CHAPTER 5

CYP2C9 polymorphism

5.1 Review of Literature

Despite the health concern, there is an increasing trend of application of new formulations of pesticides in agriculture, which are more toxic than the earlier ones. These pesticides exert the toxic effect on target and non-target organisms including humans. The pesticides, entering the human system, are detoxified through enzymatic action to hydrophilic metabolites and are eliminated, a process called biotransformation. These reactions are divided into phase I and phase II reactions (Abass et al., 2009). Cytochrome P450 (CYP) mixed function oxygenases are mainly responsible for phase I conversion. The CYP genes constitute a multigene family of CYP super family. Based on structural homology the CYP superfamily is divided into families and subfamilies. At present there are 18 families and 44 subfamilies in humans covering 57 functional CYP genes (Abass et al., 2009). A single nucleotide exchange causes allelic variants leading to non-synonymous amino acid substitution, differing only at few residues in the coding region (Schwarz, 2003). Rosemary and Adithan (2007) reviewed that oxidative biotransformation of exogenous compounds (drugs, procarcinogens, alcohols) are catalyzed by enzymes belonging to the families CYP1, CYP2 and CYP3, while others are involved in the metabolism of endogenous compounds (fatty acids, prostaglandins, steroids). CYP3A4 is the most abundant of CYP450 content in human liver (~28%) followed by CYP2C family (18%), CYP1A2 (~12%), CYP2E1 (7%), CYP2A6 (4%), CYP2D6 (1.5%) and CYP2B6 (0.2%). More than half of the drugs currently prescribed are metabolized by CYP3A (51%) followed by CYP2D6 (24%) and CYP2C (~20%) (Rosemary and Adithan, 2007).

Human cytochrome CYP2C9 gene is located on chromosome 10q24 and has an approximate length of 55 kb (Lee et al., 2002). In humans, this major enzyme of the

CYP2C subfamily constitutes approximately 20% of the hepatic cytochrome P450 enzyme (Takahashi and Echizen, 2001). The biotransformation of a huge variety of xenobiotics including the organophosphates, chlorpyrifos and parathion are found to be associated with CYP2C9 (Daly, 2003; Foxenberg et al., 2007). Mitochondrial membrane transportation is affected by organophosphate pesticides (Nakagawa and Moore, 1999). It disturbs cytochrome P450 system in human liver (Kappers et al., 2001; Sams et al., 2003) which plays a crucial role in pesticide metabolism. CYP2C9 also metabolizes approximately 10% of important therapeutic drugs (Goldstein and de Morais, 1994; Miners and Birkett, 1998; Streetman et al., 2000). There are 67 variant alleles of CYP2C9 located in the coding region (Du et al., 2016; http://www.cypalleles.ki.se/cyp2c9.htm). CYP2C9*2 and CYP2C9*3 allelic variants are most common and are associated with decreased metabolism of respective substrates (Sullivan-Klose et al., 1996; Crespi and Miller, 1997; Niemi et al., 2002).

In all ethnic population, the allelic variants of CYP2C9 namely CYP2C9*1, CYP2C9*2 and CYP2C9*3 are frequently found (Lee et al., 2002). The most characterized alleles of CYP2C9 gene are the CYP2C9*2 situated in exon 3 and CYP2C9*3 situated in exon 7. The CYP2C9*2 and CYP2C9*3 allelic variants results from CYP2C9*1 by a nucleotide substitution of $C_{430}\rightarrow T$ located in exon 3, leading to amino acid substitution of arginine by cysteine at position 144 and $A_{1075}\rightarrow C$ located in exon 7 leading to amino acid substitution of isoleucine by leucine at position 359 (Yoon et al., 2001). Studies have shown that the frequency of CYP2C9*2 allele varies significantly among the populations of north Indians and south Indians (Jose et al., 2005; Sistonen et al., 2009; Rathore et al., 2010; Yadav et al., 2010; Singh et al., 2011), while CYP2C9*3 allele was found to be equally distributed among the Indian populations (Paul et al., 2011). Enzyme activity is significantly reduced in the individuals with the allelic variants CYP2C9*2 and

CYP2C9*3 and these individuals may not be able to metabolize the substrate efficiently which in turn may lead to toxicity (Miners, 2002). Substantial reduction in metabolic activity has been found to be associated with the allele CYP2C9*3, while intermediate reduction in enzyme activity has been found to be associated with CYP2C9*2 allele in comparison to CYP2C9*1 allele (Lee et al., 2002). Both the CYP2C9 and CYP2C19 participate in the oxidative metabolism of warfarin, an anticoagulant (Kaminsky et al., 1993), diclofenac and ibuprofen, naproxen, piroxicam, aceclofenac (non steroidal anti-inflammatory agents), sulfamethoxazole and dapsone (antibacterials) (Leemann et al., 1993; Barry et al., 1997; Winter et al., 2000), H2 antagonist (Omeprazole) (Karam et al., 1996; Yamazaki et al., 1997), diazepam (benzodiazepine) (Jung et al., 1997; McGinnity et al., 1999) phenytoin (antiepileptics), rosiglitazone, tolbutamide, glyburide, glibenclamide, glimepiride, glipizide, losartan, irbesartan, torsemide, tamoxifen, fluvastatin, fluoxetine, amitriptyline (Lee et al., 2002; García-Martín et al., 2006). In a population study, Sullivan-Klose et al. (1996) have also reported the association of CYP2C9 with the metabolism of tolbutamide and the homozygotes of Leu^{359} allelic variant of CYP2C9 were identified as poor metabolizers of tolbutamide. A constant equilibrium exists between the metabolic activation and detoxification of xenobiotics in human body. Inter-individual variation is responsible for the expression and characterization of the enzymes. Oxidative desulphuration of the organophosphate parathion, chlorpyrifos and diazinon are mediated by the cytochrome P450 (Neal, 1967; Butler and Murray, 1997; Mutch et al., 1999; Sams et al., 2000).

5.1.1 CYP2C9 gene polymorphism in pesticide metabolism

Human hepatocytes when cultured for 72 h in a serum free medium on collagen with inducers namely rifampicin, dexamethasone and phenobarbital, CYP2C9 was found to be

expressed along with CYP2C8 and CYP2C19 (Raucy et al., 2002), while only CYP2C9 was expressed at an appreciable level in untreated hepatocytes. Biotransformation of organophosphorothioate insecticides (chlorpyrifos and diazinon) were investigated in human liver microsomes as well as recombinant human cytochrome P450s (Sams et al., 2004). At low substrate concentration some of the cytochromes (eg cytochromes 2C9, 2D6 and 3A4) made a minor contribution to the biotransformation of chlorpyrifos and diazinon (Sams et al., 2004). Chlorpyrifos is toxic *in vivo* due to its bioactivation to chlorpyrifos-oxon to a more potent cholinesterase inhibitor. The oxidation reaction possibly proceeds via a phospho oxythiiran intermediate in which the oxon is generated by a desulfuration reaction or the parent compound is degraded by a dearylation reaction (Chambers, 1992). Abass et al. (2009) studied the inhibition ability of 18 pesticides on CYP1A1/2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 2A4 and showed that CYP1A1/2 was most inhibited by organophosphorus insecticides followed by CYP2C8, CYP2C9, CYP2D6, CYP2A4. Organophosphates differentially inhibited different cytochromes. Deltamethrin and fenvalerate was found to potentially inhibit CYP2D6. At least one cytochrome was found to be inhibited by most of the pesticides (Abass et al., 2009).

A study was conducted to explore the DNA damage in workers occupationally exposed to organophosphate pesticides. A significantly higher DNA damage was observed in workers with CYP2D6*3 PM (poor metabolizers) and PON1 (QQ and MM) genotypes indicating that these genotypes can modulate DNA damage by gene-environment interactions (Singh et al., 2011). Enzymes involved in the metabolism of pyrethroid pesticides were studied in rat and human hepatic microsomes. In presence or absence of NADPH, rat and human hepatic microsomes were incubated with pyrethroids bifenthrin, *S*-bioallethrin, bioresmethrin, β-cyfluthrin, cypermethrin, *cis*-permethrin, and *trans*-permethrin. Rat cytochrome P450 (P450) isoforms CYP1A1, CYP1A2, CYP2C6,

CYP2C11, CYP3A1 and CYP3A2 and the human p450 isoforms CYP2C8, CYP2C9, CYP2C19 and CYP3A4 showed activity towards several pyrethroids (Scollon et al., 2009). Scollon et al. also commented that variable detoxification of pyrethroids results from species-specific difference in metabolism and these must be considered when assessing the adverse health effect of pyrethroids.

