extract the proteins from nucleic acid preparations. Usage of chilled alcohol raises the amount of DNA precipitation. Pellet shouldn't be air-dried for a long period of time. If the pellet is dried too much, it will be difficult to dissolve the DNA in any kind of solvent. Pellet is resuspending in nuclease-free water for a better result in PCR.

4.4 Optimizing the Condition of PCR

The polymerase chain reaction (PCR) is a commonly used tool for DNA amplification in molecular biology, and various PCR optimization techniques developed by molecular biologists to improve PCR performance and minimize failure. Under optimum conditions, failure to amplify can lead to multiple undefined and unwanted products being generated, even to the exclusion of the desired product. On the other end no product can be manufactured to the extreme. A typical response here is to vary one or more of many parameters which are known to contribute to the fidelity of the primers and extensions of the primers. Mg++ concentrations, buffer pH, and cycling conditions are on the list of optimisation variables. Regarding the latter, the most important temperature is annealing. The situation is even more complicated because some of the variables are fairly interdependent. For instance, because the dNTPs are direct chelate a proportional number of Mg++ ions, and an increase in dNTP concentration free Mg++ concentration available to influence the functioning of polymerase (Roux, 1995). The PCR Template DNA is one of the key ingredients for a successful PCR reaction. It is as important as the DNA primers, Taq polymerase DNA, dNTPs, and buffer reaction PCR. Inappropriate concentration for the PCR reaction template DNA results in reaction failure, as well as a higher DNA template concentration in PCR, resulting in false-positive results [43]. Too much template DNA can inhibit PCR by binding with all the primers. The Taq DNA polymerase can be activated only if the template DNA is recognized as a substratum for the PCR reaction. Although the polymerization cannot start until the primer binds to the DNA of the template (Schrader *et al.*, 2012) Taq DNA polymerase has relatively high thermostability, with an approximate half-life of 40 min at 95 ° C. It incorporates nucleotides at 70 ° C at a rate of about 60 bases per second and can amplify lengths of about 5 kb, so it is suitable without special requirements for standard PCR. The enzyme amounts with difficult templates might need to be adjusted. For example, increasing the amount of DNA polymerase may improve PCR yields when inhibitors are present in the DNA sample. Non-specific PCR products may however

appear at higher concentrations of enzymes. The PCR primers are synthetic oligonucleotides of about 15–30 bases of DNA. PCR primers are designed to bind sequences that flank the region of interest in the template DNA (via sequence complementarity). During PCR polymerase DNA extends the primers from its 3′ ends. In addition to the sequence homology, primers have to be carefully designed for PCR amplification specificity in other ways. First, the priming sequences should have melting temperatures (Tm) in the 55–70°C range, with the two primers' Tm within 5°C of each other. dNTPs are also crucial in PCR. dNTPs consist of four basic nucleotides — dATP, dCTP, dGTP, and dTTP — as new DNA strand building blocks. Typically these four nucleotides are added in equimolar amounts to the PCR reaction for optimal base incorporation (PCR Setup-Six Critical Components to Consider, 2020). The dNTPs can not be binding to the amplification site until the PCR template DNA has been recognized by the Taq as a reaction substratum (Schrader *et al.*, 2012). PCR is performed in a buffer that provides an appropriate chemical environment for the DNA polymerase activity. In general, the buffer pH is between 8.0 and 9.5 and is often stabilized by Tris-HCl. The presence in water of nucleases such as DNase and RNase can degrade valuable molecular samples, and even ruin experiments. To avoid loss of samples of DNA and RNA, it is essential to use highly pure, nuclease-free water in PCR. Thermal cycling is an essential aspect of PCR, meaning that the reaction is exposed to a series of precisely defined temperatures. The reaction mixture heats up to 95 ° C at first. This causes the melting or denature of the hydrogen bonds between the strands of the template DNA molecules. This produces two molecules of single-stranded DNA from each double helix. The mixture is refrigerated to 45-65 ° C in the step annealing. The exact temperature depends on the sequence of the priming used and the experiment 's objectives. This allows double-stranded helices to be formed between complementary DNA molecules, including annealing the primers to the template (Libretexts, 2020). A standard PCR protocol's annealing temperature is either 55 ° C, or 60 ° C. The temperature chosen depends on the primers' strand-melting temperature, and the specificity desired (Erjavec, 2019). Our annealing temperature for PCR condition was primarily 60ºC, 61ºC, 62ºC according to our primers' Tm.

