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Detection of C677T & A1298C mutations within the MTHFR gene by PCR and RFLP assays and assessment of risk factor of Hyperhomocysteinemia

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ABSTRACT

The MTHFR gene within the human genome, codes for the synthesis of Methylenetetrahydrofolate Reductase enzyme, which reduces 5,10-Methylenetetrahydrofolate to 5-Methyltetrahydrofolate, which in turn, is the major circulatory form of folate in the blood. Folate, in this form, among it's other functions, is involved in reducing the homocysteine levels in the blood, whose elevated levels lead to Hyperhomocysteinemia, causing various major disorders. Mutations within the gene lead to impairment of gene function, in turn causing the homocysteine levels to rise. The C677T and A1298C mutations are the main causative agents for MTHFR gene disruption. During the course of the project, a total of 79 samples were analyzed for the presence of these mutations. The blood samples were first subjected to PCR, giving two separate DNA fragments each responsible for either of the conditions. The fragments were then subjected to RFLP analysis to detect the mutations. The results were finally given with respect to the risk factor faced by each individual based on a molecular diagnostic point of view.

***Keywords*:** MTHFR gene; DNA methylation; Hyperhomocysteinemia

1. INTRODUCTION

The Methylenetetrahydrofolate Reductase (MTHFR) gene codes for the enzyme that catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the major circulatory form of folate in the body. The various functions of 5-methyltetrahydrofolate include participation in many complex biochemical pathways such as nucleotide synthesis, DNA methylation, methylation of proteins, neurotransmitters and phospholipids and also acting as a cofactor in the remethylation of Homocysteine to Methionine. (Van Der Put, et al.)

Homocysteine is an important substance in the blood as elevated levels of Homocysteine has been found to be the causative agent of various diseases such as; Cerebrovascular disease cerebral vein thrombosis, coronary artery disease, myocardial infarction, venous thrombosis neural tube defects leading to dementia and Alzheimer's disease osteoporosis, diabetes, complications in pregnancy, etc. (Van Der Put, et al. 1997; Mills et al. 1995; Steegers-Theunissen et al. 1994; Ramsbottom et al. 1997).

Hyperhomocysteinemia is a disorder where the homocysteine levels in the blood are high. Variations in the levels of homocysteine are attributed to both genetic and environmental factors. Variations also depend on the gender as well as on the age of the individual. The aim of the project is to analyze blood samples to detect the variations in the MTHFR gene that interfere with gene functions and lead to elevated levels of homoysteine in the blood, and to assess the risk factor of each individual depending on the genetic data obtained through the analysis.

Thus, it is expected that the individuals showing the gene variations which are considered as important risk factors for hyperhomocysteinemia to have elevated levels of homoysteine in the blood.

THE BIOCHEMISTRY OF HOMOCYSTEINE

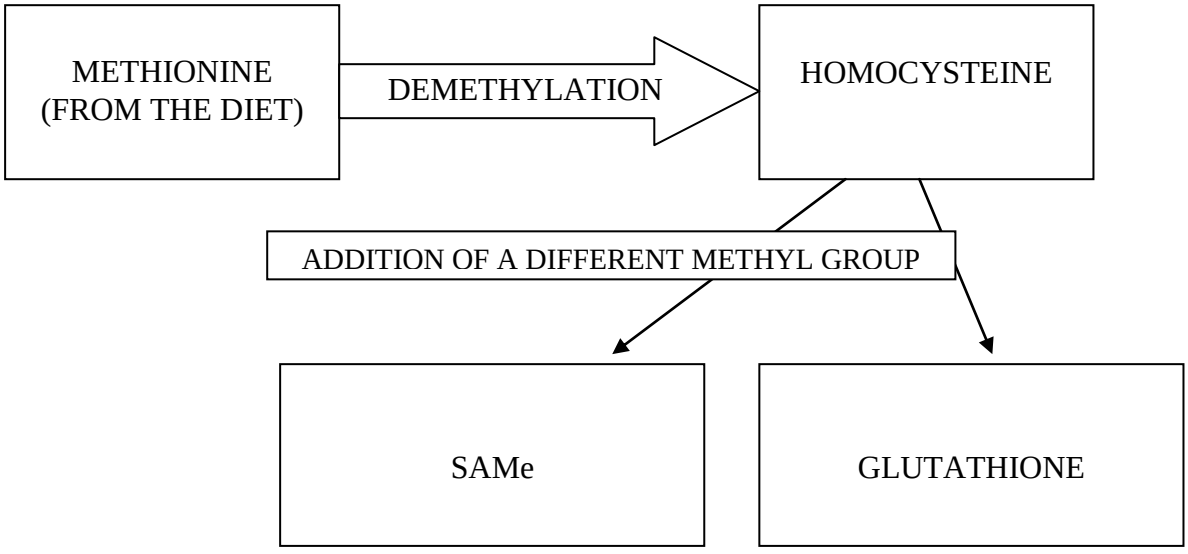

Figure 1.1. A summary of the biochemical pathway of Homocysteine

Homocysteine is an amino acid in the human blood which is produced in the body during the chemical conversion of Methionine, which is richly found in food such as fish. As Methionine is consumed, it is subjected to demethylation, where a methyl group is removed to produce Homocysteine. (Van Der Put, et al.)