Studies have also been conducted to see the effect of CYP2C9 gene polymorphism on colorectal cancer (Zhao et al., 2013). Recently, Zhao et al. (2013) showed that CYP2C9*2, CYP2C9*3 alleles were not found to be associated with colorectal cancer susceptibility. Studies on human lymphoblastoma cells exposed to chlorpyrifos have revealed that CYP1A2, 2B6, 2C9*1, 2C19 and 3A4 are responsible for dearylation and desulfuration of chlorpyrifos and the highest dearylation and desulfuration activities were displayed by CYP2C19 and 2B6 (Tang et al., 2001).

Glimepiride is a drug used to treat Type 2 diabetic patients and is metabolized by CYP2C9 (Langtry and Balfour, 1998; Elsby et al., 2012). Dutta et al. (2015) studied the association between CYP2C9 genetic polymorphism and advanced drug reaction of glimepiride. The result indicated that CYP2C9*3 polymorphism was statistically significant in individuals with adverse drug reaction. The CYP2C9*1 allele is predominant in the individuals with no adverse drug reaction, while none of the individuals showing either drug reaction or no drug reaction had CYP2C9*2 allele (Dutta et al., 2015).

For the analysis of mutations in CYP2C9 gene, several protocols are available (Wang et al., 1995; Sullivan-Klose et al., 1996; Nasu et al., 1997). The validity of those methods for the genotyping was determined in Swedish population by Yasar et al. (1999). Using the protocol described by Sullivan-Klose et al. (1996) and Nasu et al. (1997) the

frequency of CYP2C9*2 and CYP2C9*3 alleles was determined to be 0.107 and 0.074 for 430 Swedish population (Yasar et al., 1999). It has been reported that CYP2C9*2 and CYP2C9*3 polymorphisms cause a decrease in enzymatic activity and the individuals carrying these alleles have clinical implications (Crespi and Miller, 1997; Takahashi and Echizen, 2001; Schwarz, 2003). Impaired metabolic activity of the CYP2C9 is observed in affected individuals when CYP2C9*3 allele appears in homozygous condition (Schwarz, 2003). CYP2C9*3 allele has been shown to cause the largest reduction in the metabolic capacity for many substrates of CYP2C9 followed by intermediate reduction caused by CYP2C9*2 allele, while CYP2C9*1 is least reduced (Lee et al., 2002). Studies have shown that severe toxicity may be experienced by CYP2C9*3/CYP2C9*3 homozygotes while metabolizing warfarin and phenytoin. These individuals may not respond adequately to drugs, such as losartan and cyclophosphamide that may result in therapeutic failure (Yasar et al., 2002). Review of literatures reveals that CYP2C9 and other cytochrome P450 alleles can be used as a good DNA marker for genetic association studies with respect to the xenobiotic exposure.

The present study contemplates a population study of CYP2C9 polymorphism in the pesticide-exposed tea garden workers to see association between CYP2C9 polymorphisms and pesticide exposure, if any.

5.2 Materials and methods

5.2.1 Sampling area

Sampling area was same as mentioned under **section 2.2.1**

5.2.2 Characteristics of the participants

Characteristics of the participants were same as mentioned under **section 2.2.2 Characteristics of the participants.**

5.2.3 Sampling procedure

As mentioned earlier venous blood was drawn by venepuncture using 5 ml sterile, disposable syringe, transferred to EDTA containing tubes and brought to the laboratory. The blood samples were kept in -20^{o}C until further use.

5.2.4 Ethical consideration

Ethical consideration was the same as mentioned under **section 2.2.8**

5.2.5 Genomic DNA extraction

For DNA extraction, 1 ml of R.B.C. lysis buffer (155mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) was added to 1 ml of peripheral blood in a 2 ml microcentrifuge tube and mixed thoroughly, centrifuged at 5000 rpm for 6 minutes at 4^{o}C. Supernatant was discarded. Approximately 1.5 ml chilled SET buffer (0.3M Sucrose, 0.002M EDTA pH 8.0, 0.025M Tris pH 8.0) was added to it, mixed, kept for 10 minutes at -20^{o}C and centrifuged at 5000 rpm for 15 minutes at 4^{o}C. Supernatant was discarded, chilled TEN buffer (0.01M Tris pH 8.0, 0.1 mM EDTA pH 8.0, 0.1M NaCl) was added to the pellet upto 1 ml volume and SDS was added to a final concentration of 1% and kept at 50^{o}C for 10 minutes. The mixture was then kept at room temperature for 10 minutes, 5M NaCl was added to the mixture to a final concentration of 1M, mixed slowly and kept in ice for 60 minutes. The mixture was centrifuged at 11,000 rpm for 15 minutes at 4^{o}C, supernatant was collected and equal volume of Tris-saturated phenol was added to it and mixed,

centrifuged at 8000 rpm for 10 minutes at 4°C. Upper aqueous phase was collected and equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to it, mixed and centrifuged at 8000 rpm for 10 minutes at 4°C. The aqueous phase was collected and equal volume of chloroform-isoamyl alcohol (24:1) was added, centrifuged at 8000 rpm for 10 minutes at 4°C. Finally, to the aqueous phase 1/10th volume of Na-acetate (3M pH 5.2) and twice the volume of chilled ethanol was added, kept at -20°C for 1 hour. DNA was peletted by centrifugation at 15,000 rpm for 15 minutes at 4°C. Pellet was washed with chilled 70% ethanol by centrifuging at 15,000 rpm for 15 minutes at 4°C. DNA was dried and dissolved in 25 µl Tris-EDTA pH 8.0 (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0). DNA samples were kept at -20°C until further use.

5.2.6 PCR amplification

CYP2C9 region of DNA was amplified using the polymerase chain reaction (PCR). Amplification was carried out in a thermalcycler (primus ^{advanced} 96 of PEQLAB) using the appropriate primers (**Table 5.1**) and PCR cycling conditions (**Table 5.2**).

Table 5.1: CYP2C9 specific primer sequences.

Primer Name	**Sequence 5' → 3'**	**Detection**	**Amplicon size (bp)**	**Reference**
1F	cac tgg ctg aaa gag cta aca gag	CYP2C9*2 *Ava*II, 37°C Wt: 296, 79; variant: uncut	375	Aynacioglu et al. (1999); Burian et al. (2002)
1R	gtg ata tgg agt agg gtc acc cac			
3F	aat aat aat atg cac gag gtc cag aga tGc	CYP2C9*3 *Nsi*I, 37°C Wt: 112,29; variant: uncut	141	Sullivan-Klose et al. (1996)
3R	gat act atg aat ttg gga ctt c			
4F	aat aat aat atg cac gag gtc cag agG tac	CYP2C9*3 *Kpn*I, 37°C Wt: uncut; variant: 111,30	141	Sullivan-Klose et al. (1996)
4R	gat act atg aat ttg gga ctt c			

Table 5.2: PCR cycling condition for detection of CYP2C9*2 and CYP2C9*3 allele.

Cycling Condition	**CYP2C9*1/CYP2C9*2 using *Ava*II restriction enzyme**	**CYP2C9*1/CYP2C9*3 using *Nsi*I restriction enzyme**	**CYP2C9*1/CYP2C9*3 using *Kpn*I restriction enzyme**
Lid temperature	105^{o}C	105^{o}C	105^{o}C
Initial denaturation	95^{o}C, 10 min	95^{o}C, 5 min	95^{o}C, 5 min
Number of cycle(s)	45	35	35
Denaturation	95^{o}C, 5 secs	95^{o}C, 45 secs	95^{o}C, 45 secs
Annealing	67^{o}C, 10 secs	58^{o}C, 20 secs	58^{o}C, 20 secs
Extension	72^{o}C, 15 secs	72^{o}C, 2 minutes	72^{o}C, 2 minutes
Final extension step	72^{o}C, 5 min	72^{o}C, 5 min	72^{o}C, 5 min
Store forever	4^{o}C	4^{o}C	4^{o}C

5.2.7 Preparation of reaction mixture

The PCR reaction was set in 0.2 ml PCR tubes. For several parallel reactions, a master mix was prepared by multiplying the quantity of each reagent for 25 µl reaction mixture as shown in **Table 5.3**. Master mix containing water, buffer, dNTPs, primers and Taq DNA polymerase was dispensed in individual tubes and the template DNA was added to each tube. The reaction mixture was gently vortexed followed by brief centrifugation and placed in a thermalcycler.