We were supposed to optimize the annealing temperature. At first, trial of optimizing the PCR condition, we set our annealing temperature at 60ºC, and the annealing time was 30 seconds. But at this annealing temperature no bands were found (**Fig 3.4**). According to our primers' Tm, it was supposed to get bands. Annealing time at the first trial was 30 seconds. An approx. time to anneal is 40 seconds up to 1 minute for oligonucleotide diffusion and template binding is usually sufficient. Increasing the time usually doesn't have much effect because the template was binding and saturated by oligonucleotides. In contrast, the temperature of the annealing follows an optimal curve. If it is too high or too low this temperature has a strong negative effect on PCR efficiency. The reason behind not getting any bands is probably the PCR components weren't mixed properly. Or somehow any of the components got contaminated. If the DNA sample is somehow contaminated, then also no bands will be found. At the second trial of optimization, we set the annealing temperature at 59ºC and this time non-specific bands were found (**Fig 3.5**). The Annealing temperature was 30 seconds again this time. Non-specific bands were found because primer could have dissociated and rejoined together causing primer dimer. The water purity also had to be checked as it might be contaminated during pipetting. To avoid non-specific bands, PCR cycles should be kept in less than 30. Because each cycle increases the risk of error. Our PCR cycle was 32 and we couldn't optimize the cycle because of this COIV-19 pandemic. At third and last trial of optimization we set the annealing temperature at 58ºC and extended the extension time from 15 seconds to 30 seconds. The next step in PCR after annealing of the primers is to extend the 3′ end of the primers, complementing the template. In this step, the DNA polymerase activity of 5′ to 3′ incorporates dNTPs and synthesizes the strands of the daughter. There is no problem with the extension time. But at the third trial no bands were found (**Fig 3.6**). Maybe we had to raise the annealing temperature to 62ºC to get bands. Our optimization step was incomplete due to the pandemic situation.

4.5 *In silico* Primer Designing

The first target of this project was to design Tetra primers to detect SNP in the ARMS PCR technique. WASP (Web-based Allele-Specific Primer) theory was implemented to design the tetra primers. The result is shown in **Fig 3.3.** In general, the optimum length of primers for PCR is 18-22 bp (Abd-Elsalam, 2003). In this project, our designed tetra primers were in this length. For an ideal PCR condition, GC% should be between 40% to 60% (Abd-Elsalam, 2003). Primers with 40% to 60% GC content ensure stable binding of primer with the template. In our designed primer, GC content was in this range.

Future Perspective

We have developed a SNP genotyping tool in this research project whether an individual is wild type homozygous, homozygous mutant, or heterozygous mutant. Because of this pandemic condition with COVID-19, we did not carry out our experiment anymore. But if we could continue our experiment, we might genotype 100 samples and if we got 25 heterozygous mutants, for example, from that genotyping. This means in the population of Bangladesh, in this locus of this 25% SNP was heterozygous, which means that some enzymes did not work efficiently for this SNP and if this 25% were given this drug, they could not properly metabolize this drug. If he/she were unable to metabolize this drug, the concentration of this drug in the blood would increase and the half-life of this drug would increase. So, this 25% shouldnot be given this drug and if given, should be given at reduced doses. If mutant homozygous, the drug shouldn't be given, and if mutant heterozygous, the drug should be given in reduced doses. This developed genotyping method has immense application in personalized medicine. To know the appropriate doses of a medicine for an individual, DNA will be collected from that individual and extracted and then the sample will be run through this method. If the output is wild type homozygous, then the individual should be given the drug at normal doses. If the output is mutant homozygous, the individual shouldn't be given the drug, and if the individual is mutant heterozygous, he should be given the drug at reduced doses.