Homocysteine is an important intermediate compound during this biochemical pathway, and as the body adds a different methyl group to homocysteine, S-Adenosyl Methionine (SAMe) is produced. It is an antidepressant and also has anti arthritic properties. Apart from these functions, it also assists to reduce the blood homoysteine levels. As the SAMe levels increase, the body produces another important compound, Glutathione; this is the best anti ageing agent in the body and is also a detoxifier. Reduced levels of Glutathione have been shown to be a major cause for death of common causes. (The Homocysteine information site)

The biochemical pathway is summarized in the following diagram (Fig. 1.1).

MEASURING HOMOCYSTEINE

The concentration of Homoysteine levels is measured in u/mol per litre of plasma. According to reports from the Homocysteine information site, overall levels of Homocysteine of the world's population are reported to be high. Even so, there is no specific concentration level in the blood which can be considered as perfectly safe.However, a safe level is generally considered to be below 9 umol/l. levels exceeding 14 umol/l are considered to put the individual at a great risk of major disorders.

The following graph shows the association between the increase of coronary artery disease (CAD) and the concentration of Homocysteine (Hcy) in the blood.

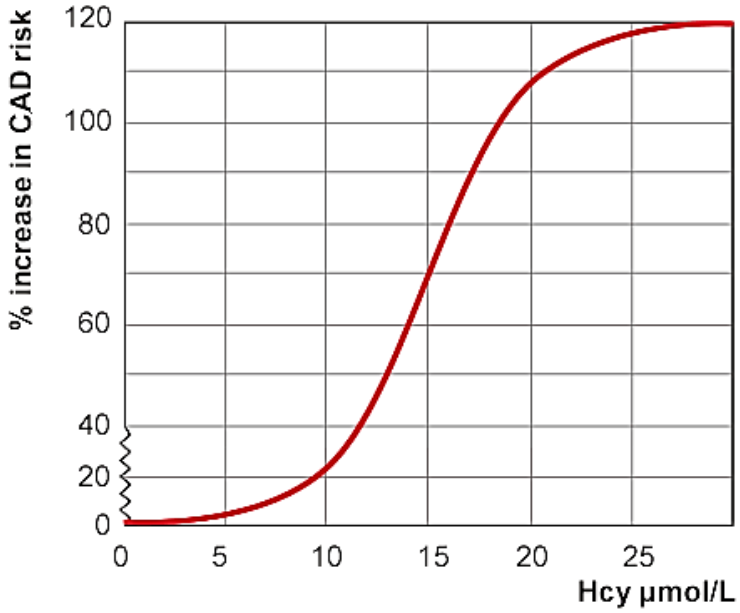

Figure 1.2. A graph showing the tendency of coronary artery diseases to increase with levels of Homocysteine in the blood (the Homocysteine information site)

THE GENETICS OF HOMOCYSTEINE

Homocysteine levels in the blood are influenced by both environmental and genetic factors, as stated earlier. Genetic factors involve point mutations in the MTHFR gene. The MTHFR gene is roughly 19300bp long and is located on the short arm of the 1st pair of chromosomes at position 36.3. The cDNA sequence is 2.2 kilo bases in length and consists of 11 exons. Alternative splicing of the gene is observed in humans. The major product of the MTHFR gene in humans is a 77-kDa protein. (Genetics Home Reference)

Molecular Location on chromosome 1: base pairs 11,769,246 to 11,788,568

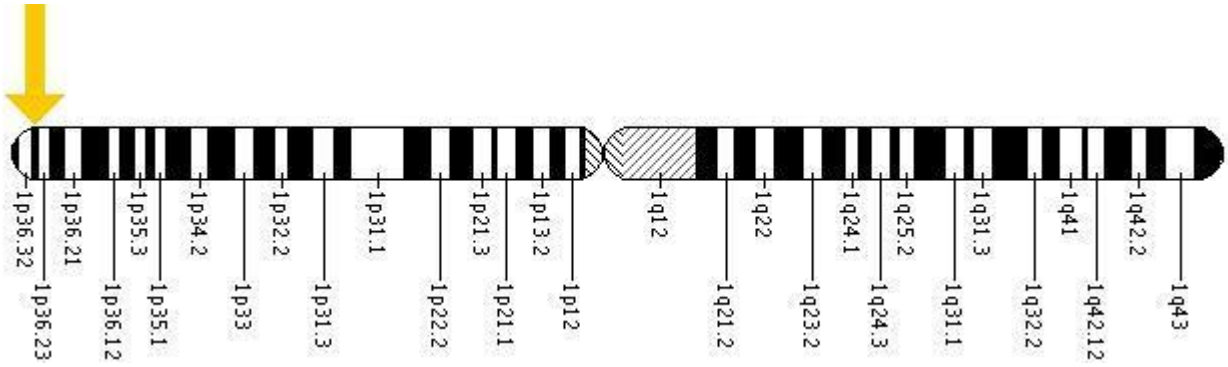

Figure 1.3. The location of the MTHFR gene on the first pair of chromosomes

The coding sequence of the MTHFR gene is as given below. (GENBANK Acc no. DQ053700)