Table 5.3: Composition of reaction mixture for detection of CYP2C9*2 and CYP2C9*3 allele.

Reagent	Final Concentration		Quantity for 25 µl reaction mixture	
	CYP2C9*2	CYP2C9*3	CYP2C9*2	CYP2C9*3
Sterile deionized water			13.6 µl	14.3 µl
5X Taq buffer	1X	1X	5 µl	5 µl
dNTP mix	200 µM	200 µM	0.5 µl	0.5 µl
25mM $MgCl_2$	2.5 mM	3 mM	2.5 µl	3 µl
1F Primer	1 µM	0.4 µM	1 µl	0.4 µl
1R Primer	1 µM	0.4 µM	1 µl	0.4 µl
Taq DNA polymerase	5 U/ λ	5 U/ λ	0.4 µl	0.4 µl
Template DNA	90 ng	90 ng	1 µl	1 µl

5.2.8 Amplification check by Agarose gel electrophoresis

The amplified PCR products were checked by separating on 2.5% (for 1F 1R primers) and 3% (for 3F 3R and 4F 4R primers) agarose gel containing 0.5 µg/ml ethidium bromide. The electrophoresis was carried out in TAE (40 mM Tris, 40 mM acetic acid, 1 mM EDTA pH 8.0) containing buffer with 100 bp DNA ladder as size marker. Gels were viewed on Gel Documentation system (Spectroline of Bio-vision) and photographed with transmitted UV light (**Figure 5.1**).

DNA sequencing of representative samples were done to validate the PCR-RFLP protocol (**Figures 5.2, 5.3, 5.4**).

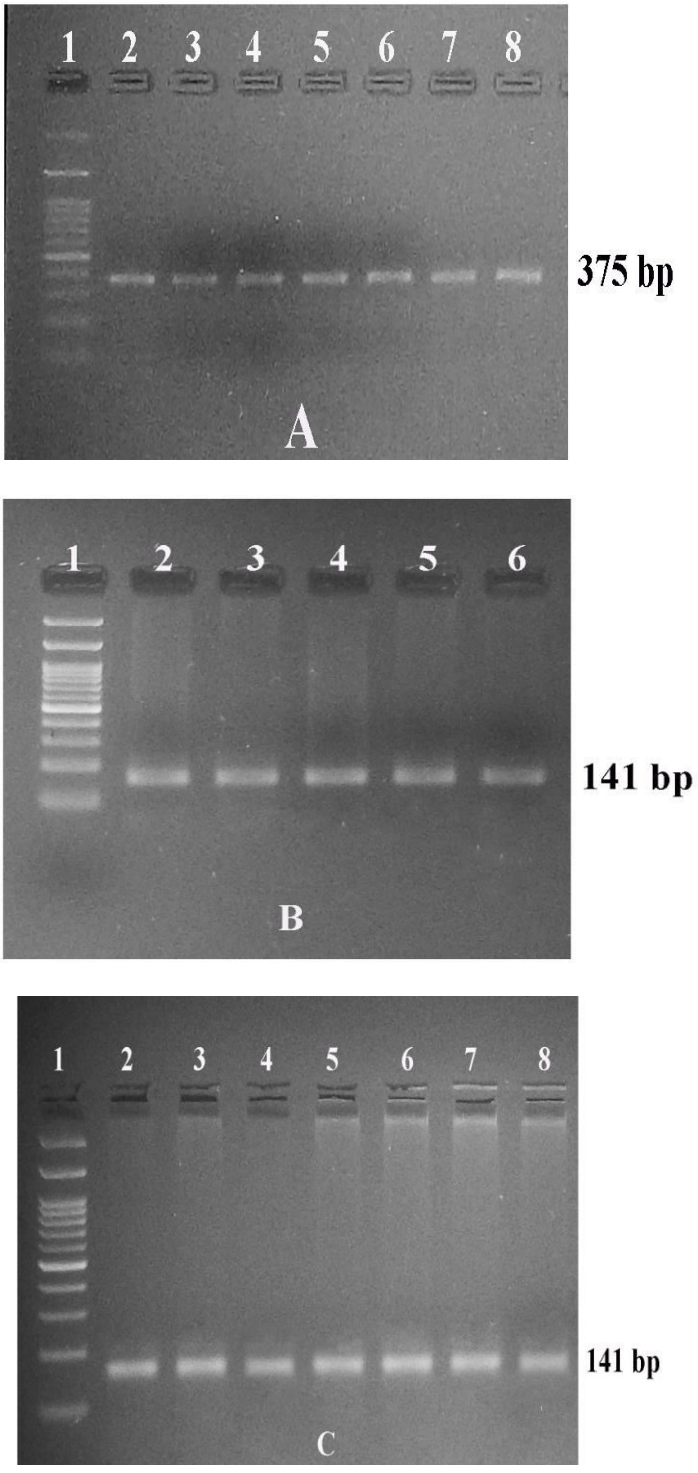
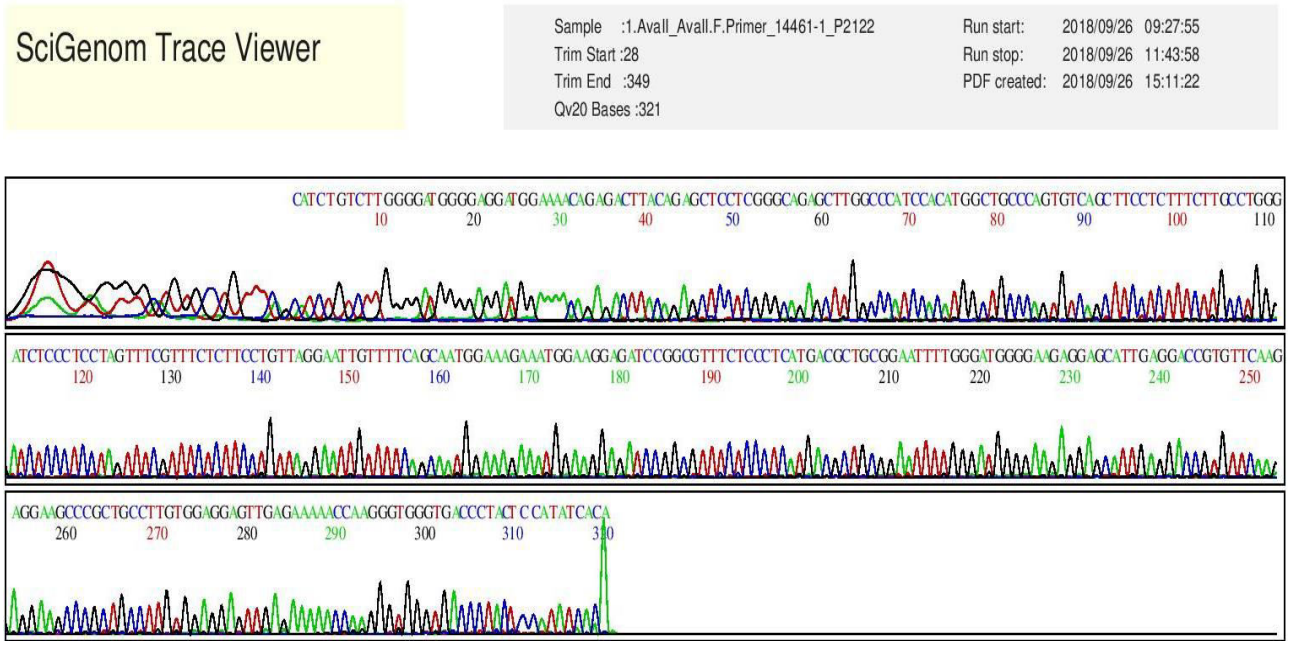


Figure 5.1: Gel showing PCR amplicons using Primer sets, **(A)** 1F and 1R **(B)** 3F and 3R **(C)** 4F and 4R (as shown in **Table 5.1**). Lane 1 in each gel indicates 100 bp DNA marker.