Conclusion

Pharmacology is drug research and its impact on living organisms. It is the study of the response of the body to the drugs. In this research project, we have chosen drugs specifically for sulfonylureas. We created an SNP genotyping method to determine whether an individual is homozygous or heterozygous or homozygous of the wild type or mutant. Drugs to sulfonylureas are metabolized by the enzyme CYP2C9. We tried to figure out the SNP 's effect on the enzyme 's function. We selected clinically significant SNP (rs1799853) based on its heterozygosity. The majority of SNPs have no health or growth impacts. However, some of those genetic variations have proven to be very significant in human health research. Studies have identified SNPs that can help predict an individual's reaction to some medicines, vulnerability to environmental factors such as pollutants, and the likelihood of contracting different diseases. We developed a Tetra Primer ARMS PCR technique to detect SNP at a particular locus. This technique is very cost-effective and easy to execute. Yet the other side of this approach is that it's a method that takes time. This method has been developed for a routine diagnostic checkup as some SNPs have severe effects on drug response. Using this Tetra Primer ARMS PCR technique, individuals will diagnose their SNPs and know their correct doses of the drug.

CHAPTER 5

BIBLIOGRAPHY

Bibliography

1. Abd-Elsalam, K. A. (2003). Bioinformatic tools and guideline for PCR primer design. african Journal of biotechnology, 2(5), 91-95.
2. Ahmadian, A., Gharizadeh, B., Gustafsson, A. C., Sterky, F., Nyrén, P., Uhlén, M., & Lundeberg, J. (2000). Single-nucleotide polymorphism analysis by pyrosequencing. Analytical biochemistry, 280(1), 103-110.
3. Aquilante, C. L. (2010). Sulfonylurea pharmacogenomics in Type 2 diabetes: the influence of drug target and diabetes risk polymorphisms. Expert review of cardiovascular therapy, 8(3), 359-372.
4. Aquilante, C. L. (2010). Sulfonylurea pharmacogenomics in Type 2 diabetes: the influence of drug target and diabetes risk polymorphisms. Expert review of cardiovascular therapy, 8(3), 359-372.
5. Aquilante, C. L., & Lam, Y. F. (2013). The Role of Pharmacogenomics in Diabetes, HIV Infection, and Pain Management. In Pharmacogenomics (pp. 247271). Academic Press.
6. Aylor, D. L., Valdar, W., Foulds-Mathes, W., Buus, R. J., Verdugo, R. A., Baric, R. S., ... &Gralinski, L. E. (2011). Genetic analysis of complex traits in the emerging Collaborative Cross. Genome research, 21(8), 1213-1222.
7. Becker, M. L., Visser, L. E., Trienekens, P. H., Hofman, A., Van Schaik, R. H. N., & Stricker, B. (2008). Cytochrome P450 2C9* 2 and* 3 polymorphisms and the dose and effect of sulfonylurea in type II diabetes mellitus. Clinical Pharmacology & Therapeutics, 83(2), 288-292.
8. Bishop, J. R. (2009). Pharmacogenetics. ScienceDirect.com | Science, health and medical journals, full text articles and books. https://www.sciencedirect.com/topics/medicine-anddentistry/pharmacogenetics
9. Chasman, D. I., Posada, D., Subrahmanyan, L., Cook, N. R., Stanton Jr, V. P., &Ridker, P. M. (2004). Pharmacogenetic study of statin therapy and cholesterol reduction. Jama, 291(23), 2821-2827.