ATGTGTCGGGGGTGTGGCTGCCTGCCCCCTGATGCTCCCTGCCCCACCCTGTGCAGTAGG
AACCCAGCCATGGTGAACGAAGCCAGAGGAAACAGCAGCCTCAACCCCTGCTTGGAGGGC
AGTGCCAGCAGTGGCAGTGAGAGCTCCAAAGATAGTTCGAGATGTTCCACCCCGGGCCTG
GACCCCGAGCGGCATGAGAGACTCCGGGAGAAGATGAGGCGGCGATTGGAATCTGGTGAC
AAGTGGTTCTCCCTGGAATTCTTCCCTCCTCGAACTGCTGAGGGAGCTGTCAATCTCATC
TCAAGGTTTGACCGGATGGCAGCAGGTGGCCCCCTCTACATAGACGTGACCTGGCACCCA
GCAGGTGACCCTGGCTCAGACAAGGAGACCTCCTCCATGATGATCGCCAGCACCGCCGTG
AACTACTGTGGCCTGGAGACCATCCTGCACATGACCTGCTGCCGTCAGCGCCTGGAGGAG
ATCACGGGCCATCTGCACAAAGCTAAGCAGCTGGGCCTGAAGAACATCATGGCGCTGCGG
GGAGACCCAATAGGTGACCAGTGGGAAGAGGAGGAGGGAGGCTTCAACTACGCAGTGGAC
CTGGTGAAGCACATCCGAAGTGAGTTTGGTGACTACTTTGACATCTGTGTGGCAGGTTAC
CCCAAAGGCCACCCCGAAGCAGGGAGCTTTGAGGCTGACCTGAAGCACTTGAAGGAGAAG
GTGTCTGCGGGAGCCGATTTCATCATCACGCAGCTTTTCTTTGAGGCTGACACATTCTTC
CGCTTTGTGAAGGCATGCACCGACATGGGCATCACTTGCCCCATCGTCCCCGGGATCTTT
CCCATCCAGGGCTACCACTCCCTTCGGCAGCTTGTGAAGCTGTCCAAGCTGGAGGTGCCA
CAGGAGATCAAGGACGTGATTGAGCCAATCAAAGACAACGATGCTGCCATCCGCAACTAT
GGCATCGAGCTGGCCGTGAGCCTGTGCCAGGAGCTTCTGGCCAGTGGCTTGGTGCCAGGC
CTCCACTTCTACACCCTCAACCGCGAGATGGCTACCACAGAGGTGCTGAAGCGCCTGGGG
ATGTGGACTGAGGACCCCAGGCGTCCCCTACCCTGGGCTCTCAGCGCCCACCCCAAGCGC
CGAGAGGAAGATGTACGTCCCATCTTCTGGGCCTCCAGACCAAAGAGTTACATCTACCGT
ACCCAGGAGTGGGACGAGTTCCCTAACGGCCGCTGGGGCAATTCCTCTTCCCCTGCCTTT

GGGGAGCTGAAGGACTACTACCTCTTCTACCTGAAGAGCAAGTCCCCCAAGGAGGAGCTG
CTGAAGATGTGGGGGGAGGAGCTGACCAGTGAAGAAAGTGTCTTTGAAGTCTTTGTTCTT
TACCTCTCGGGAGAACCAAACCGGAATGGTCACAAAGTGACTTGCCTGCCCTGGAACGAT
GAGCCCCTGGCGGCTGAGACCAGCCTGCTGAAGGAGGAGCTGCTGCGGGTGAACCGCCAG
GGCATCCTCACCATCAACTCACAGCCCAACATCAACGGGAAGCCGTCCTCCGACCCCATC
GTGGGCTGGGGCCCCAGCGGGGGCTATGTCTTCCAGAAGGCCTACTTAGAGTTTTTCACT
TCCCGCGAGACAGCGGAAGCACTTCTGCAAGTGCTGAAGAAGTACGAGCTCCGGGTTAAT
TACCACCTTGTCAATGTGAAGGGTGAAAACATCACCAATGCCCCTGAACTGCAGCCGAAT
GCTGTCACTTGGGGCATCTTCCCTGGGCGAGAGATCATCCAGCCCACCGTAGTGGATCCC
GTCAGCTTCATGTTCTGGAAGGACGAGGCCTTTGCCCTGTGGATTGAGCGGTGGGGAAAG
CTGTATGAGGAGGAGTCCCCGTCCCGCACCATCATCCAGTACATCCACGACAACTACTTC
CTGGTCAACCTGGTGGACAATGACTTCCCACTGGACAACTGCCTCTGGCAGGTGGTGGAA
GACACATTGGAGCTTCTCAACAGGCCCACCCAGAATGCGAGAGAAACGGAGGCTCCATGA