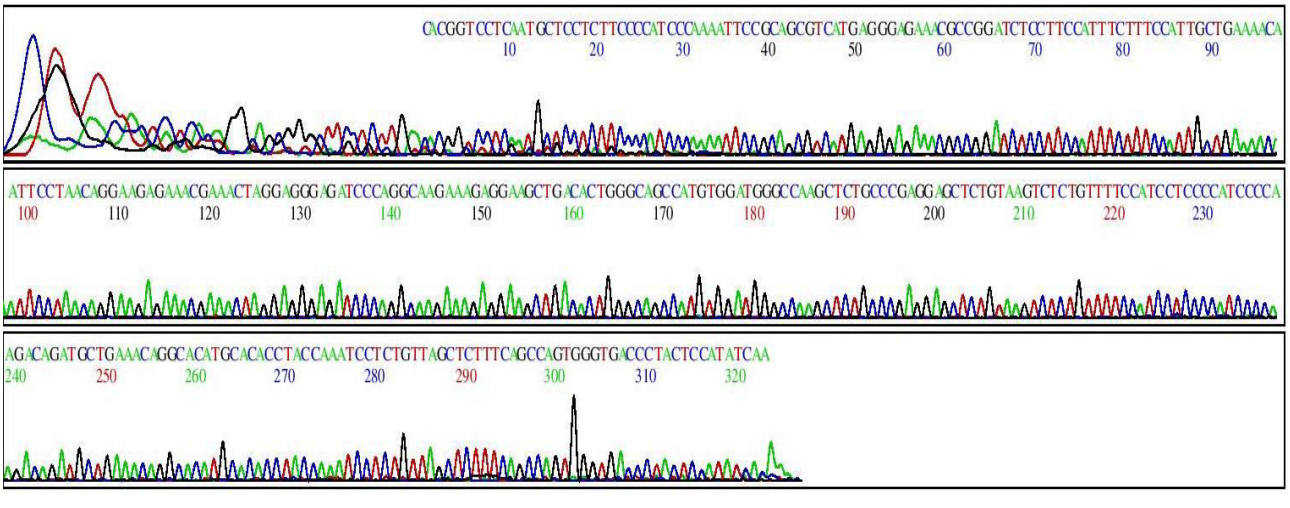


Figure 5.2: Chromatogram of PCR product sequence (exon 3, *Ava*II recognition site) generated by automated sequence analyzer showing 4 peaks of four nucleotides.

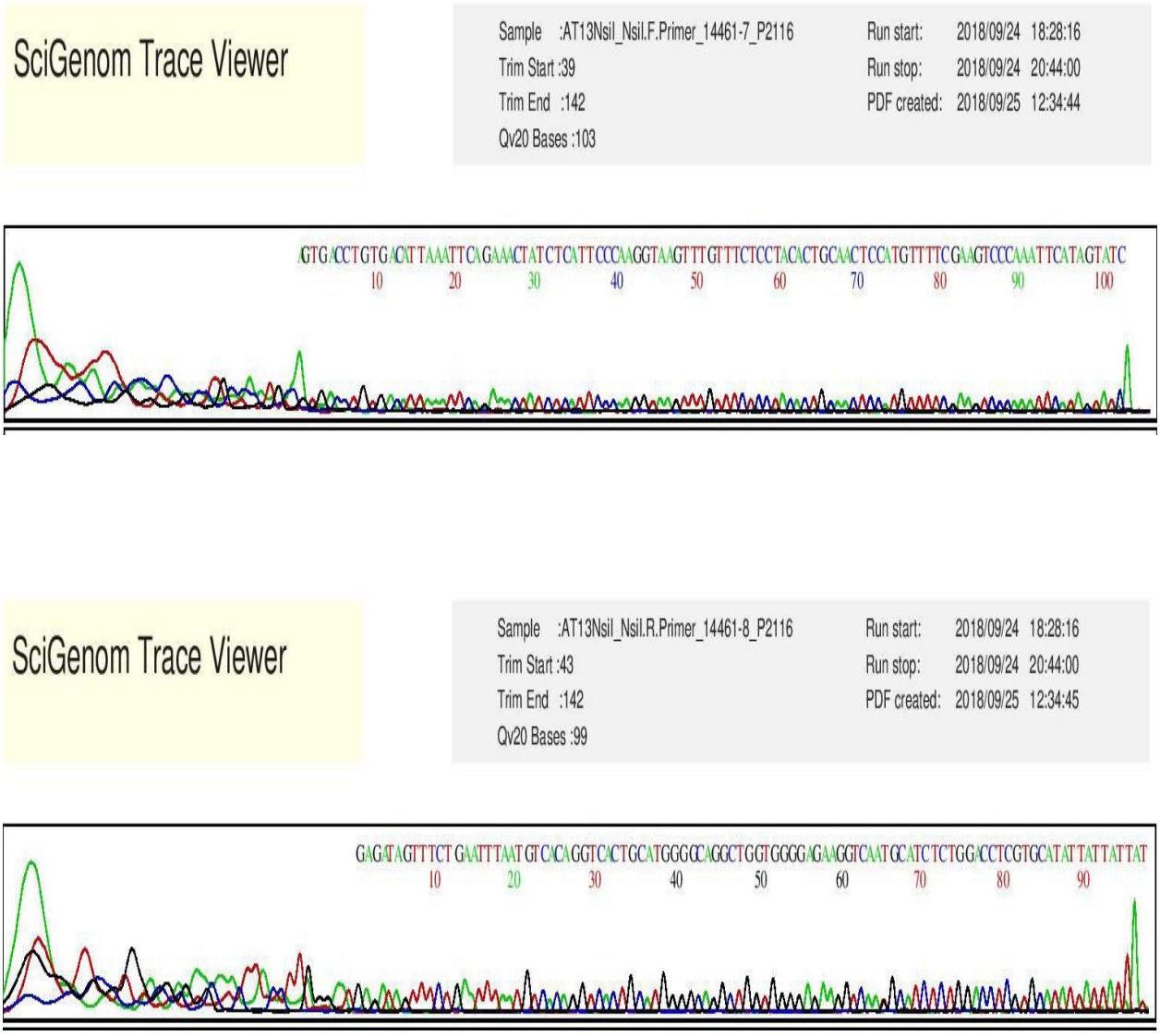


Figure 5.3: Chromatogram of PCR product sequence (exon 7, *Nsi*I recognition site) generated by automated sequence analyzer showing 4 peaks of four nucleotides.