10. Davis, S. N. (2004). The role of glimepiride in the effective management of type 2 diabetes. Journal of Diabetes and its Complications, 18(6), 367-376.
11. DeFronzo, R. A. (1999). Pharmacologic therapy for type 2 diabetes mellitus. Annals of internal medicine, 131(4), 281-303.
12. Drug absorption - Clinical pharmacology - MSD manual professional edition. (2019, May). MSD Manual Professional Edition. https://www.msdmanuals.com/professional/clinicalpharmacology/pharmacokinetic s/drug-absorption.
13. Erjavec, M. S. (2019). Annealing Temperature of 55° C and Specificity of Primer Binding in PCR Reactions. In Synthetic Biology-New Interdisciplinary Science. IntechOpen.
14. Eum, S., Lee, A. M., & Bishop, J. R. (2016). Pharmacogenetic tests for antipsychotic medications: clinical implications and considerations. Dialogues in clinical neuroscience, 18(3), 323.
15. Fan, J. B., Oliphant, A., Shen, R., Kermani, B. G., Garcia, F., Gunderson, K. L., ... &Galver, L. (2003, January). Highly parallel SNP genotyping. In Cold Spring Harbor symposia on quantitative biology (Vol. 68, pp. 69-78). Cold Spring Harbor Laboratory Press.
16. Fodor, A., Cozma, A., Suharoschi, R., Sitar-Taut, A., & Roman, G. (2019). Clinical and genetic predictors of diabetes drug's response. Drug metabolism reviews, 51(4), 408-427.
17. Furman, B. L. (2007). Sulfonylurea. ScienceDirect.com | Science, health and medical journals, full text articles and books. https://www.sciencedirect.com/topics/agriculturaland-biologicalsciences/sulfonylurea.
18. Ghosh, M. (2007). Fundamentals of experimental pharmacology. Indian Journal of Pharmacology, 39(4), 216-216.
19. Harvard Health Publishing. (2019, September 24). Type 2 diabetes mellitus. Harvard Health. https://www.health.harvard.edu/a_to_z/type-2-diabetes-mellitus-ato-z
20. Jehan, T., &Lakhanpaul, S. (2006). Single nucleotide polymorphism (SNP)–methods and applications in plant genetics: a review.

21. Kirchheiner, J. Impact of CYP2C9 and CYP2C19 polymorphisms on tolbutamide kinetics and the insulin and glucose response in healthy volunteers. Pharmacogenetics 12, 101–109 (2002)

22. Kirchheiner, J. Impact of CYP2C9 amino acid polymorphisms on glyburide kinetics and on the insulin and glucose response in healthyvolunteers. Clin. Pharmacol. Ther. 71, 286–296 (2002)

23. Kwok P-Y & Chen X (2003) Detection of single nucleotide polymorphisms. Curr Issues Mol Biol 5:43–60.

24. Kwok, P. Y. (2001). Methods for genotyping single nucleotide polymorphisms. Annual review of genomics and human genetics, 2(1), 235-258.

25. Lazar, A., Tomalik-Scharte, T., & Fuhr, U. (2004). Applications of genotyping and phenotyping for clinically-relevant polymorphisms of drug metabolizing enzymes and drug transporters. Handbook of Analytical Separation, 5, 321-354.

26. Lazar, A., Tomalik-Scharte, T., & Fuhr, U. (2004). Applications of genotyping and phenotyping for clinically-relevant polymorphisms of drug metabolizing enzymes and drug transporters. Handbook of Analytical Separation, 5, 321-354.

27. Libretexts. (2020, July 14). 8.2: Isolating or Detecting a Specific Sequence by PCR. Retrieved July 15, 2020, from https://bio.libretexts.org/Bookshelves/Genetics/Book:_Online_Open_Genetics_(Nickle_and_Barrette-

28. Lieberman M, Marks AD. Chapter 12. Structure of the nucleic acids. In: Mark's Basic Medical Biochemistry. A Clinical Approach. 3rd ed. Lieberman M, Marks AD eds. Wolters Kluwer-Lippincott Williams & Wilkins. Philadelphia, PA. 2009:199-215.