Figure 1.4. The coding sequence of the MTHFR gene

VARIATIONS WITHIN THE MTHFR GENE

As with any other gene, the MTHFR gene is also prone to mutations. Specific mutations have the capacity to disrupt the functions of the gene, which in turn lead to the disruption of the enzyme or even the total loss of enzymatic activity. The various mutation conditions and the disorders they lead to are discussed below.

Nearly 24 different mutations in the MTHFR gene have been found responsible in causing a disorder known as *homocysteinuria.* (U.S. National Library of Medicine)

Here, due to amino acid substitution, the MTHFR gene is rendered non functional, leading to elevated levels of Homocysteine in the blood, where the excess Homocysteine is excreted out with urine. However, according to the latest findings and reports, researchers have still not been successful in how variations in the levels of Homocysteine and methionine lead to this condition.Apart from the mutations related to homocysteinuria, there are 2 other major mutations that cause elevated levels of Homocysteine in the blood. (Van Der Put, et al. 1997)

The C677T variant occurs when a cytosine residue at the 677th position is changed to a thymine residue. This causes the production of a form of MTHFR enzyme that has a reduced activity at higher temperatures, i.e. a thermo labile enzyme. Individuals with thermo labile Methylenetetrahydrofolate Reductase tend to have an increased level of Homocysteine in the blood, a condition known as Hyperhomocysteinemia.

Individuals with the C677T mutation have a high risk of suffering from cardiovascular diseases, coronary heart diseases, Alzheimer's disease & strokes in adults. The mutation also leads to birth defects such as spina bifida and other NTDs (Neural Tube Defects). (Van Der Put, et al. 1997, Medical Research Council [MRC] Vitamin Study Group 1991; Czeizel and Dudas 1992)

However, according to the Genetics Home Reference site, there is no conclusive evidence that genetic factors alone lead to disorders related to hyperhomocysteinemia. Variations in the levels of Homocysteine in the blood are also attributed to environmental conditions.

Studies show that the C677T mutation in it's homozygous form alone or as a compound heterozygote, which involves both C677T and an A1298C condition (where an Adenine residue changes to a Cytosine residue at the 1298th position) lead to the disruption of the MTHFR gene (Van der Put et al. 1995, 1996*b*, 1997) and causes a drastic reduction of the MTHFR enzyme. This in turn, leads to an elevation of Homocysteine in the blood.

It has been observed that the said homozygous C677T condition results in a 70% reduction in the MTHFR gene (Frosst, P., et al.,1995). Similar effects are observed in the compound heterozygote condition as well. These individual show almost twice the normal levels of Homocysteine in the blood. The creation of a test for the detection of the above mutations makes use of the fact that the C677T condition results in the creation of a restriction endonuclease site whereas the A1298C condition results in the abolition of a restriction site. (Van Der Put, et al. 1997) Thus, taking these facts into account, PCR amplification was initially performed to obtain suitable fragments containing the sites of the mutations. These products were then subjected to restriction digestion. The band patterns were observed on an agarose gel and a result was given. Based on the combined results of both mutation conditions, the individuals were determined to be normal or to be at risk.

The procedures and techniques followed in the study are given in detail in the following chapters.

2. MATERIALS AND METHODS

REAGENTS, ENZYMES, GLASSWARE AND PLASTICWARE

The general reagents of molecular biology grade used in the study were supplied mainly by Promega Corporation, USA and Sigma, USA. The restriction enzymes were purchased from Promega Corporation, USA. Primers for the PCR amplification were ordered from Sigma, USA. The glassware and plastic ware used for molecular biological studies, including PCR tubes, micropipette tips, centrifuge tubes, etc were sterilized by appropriate methods or were made available pre sterilized.

2.1.1. Biological samples

2.1.2. Human Blood

Human whole blood samples were collected into sterile BD vacutainer® pink top vials containing EDTA to prevent clotting. Sample labels from TO02 to TO80 were obtained with voluntary informed consent from different individuals and were supplied by the University of Sri Jayawardenapura, Colombo, Sri Lanka for molecular biological analysis.
The blood samples obtained were stored at 4 degrees C.