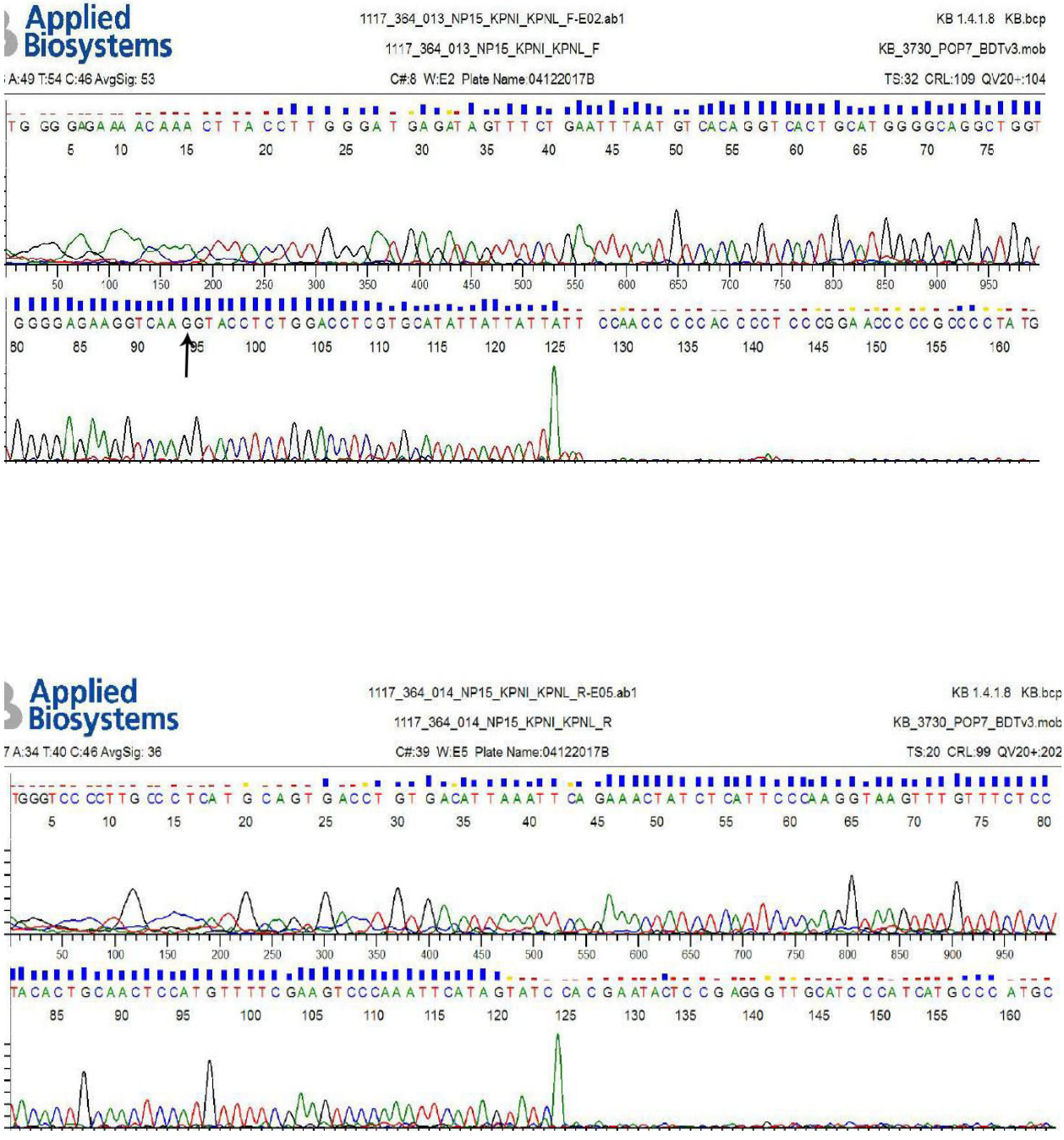


Figure 5.4: Chromatogram of PCR product sequence (exon 7, *Kpn*I recognition site) generated by automated sequence analyzer showing 4 peaks of four nucleotides (arrow indicates a variant for CYP2C9*3 allele).

5.2.9 Restriction digestion of PCR products

Aliquotes of each PCR product (10 µl) were digested with restriction endonucleases (*Ava*II for CYP2C9*2 and *Nsi*I and *Kpn*I for CYP2C9*3) at 37^{o}C overnight for complete digestion. The DNA fragments were electrophoresed either on 2.5% agarose gel to detect CYP2C9*2 allele or 3% agarose gel to detect CYP2C9*3 allele. Bands were detected by viewing on Gel Documentation system (Spectroline of Bio-vision) and photographed with transmitted UV light (**Figures 5.5, 5.6 and 5.7**).

Table 5.4: Composition of reaction mixture for restriction digestion.

Reagent	Final Concentration	Quantity for 20 µl reaction mixture
Sterile deionized water		4.6 µl
Buffer	2X	4 µl
BSA	0.2 mg/ml	0.4 µl
Restriction enzyme	1U/ µl	1 µl

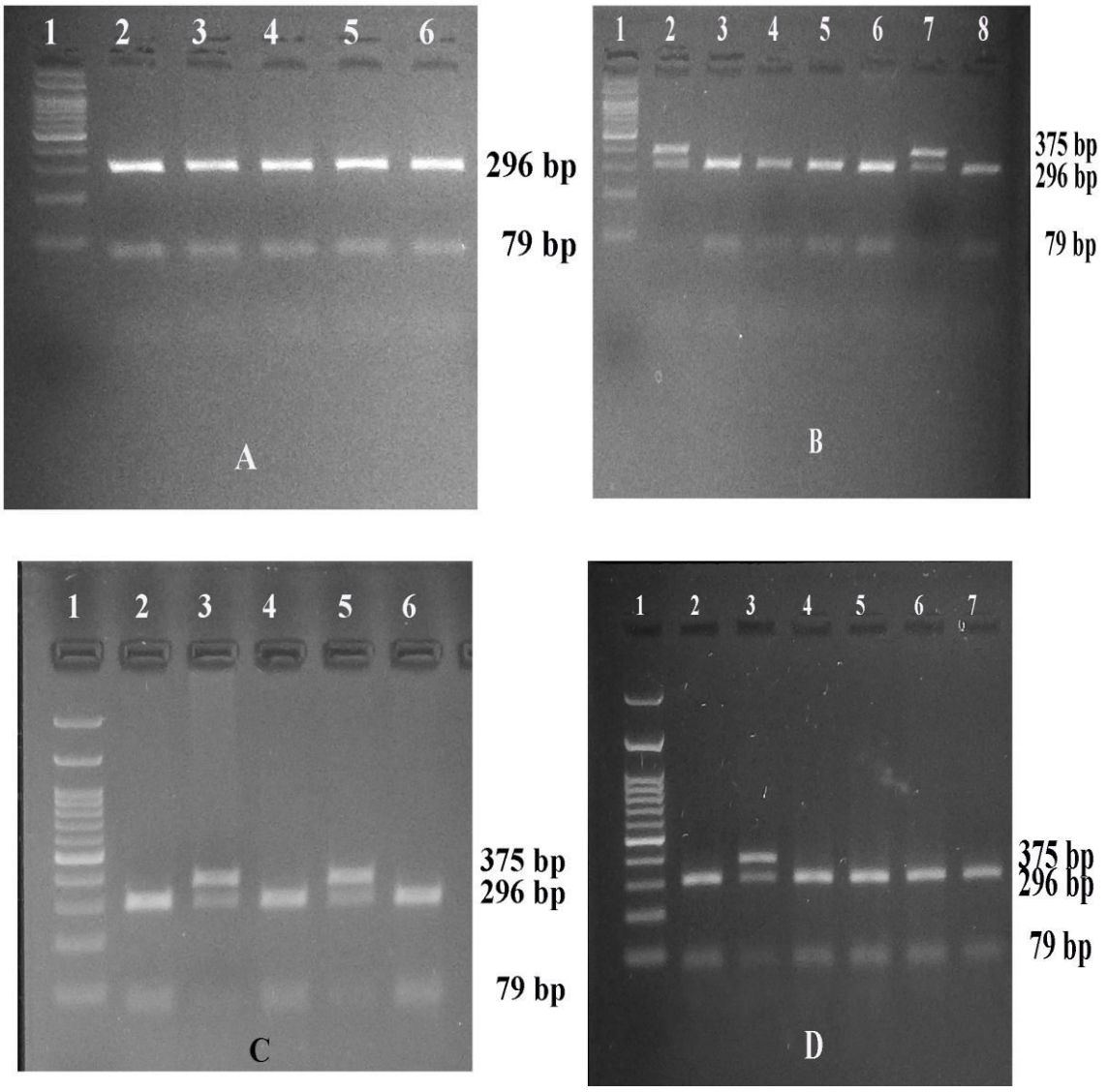


Figure 5.5: PCR-RFLP analysis of CYP2C9 gene using *Ava*II
(**A)** Lanes 2-6 indicate CYP2C9*1/*1 (296 bp and 79 bp).
(B) Lanes 2,7 indicate CYP2C9*1/*2 (375 bp, 296 bp and 79 bp), Lanes 3,4,5,6,8 indicate CYP2C9*1/*1.
(C) Lanes 2,4,6 indicate CYP2C9*1/*1, Lanes 3,5 indicate CYP2C9*1/*2.
(D) Lanes 2,4,5,6,7 indicate CYP2C9*1/*1, Lane 3 indicates CYP2C9*1/*2.
Lane 1 denotes 100 bp DNA marker (A, B, C and D).