29. Little, S. (1995). Amplification-refractory mutation system (ARMS) analysis of point mutations. Current protocols in human genetics, 7(1), 9-8.

30. Luikart, G., Allendorf, F. W., Cornuet, J. M., & Sherwin, W. B. (1998). Distortion of allele frequency distributions provides a test for recent population bottlenecks. Journal of heredity, 89(3), 238-247.

31. McCarroll, S. A., Kuruvilla, F. G., Korn, J. M., Cawley, S., Nemesh, J., Wysoker, A., ... & Elliott, A. L. (2008). Integrated detection and populationgenetic analysis of SNPs and copy number variation. Nature genetics, 40(10), 1166.

32. Mcdonnell, A. M., & Dang, C. H. (2013). Basic review of the cytochrome p450 system. Journal of the advanced practitioner in oncology, 4(4), 263.

33. Meyer, U. A. (2000). Pharmacogenetics and adverse drug reactions. The Lancet, 356(9242), 1667-1671.

34. Miller, D., Brinkworth, M., & Iles, D. (2010). Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. Reproduction, 139(2), 287-301.

35. Mohiuddin, A. K. (2019). Diabetes fact: Bangladesh perspective. Community and Public Health Nursing, 39.

36. Muotri, A. R., Chu, V. T., Marchetto, M. C., Deng, W., Moran, J. V., & Gage, F. H. (2005). Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition. nature, 435(7044), 903-910.

37. Parvez, B., Vaglio, J., Rowan, S., Muhammad, R., Kucera, G., Stubblefield, T., ... & Darbar, D. (2012). Symptomatic response to antiarrhythmic drug therapy is modulated by a common single nucleotide polymorphism in atrial fibrillation. Journal of the American College of Cardiology, 60(6), 539-545.

38. PCR Setup-Six Critical Components to Consider. (n.d.). Retrieved July 15, 2020,fromhttps://www.thermofisher.com/us/en/home/life-science/cloning/cloning-learningcenter/invitrogen-school-of-molecular-biology/pcr-education/pcr-reagents-enzymes/pcrcomponent-considerations.html

39. Pharmacology archives. (n.d.). ExploreHealthCareers.org. https://explorehealthcareers.org/field/pharmacology/

40. Pratt, V. M., Del Tredici, A. L., Hachad, H., Ji, Y., Kalman, L. V., Scott, S. A., &Weck, K. E. (2018). Recommendations for clinical CYP2C19 genotyping allele selection: a report of the association for molecular pathology. The Journal of Molecular Diagnostics, 20(3), 269-276.

41. Roux, K. H. (1995). Optimization and troubleshooting in PCR. Genome Research, 4(5), S185-S194.

42. Schrader, C., Schielke, A., Ellerbroek, L., &Johne, R. (2012). PCR inhibitors–occurrence, properties and removal. Journal of applied microbiology, 113(5), 1014-1026.

43. Seltzer HS: Drug-induced hypoglycemia. A review based on 473 cases. Diabetes 21:955966, 1982

44. Simmons,T. E., Joffe, D L. (Nov 16, 2019) BSPharm Sulfonylureas' Association with Major Adverse Effects

45. Vannucchi, A. M., Pancrazzi, A., Bogani, C., Antonioli, E., & Guglielmelli, P. (2006). A quantitative assay for JAK2 V617F mutation in myeloproliferative disorders by ARMSPCR and capillary electrophoresis. Leukemia, 20(6), 10551060.

46. What are sulfonylureas? (2020, April 13). Latest Medical News, Clinical Trials, Guidelines - Today on Medscape. https://www.medscape.com/answers/117853-6501/what-aresulfonylureas

47. Zanger, U. M., & Schwab, M. (2013). Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacology & therapeutics, 138(1), 103-141.