ISOLATION OF DNA FROM HUMAN BLOOD USING THE CHELEX EXTRACTION METHOD

20 ul of the blood was taken in a 1.5 ml centrifuge tube containing 1ml of autoclaved distilled water after mixing thoroughly by vortexing. The mixture was kept aside for 30 minutes for lysis of blood cells. The contents of the tubes were mixed by inverting halfway

through the process. Then, the tubes were centrifuged at 13000 rpm for 3 minutes, using an eppendorf micro centrifuge to obtain the leukocyte pellet. The supernatant was discarded to leave about 30 ul of liquid remaining in the tube. The pellet was washed with 1ml of autoclaved distilled water and then was broken by tapping the tube and vortexing appropriately. The washing steps were repeated twice and after the last wash, the supernatant was carefully pipetted out as not to disturb the pellet.

200 ul of 5% chelex 100 was added to each tube containing the pellet and the tubes were incubated at 56 degrees C for 20 minutes in a dry bath. The tubes were then taken out, vortexed thoroughly for 7 to 10 seconds. The tubes were then again incubated at 100 degrees C for 8 minutes.

The tube contents were again mixed thoroughly at high speed for 7 to 10 seconds. Centrifugation was then done at 12000 rpm for 2 minutes.

The extractions were stored at -70 degrees C.

POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION OF REGIONS OF INTEREST

The standard protocols followed were obtained from the St. Joseph's Hospital and Medical Centre DNA Diagnostic laboratory, Phoenix, Arizona, USA.

PCR was used in order to selectively amplify regions of interest within the MTHFR gene which would later be used for analysis of C677T and A1298C mutations.

The region responsible for the C677T mutation was amplified using the Homocysteine MT-1 and MT-2 primers, and that for the A1298C mutation was amplified using the Homocysteine MT-3 and MT-4 primers. All primers were purchased from Sigma Corporation, USA.

MT-1 and MT-2 primers

The oligonucleotide primers flank a region of 198bp within the MTHFR gene .The primer sequences are given below.

MT-1 - 5'- TGAAGGAGAAGGTGTCTGCGGGA – 3'
MT-2 - 5'- AGGACGGTGCGGTGAGAGTG – 3'

MT-3 and MT-4 primers

The oligonucleotide primers flank a region of 163bp within the MTHFR gene. The sequences are given below.

MT-3 - 5'- CTTTGGGGAGCTGAAGGACTACTAC -3'
MT-4 - 5'- CACTTTGTGACCATTCCGGTTTG – 3'

PCR assay conditions for amplification.

The reaction mixture for the PCR amplification used is as follows.

10X PCR buffer (containing $MgCl_2$) - 2.5 ul
2 mM dNTP - 2.5 ul

Forward primer (10 uM)	- 1.0 ul (0.4 umoles)
Reverse primer (10 uM)	- 1.0 ul (0.4 umoles)
Sterile distilled water	- 12.8 ul
Taq DNA polymerase (5units/ul)	- 0.4 ul (2 units)
Sample DNA extraction	- 5.0 ul (unknown concentration)
Total	-25.0ul

The PCR reaction mixture was prepared using a separate set of pipettes and micropipette tips in a UV sterilized chamber. In instances where more than one reaction tube was prepared, a master mixture was initially prepared out of which 20 ul were pipetted out into each tube containing the extracted DNA. A negative control was also used in certain cases to confirm the absence of contaminating DNA.

PCR programme

The amplification programme used is as given below.

Step 1	Initial denaturation at 94 degrees C for 3 minutes.
Step 2	35-40 cycles of:
	Denaturation at 94 degrees C for 30 seconds
	Primer annealing at 65 degrees C for 30 seconds
	Primer extension at 72 degrees C for 30 seconds
Step 3	Final primer extension at 72 degrees C for 4 minutes. .

The protocol was optimized for maximum performance and yield and the given conditions were found to be best suited for amplification.The same conditions were used with both sets of primers, i.e. (MT-1 & MT-2) and (MT-3 & MT-4) except for using an increased cycle number of 40 for the (MT-3 & MT-4) primers.

VISUALIZATION OF PCR PRODUCTS BY AGAROSE GEL ELECTROPHORESIS

The PCR amplified DNA fragments were checked by 2% Agarose gel electrophoresis with an aliquot of 8 ul of the PCR product along with 2ul of the gel loading dye. A DNA ladder of either 50 bp or 100 bp (Promega, USA) was used to verify the band size.

The gel was applied an initial 50V which was later increased to 100V as the DNA started migrating. Photographs were taken under the UV transilluminator which were then visually analyzed for the expected band size. (please refer chapter 03-Results and Discussion for gel photos)

PCR AMPLIFICATION OF DMD INTERNAL CONTROL

According to standard protocol, an internal control was used for the restriction digestion of the fragments for C677T mutation detection. This was done in order to avoid misinterpretation of the RFLP results, as will be explained later.

For this, DNA extracted from human male blood samples of individuals suffering from Duchenne Muscular Dystrophy (DMD) using the chelex DNA extraction protocol was used. The sample was amplified using the DMD 48 primer whose sequence is given below. A band size of 574bp was expected.