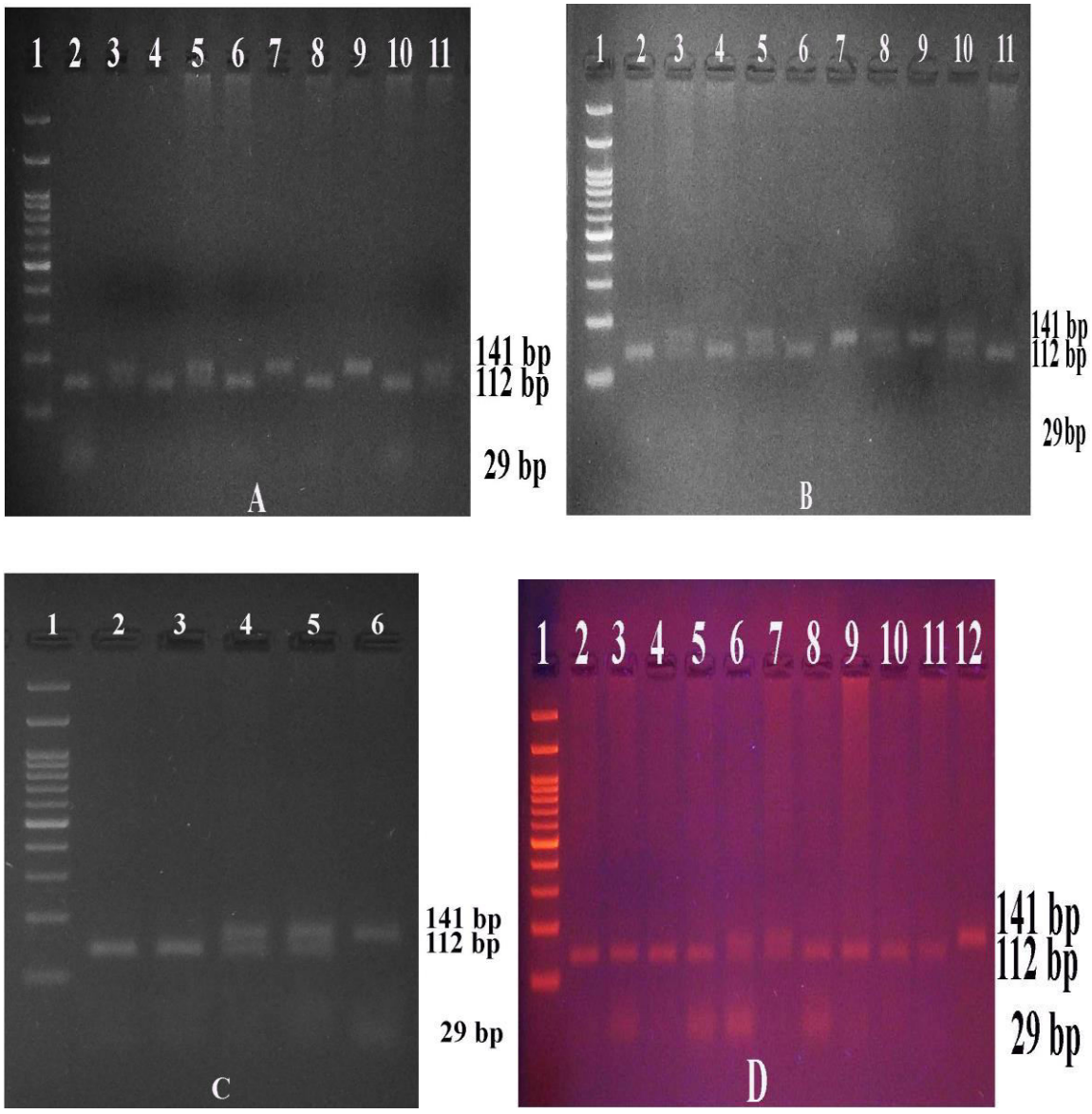


Figure 5.6: PCR-RFLP analysis of CYP2C9 gene using *Nsi*I.
(A) Lanes 2,4,6,8,10 indicate CYP2C9*1/*1 (112 bp and 29 bp), Lanes 3,5,11 indicate CYP2C9*1/*3 (141 bp, 112 bp and 29 bp), Lanes 7,9 indicate CYP2C9*3/*3 (141 bp).
(B) Lanes 2,4,6,11 indicate CYP2C9*1/*1. Lanes 3,5,8,10 indicate CYP2C9*1/*3. Lanes 7,9 indicates CYP2C9*3/*3.
(C) Lanes 2,3 indicate CYP2C9*1/*1. Lanes 4,5 indicate CYP2C9*1/*3, Lane 6 indicates CYP2C9*3/*3.
(D) Lanes 2,3,4,5,8,9,10,11 indicate CYP2C9*1/*1, Lanes 6,7 indicate CYP2C9*1/*3, Lane 12 indicates CYP2C9*3/*3.
Lane 1 denotes 100 bp DNA marker (A, B, C and D).

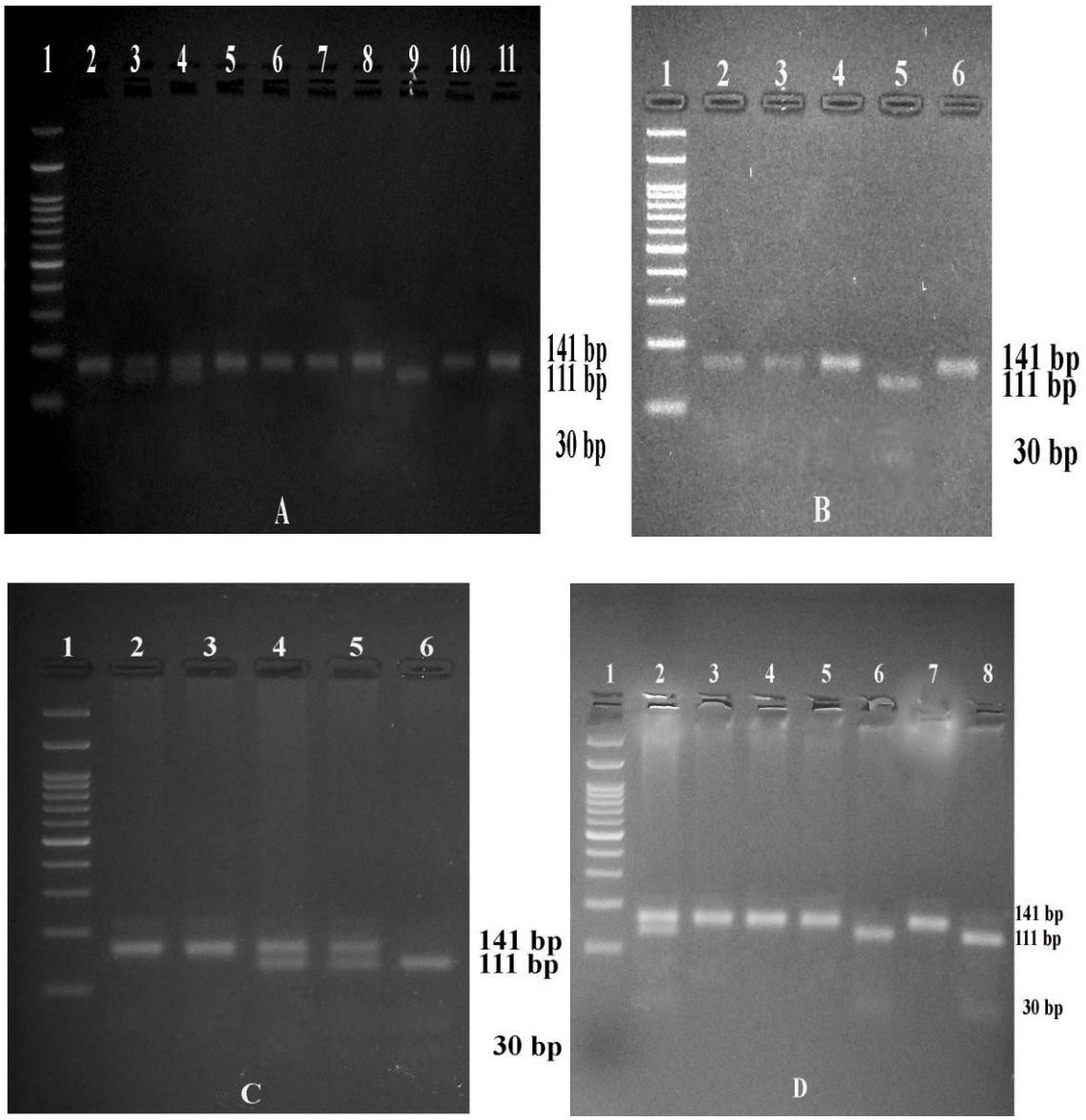


Figure 5.7: PCR-RFLP analysis of CYP2C9 gene using *Kpn*I.
(A) Lanes 2,5,6,7,8,10,11 indicate CYP2C9*1/*1 (141 bp), Lanes 3,4 indicate CYP2C9*1/*3 (141 bp, 111 bp and 30 bp), Lane 9 indicates CYP2C9*3/*3 (111 bp and 30 bp).
(B) Lanes 2,3,4,6 indicate CYP2C9*1/*1, Lane 5 indicates CYP2C9*3/*3.
(C) Lanes 2,3 indicate CYP2C9*1/*1, Lanes 4,5 indicate CYP2C9*1/*3, Lane 6 indicates CYP2C9*3/*3.
(D) Lane 2 indicates CYP2C9*1/*3, Lanes 3,4,5,7 indicate CYP2C9*1/*1, Lanes 6,8 indicate CYP2C9*3/*3.
Lane 1 denotes 100 bp marker (A, B, C and D).

5.2.10 Detection of restriction digestion product

Interpretation of CYP2C9*2 allele was done according to Burian et al. (2002). PCR with 1F and 1R primer produced a 375 bp amplicon. Samples containing CYP2C9*1 allele produced 296 bp and 79 bp after digestion with *Ava*II, CYP2C9*2 allele was not cleaved and remained as 375 bp. Samples heterozygous for CYP2C9*1/*2 allele produced bands of 375 bp, 296 bp and 79 bp (**Figure 5.8**).

Interpretation of CYP2C9*3 allele was done according to Sullivan-Klose et al. (1996). Both the primer sets 3F 3R and 4F 4R produced 141 bp amplicon. When digested with *Nsi*I and *Kpn*I samples containing CYP2C9*1 allele produced 112 bp and 29 bp after digestion with *Nsi*I, CYP2C9*3 allele was not cleaved. Samples heterozygous for CYP2C9*1/*3 allele produced bands of 141 bp, 112 bp and 29 bp (**Figure 5.9**). Samples containing CYP2C9*3 allele produced 111 bp and 30 bp after digestion with *Kpn*I, CYP2C9*1 allele was not cleaved by *Kpn*I. Samples heterozygous for CYP2C9*1/*3 alleles produced bands of 141 bp, 111 bp and 30 bp (**Figure 5.10**).

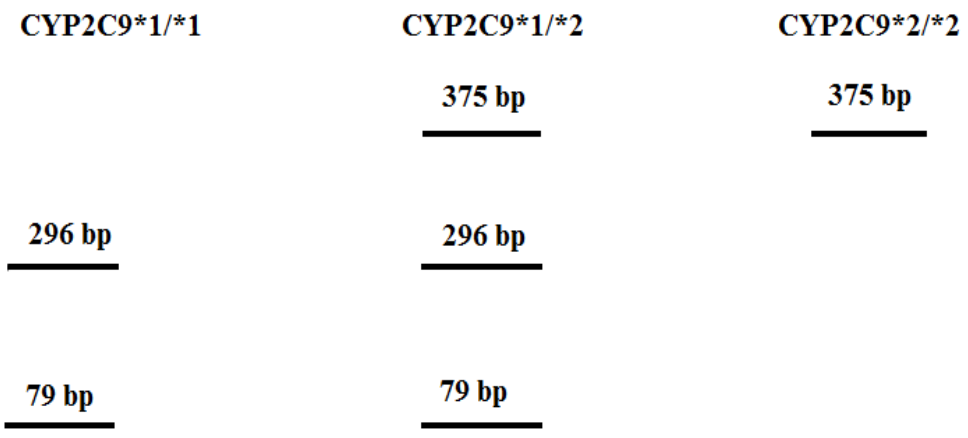


Figure 5.8: *Ava*II RFLP pattern of the PCR product produced by primer set 1F 1R.

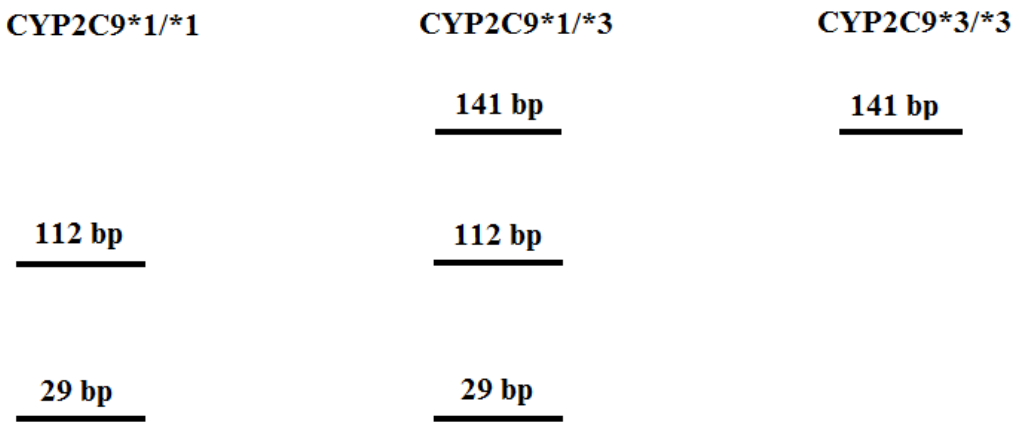


Figure 5.9: *Nsi*I RFLP pattern of the PCR product produced by primer set 3F 3R.

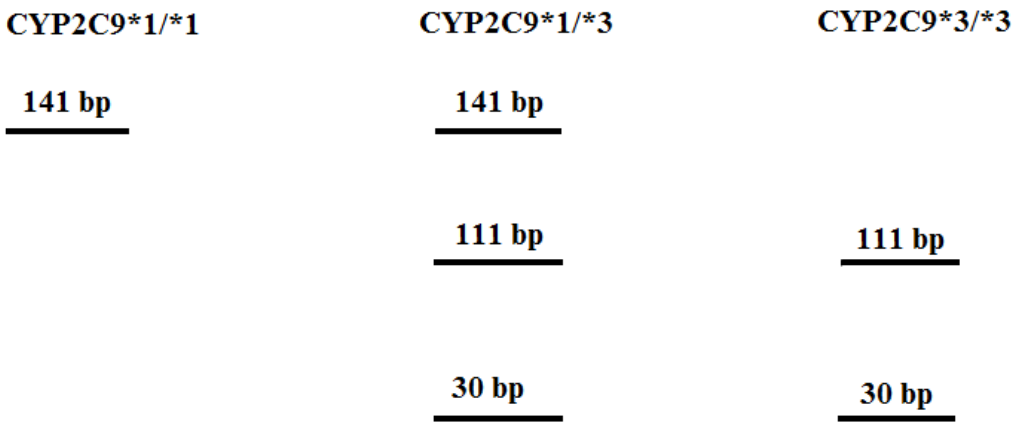


Figure 5.10: *Kpn*I RFLP pattern of the PCR product produced by primer set 4F 4R.

5.2.11 Statistical analysis

Genotype and allele frequencies were calculated according to Hardy-Weinberg equilibrium. χ^2 test was done to see whether the population surveyed were in equilibrium.

Allele frequency was calculated according to the formula given below:

Allele frequency = Genotype frequency of Homozygotes + ½ X Genotype frequency of Heterozygote (Strickberger, 1999).

5.3 Results

A total of 224 individuals were genotyped for CYP2C9 gene using exon 2 forward, intron 3 reverse primer (for detection of CYP2C9*2 allelic variant) and exon 7 forward, intron 7 reverse primer (for detection of CYP2C9*3 allelic variant) and PCR RFLP. Out of 224 individuals, 94 were pesticide exposed tea garden workers and rest 130 were considered as non-tea garden workers which included 60 control subjects, 39 smokers and 31 alcohol consumers. They were not directly exposed to pesticides and resided in areas away from the pesticide-exposed ones. The 94 pesticide exposed individuals were directly involved in pesticide spraying/mixing or tea leaf plucking. The results obtained from the analysis of CYP2C9 gene are represented in **Table 5.5 and 5.6**. 94 tea garden workers (exposed to pesticides) and 130 non-tea garden workers (not exposed to pesticides) were analysed to estimate CYP2C9 allelic variants compared to wild type. Analysis revealed the presence of all the genotypes except CYP2C9*2/*2 (**Table 5.6**).

In case of non-tea garden worker population, PCR analysis showed that CYP2C9*1 is the most predominant allele and is present at a frequency of 0.87695 (78.46% of individuals). Sequences containing CYP2C9*2 and CYP2C9*3 were also studied. 2.30% of the individuals were found to be homozygous for CYP2C9*3 with an allele frequency of 0.09615. No homozygotes for CYP2C9*2 allele were detected. Genotyping revealed that one individual was heterozygous for both mutations hence was interpreted as CYP2C9*2/*3 genotype as there is no evidence of linkage between CYP2C9*2 and

CYP2C9*3 allele on the same chromosome (Stubbins et al., 1996). The CYP2C9*2 allele was found to be least frequent with a frequency of 0.02690 (**Table 5.5**). About 0.77% of individuals were heterozygous for CYP2C9*2/*3 alleles. 4.62% of individuals were heterozygous for CYP2C9*1/*2 allele, while 13.85% of non-tea garden worker population were found to be heterozygous for CYP2C9*1/*3 alleles, respectively (**Table 5.6**). The observed and expected frequency distribution indicates that the results are in good agreement with that of the expected calculated by the Hardy-Weinberg law. χ^2 value was found to be 3.661 ($p < 0.05$).