DMD 48 (forward primer) -5' TTGAATACATTGGTTAAATCCCAACAT -3'
DMD 48 (reverse primer) -5' CTTGAATAAAGTCTTCCTTACCACAC -3'

The reaction mixture for the PCR amplification used was as follows.

10X PCR buffer (containing MgCl2)	- 2.5 ul
5 mM dNTP	- 1.25 ul
Forward primer (5 uM)	- 1.0 ul (0.2 umoles)
Reverse primer (5 uM)	- 1.0 ul (0.2 umoles)
Sterile distilled water	- 13.0 ul
Taq DNA Polymerase (5 units/ul)	- 0.25 ul (1.25 units)
Sample DNA extraction	- 3.0 ul (concentration unknown)
Total	- 25.0 ul

PCR programme

The amplification programme used is as given below

Step 1	Initial denaturation at 94 degrees C for 5 minutes.
Step 2	35 cycles of:
	Denaturation at 94 degrees C for 30 seconds
	Primer annealing and extension at 65 degrees C for 7 minutes

The given programme was optimized for maximum efficiency and yield in the laboratory and was performed using a Roboycler Gradient machine of the STRATAGENE Corporation, USA.

Visualization of PCR products by agarose gel electrophoresis

The PCR products thus obtained were subjected to gel electrophoresis and the samples yielding an expected band size of 574bp were used in the restriction digestion assay. (please refer Results and Discussion for gel photos)

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ASSAY

The standard protocols followed were obtained from the St. Joseph's Hospital and Medical Centre DNA Diagnostic laboratory, Phoenix, Arizona, USA

The obtained DNA fragments of the desired length were then subjected to restriction enzyme digestion in a technique known as Restriction Fragment Length Polymorphism (RFLP). Here, the particular fragment is digested using a specific restriction digestion enzyme which yields band sizes of varying lengths, depending on the condition of the fragment. Thus a final result could be given depending on the digestion result.

Enzymes used

Two different enzymes are used in the RFLP assay, both provided by the Promega Corporation as a kit. The enzymes were:

Hinf I – for C677T mutation detection
Mbo II- for A1298C mutation detection

The enzymes were supplied in the following manner:

- Hinf I was supplied as 1000 units of the enzyme in a storage buffer containing 10mM Tris-HCl (pH 7.3), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA and 50% glycerol.
- Mbo II was supplied as 10 units of the enzyme in a storage buffer containing 10mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA and 50% glycerol.

Hinf I

Hinf I is a restriction endonuclease enzyme obtained from *Haemophilus influenzae* and cuts the DNA strand at the recognition site given below.

5'------G▼ANTC------3'
3'------CTNA▲G------5'

Mbo II

Mbo II is a restriction enzyme found in *Moraxella bovis* and cuts the DNA strand at the following recognition site.

5'------GAAGA(N)8▼------3'
3'------CTTCT(N)7▲ ------5'

Both enzymes were supplied along with:

Buffer B

The buffer has a concentration of 10X and a composition of 60 mM Tris-HCl (pH 7.5), 500 mM NaCl, 60 mM MgCl2 and 10 mM DTT at 37 degrees C.

BSA

Acetylated BSA is added in all Promega restriction enzyme reactions to a final concentration of 0.1 mg/ml. BSA has shown to enhance the enzyme activity.

MULTI-CORE Buffer

Composition of 250 mM Tris acetate(pH 7.8), 1M potassium acetate, 100 mM magnesium acetate and 10 mM DTT.
However, in this analysis, the multi core buffer was not used, as the 10X buffer B alone proved to be more efficient.

C677T MUTATION DETECTION

The PCR products obtained by using the primers MT-1 and MT-2 were used to detect the C677T mutation.

For this assay, an internal control was required. As mentioned earlier, a DMD internal control was used. This was necessary, as the original PCR product and the digested product yielding a positive result for homozygous wild type genotype were of the same band size (198bp); therefore, an inactivation of the restriction enzyme would not be noticed and would lead to a misinterpretation of the result obtained.

The digestion reaction mixture was prepared according to the standard protocol provided by Promega (USA) who supplied the enzyme kit.The reaction mixture was prepared as follows;

Sterile distilled water	-8.3 ul
10X Buffer B	-2.0 ul
BSA	-0.2 ul (2 x 10^{-5} mg)
Hinf I	-0.5 ul (5 units)
Sample DNA	-8.0 ul
DMD PCR product	-1.0 ul
Total	20.0 ul

The reaction mixture thus prepared was kept in an incubator maintained at 37 degrees C overnight. The following day, the mixture was taken out and was subjected to gel electrophoresis. Fragments of digested DNA thus obtained were visually analyzed and a result was given accordingly. The reference table followed is given below, which gives the respective genotypes for each fragment combination.

Table 2.1. Expected bands for each genotype at position 677

677 GENOTYPE	HOMOZYGOUS WILD TYPE 677CC	HETEROZYGOUS MUTANT 677CT	HOMOZYGOUS MUTANT 677TT	DMD 48 CONTROL DIGEST
FRAGMENT SIZES				236 bp
	198 bp	198 bp		
				189 bp
		175 bp	175 bp	
				81 bp
		23 bp	23 bp	

Visualization of digestion products by agarose gel electrophoresis

The digested products were loaded on a 2% agarose gel and were subjected to electrophoresis, along with a 50 bp DNA ladder, provided by Promega (USA). An initial voltage of 50V was supplied till the DNA moved out of the wells and started migrating and the voltage was increased to 100V. An early photograph of the gel was taken as soon as the loading dye became visible just outside of the wells. (Refer-Results and Discussion for gel photos)

A result was given based on the table above.

A1298C MUTATION DETECTION

The PCR products obtained by using the primers MT-3 and MT-4 were used to detect the A1298C mutation. For this assay, no internal control was required. The digestion reaction mixture was prepared according to the standard protocol provided by Promega (USA) who supplied the enzyme kit.

The reaction mixture was prepared as follows.

Sterile distilled water	-5.44 ul
Buffer B	-1.40 ul
BSA	-0.15 ul ($1.5x10^{-5}$ mg)
Mbo II	-0.01 ul (0.1 units)
Sample DNA	-8.00 ul
Total	-15.0 ul

The reaction mixture was incubated overnight at 37 degrees C and the mixture was subjected to gel electrophoresis. The results were given according to the following table.

Table 2.2. Expected bands for each genotype at position 1298

1298 GENOTYPE	HOMOZYGOUS WILD TYPE 1298AA	HETEROZYGOUS MUTANT 1298AC	HOMOZYGOUS MUTANT 1298CC
FRAGMENT SIZE		84 bp	84 bp
	56 bp	56 bp	
	31 bp	31 bp	31 bp
	30 bp	30 bp	30 bp
	28 bp	28 bp	
	18 bp	18 bp	18 bp

Visualization of digestion products by agarose gel electrophoresis

The digested products were loaded on a 2% agarose gel and were subjected to electrophoresis, along with a 50 bp DNA ladder, provided by Promega (USA). An initial voltage of 50V was supplied till the DNA moved out of the wells and started migrating and the voltage was increased to 100 V. An early photograph of the gel was taken as soon as the loading dye became visible just outside of the wells. (Refer -Results and Discussion for gel photos)

A result was given based on the table above.

3. RESULTS AND CONCLUSIONS

PCR AMPLIFICATION RESULTS

The PCR amplification was performed in order to obtain DNA fragments containing the regions responsible for the C677T and A1298C mutations, using the (MT-1 & MT-2) and (MT-3 & MT-4) primers respectively.

The regions responsible for C677T and A1298C mutations shall be referred to as system 1 and system 2 respectively from here onwards, for the sake of convenience.

During the initial stages, the PCR for system 1 yielded strong bands. All samples gave satisfactory results. However, the PCR assays for system 2 gave weak bands, and as a result, were not found suitable to be subjected to restriction digestion. Therefore, the PCR conditions had to be modified, thus the cycle number for system 2 was increased to 40 from the previous 35, during the latter stages. The PCR program has been given in detail in the previous section. This modification gave a satisfactory yield.

The PCR amplification done to obtain the DMD internal control gave satisfactory results as well, with bands of 574bp. However, only a limited number of amplifications were done, as the internal control was discontinued, as will be explained later.

The gel photographs obtained for each PCR assay are shown below.

PCR assay results for the amplification of MTHFR gene segments

The following figures show the gel photographs obtained using the 2 sets of primers as described.