Similarly, CYP2C9 genotyping was performed on 94 pesticide exposed workers population. CYP2C9*1 was the most frequent allele present with a frequency of 0.87235 (77.66% of individuals). 2.13% of individuals were homozygous for CYP2C9*3 allele giving an allele frequency of 0.10105. Like non-tea garden workers no homozygotes were found for CYP2C9*2 in worker population. Genotyping revealed that one individual was heterozygous for CYP2C9*2/*3 genotype with a frequency of 1.06%. The CYP2C9*2 allele was found to be least frequent with a frequency of 0.02660. 4.26% of tea garden worker population was observed to be heterozygous for CYP2C9*1/*2 alleles and 14.89% were heterozygous for CYP2C9*1/*3 alleles (**Table 5.6**). The allele frequency in pesticide exposed worker population is comparable to those of non-tea garden worker (**Table 5.5**). The observed and expected frequency distribution indicates that the results are in good agreement with that of the expected as calculated using the Hardy-Weinberg law with a χ^2 value of 1.8 ($p < 0.05$).

Table 5.5: Allele frequency of non-tea garden worker and tea garden worker populations.

Allele	**Allele frequency**	
	Non-Worker	**Worker**
CYP2C9*1	0.87695	0.87235
CYP2C9*2	0.02690	0.02660
CYP2C9*3	0.09615	0.10105

Table 5.6: Observed and expected frequency distribution and number in non-tea garden workers and tea garden workers.

	CYP2C9 allelic variants						
	***1/*1**	***1/*2**	***1/*3**	***2/*3**	***2/*2**	***3/*3**	**Total**
Non Worker							
Observed no. (N=130)	102 (78.46%)	6 (4.62%)	18 (13.85%)	1 (0.77%)	nil	3 (2.30%)	130
Expected no. (N=130)	100 (76.92%)	6.1 (4.69%)	21.9 (16.85%)	0.7 (0.54%)	0.1 (0.08%)	1.2 (0.92%)	130
χ^2	**0.040**	**0.002**	**0.690**	**0.129**	**0.100**	**2.700**	**3.661**
Worker							
Observed no. (N=94)	73 (77.66%)	4 (4.26%)	14 (14.89%)	1 (1.06%)	nil	2 (2.13%)	94
Expected no. (N=94)	71.5 (76.06%)	4.3 (4.58%)	16.5 (17.55%)	0.6 (0.64%)	0.1 (0.11%)	1 (1.06%)	94
χ^2	**0.03**	**0.02**	**0.38**	**0.27**	**0.10**	**1.00**	**1.8**

The results are in good agreement with the expected number calculated using Hardy-Weinberg law.

5.4 Discussion

Cytochrome P450 enzyme system is known to be inhibited by certain pesticides for example parathion, carbaryl, chlorpyrifos etc. resulting in free radical production which causes DNA damage (Butler and Murray, 1997; Dinsdale and Verschoyle, 2001). The biotransformation of a huge variety of xenobiotics including the organophosphates (chlorpyrifos and parathion) is associated with CYP2C9 (Daly, 2003; Foxenberg et al., 2007). A number of exogenous compounds namely drugs, procarcinogens, alcohols etc. are catalyzed by enzymes belonging to the families CYP1, CYP2 and CYP3, while others metabolize fatty acids, prostaglandins, steroids (endogenous compounds) (van der and Steijns, 1999; Review by Rosemary and Adithan, 2007). In view of the above, a genotyping of the pesticide exposed tea garden worker population and non-exposed population was carried out to see if any particular allelic variant (CYP2C9*2 and CYP2C9*3) in addition to wild type was present at a greater frequency in any particular group (pesticide exposed tea garden workers and non-exposed individuals).

The wild type allele CYP2C9*1 was most frequent in both the populations i.e., tea garden workers and non-tea garden workers having a frequency of 0.87235 and 0.87695, respectively (**Table 5.5**) which is comparable to the findings of Seng et al. (2003) who had reported the CYP2C9*1 allele frequency to be 87.50% in Indians. The CYP2C9*2 mutant allele was least frequent in both the populations whereas, CYP2C9*3 was slightly higher than CYP2C9*2 allele in tea garden worker as well as non-exposed individuals. The results also indicated that the pesticide induced damage has no association with any of the alleles found. Our results showing allele frequency of 0.09615 and 0.10105 in the non-tea garden worker and the tea garden worker population strongly corroborate the results of Seng et al. (2003) with a frequency of 0.0815 for CYP2C9*3 allele. The

frequency of CYP2C9*2 allele was much low in the present study compared to 0.0435 reported by Seng et al. (2003) in Indian population. The high frequency by Seng et al. (2003) may be due to association with patients on warfarin treatment.

In comparison to CYP2C9*1, the alleles CYP2C9*2 and CYP2C9*3 have low metabolic activities (Lee et al., 2002). Singh et al. (2011, 2012) have shown that CYP2C9*1, CYP2C9*2 and CYP2C9*3 were not significantly associated with DNA damage related to pesticide exposure. However, CYP2C9*3 alleles may result in substantial reduction in metabolic activities, while CYP2C9*2 allele produces intermediate reduction with GSTM1 null genotypes and NAT2 slow acetylation genotypes (Singh et al., 2012).

CYP2C9 genetic polymorphism was studied in south Indian population (346 subjects) which revealed that the frequencies of CYP2C9*1, CYP2C9*2 and CYP2C9*3 were 0.88, 0.04 and 0.08, respectively. Different CYP2C9 genotypes CYP2C9*1/*1, CYP2C9*1/*2, CYP2C9*1/*3, CYP2C9*2/*2, CYP2C9*2/*3, CYP2C9*3/*3 were reported with their corresponding frequencies 0.78, 0.05, 0.15, 0.01, 0.01 and 0.00 (Jose et al., 2005) which are comparable to the results of the present study except that no homozygotes for CYP2C9*2/*2 was detected. On the other hand CYP2C9*3/*3 homozygotes were present at low frequency in contrast to the result obtained by Jose et al. (2005). Recent studies have reported the presence of CYP2C9*2 and CYP2C9*3 alleles with a frequency of 0.006 and 0.09 and 0.05 and 0.11 in the south and north Indian populations, respectively. There existed a significant difference in the CYP2C9*2 allele between the south Indian and north Indian population (Nahar et al., 2013).

Chaudhary et al. (2016) genotyped CYP2C9 gene in 89 epileptic children in order to see the association with drug phenytoin. CYP2C9*1, CYP2C9*2 and CYP2C9*3 allelic frequencies were 85.4%, 4.5% and 10.1%, respectively. CYP2C9*3 allelic group showed

significantly higher serum phenytoin compared to wild variant. In their study also CYP2C9*3 was more common than CYP2C9*2 which is similar to the result observed in the present study. Earlier in a similar study Kesavan et al. (2010) had reported the association of CYP2C9*3 with higher risk of phenytoin induced toxicity in epileptic patients (Kesavan et al., 2010). Swar et al. (2016) have also shown a significant association between phenotype and genotype with respect to flurbiprofen metabolism and reported comparatively high frequency of CYP2C9*3 allele (9.5%), while CYP2C9*2 was 4.3%. The CYP2C9*1 and CYP2C9*3 frequencies reported by Swar et al. (2016) corroborate the findings of the present study. The discussion indicates that in the Indian population CYP2C9*1 is most frequent and other two alleles exist in variable frequency.

Studies from other workers have yielded variable results. Genotyping of CYP2C9 in Caucasian population of clinical trial volunteers have shown highest to lowest frequency in order of CYP2C9*1 > CYP2C9*2 > CYP2C9*3 where the CYP2C9*1 frequency though lower but comparable to the result of the present study (Stubbins et al., 1996). Sullivan-Klose et al. (1996) had reported that Cys^{144} (CYP2C9*2) and Leu^{359} (CYP2C9*3) alleles are rarer in Caucasian-American and African-American population which also supports the rarer frequency of CYP2C9*2 and CYP2C9*3 allele observed in the present study. Among Asian, Cys^{144} (CYP2C9*2) was not found in Chinese-Taiwanese population (Sullivan-Klose et al., 1996). Other studies have also shown that CYP2C9*2 and CYP2C9*3 alleles are in lower frequency in different population namely Japanese population (Nasu et al., 1997), Swedish population (Yasar et al., 1999) and unrelated Turkish subjects (Aynacioglu et al., 1999).

The observed and expected frequency distribution of pesticide exposed tea garden worker population and non-tea garden workers are in accordance with Hardy-Weinberg law

(**Table 5.6**) and the allele frequencies are comparable between the two groups (**Table 5.5**). It can be assumed that none of the alleles is apparently associated with the pesticide exposure and related toxicity.