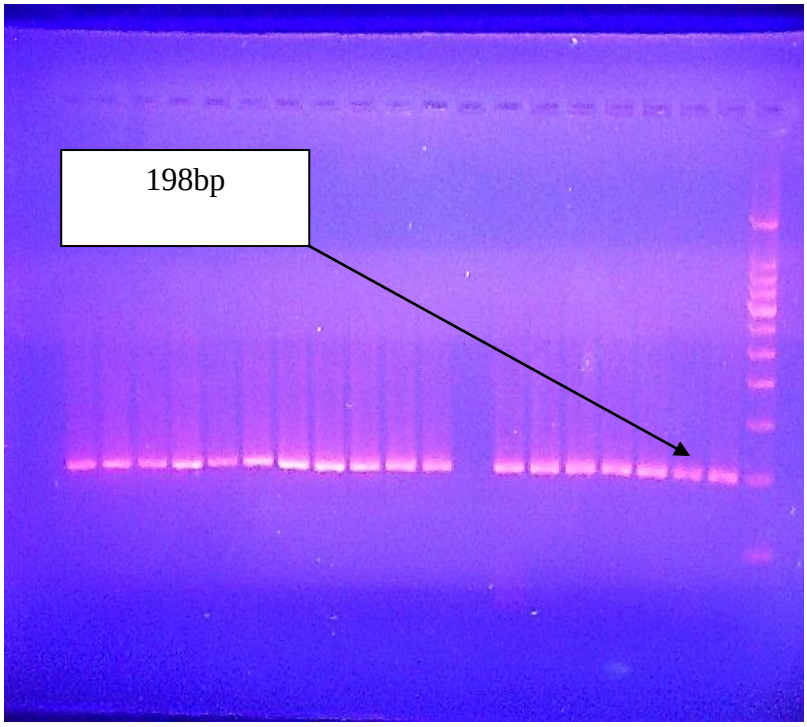

Figure 3.1. A gel photograph showing amplified DNA fragments of 198 bp using the MT-1 and MT-2 primers (with a 100 bp ladder)

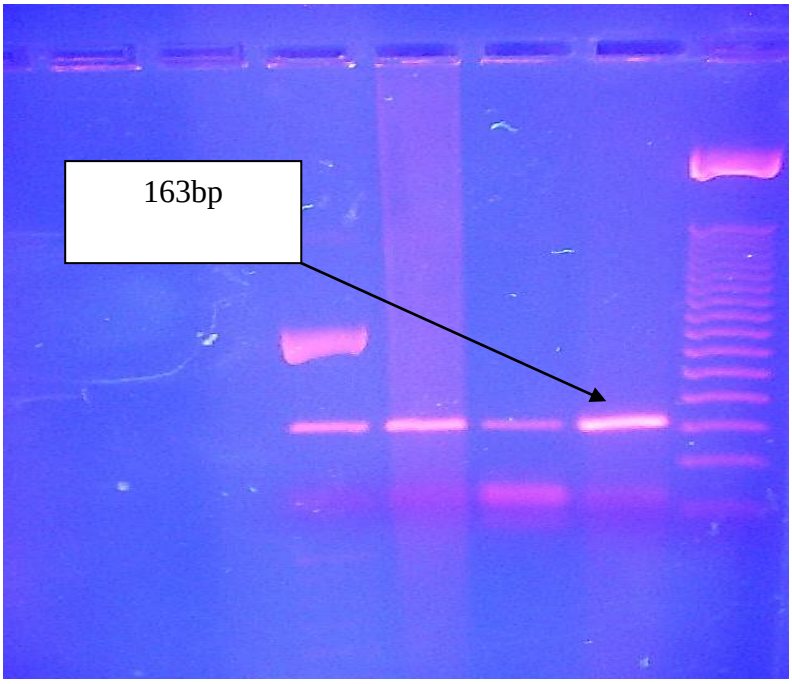

Figure 3.2. A gel photograph showing amplified DNA fragments of 163 bp using the MT-3 and MT-4 primers (with a 50 bp ladder)

PCR assay results for amplification of DMD internal control

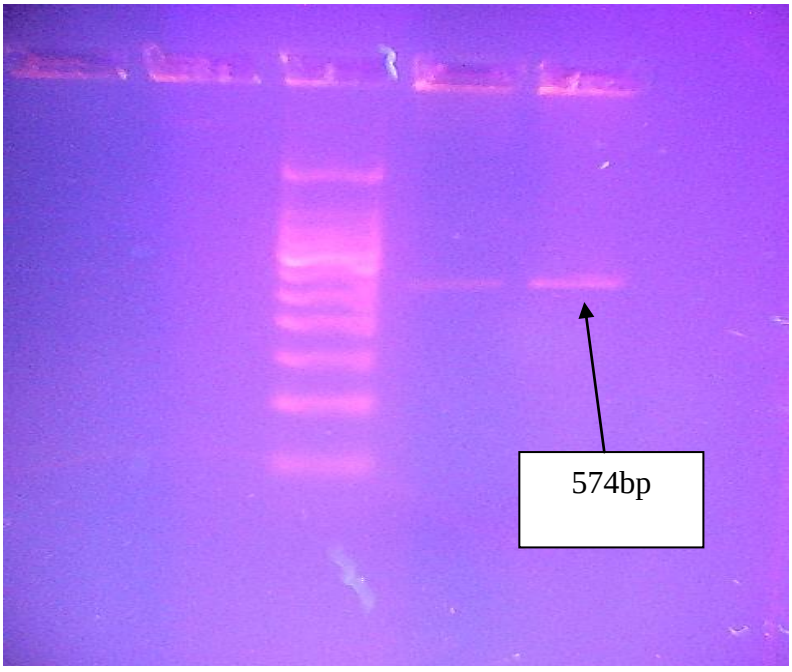

Figure 3.3. A gel photograph showing 2 PCR products of DMD internal control with the expected band size of 574bp. (with a 100bp ladder)

RFLP ASSAY RESULTS

The assay was performed using the Promega® restriction enzymes, as mentioned earlier. The PCR products of system 1 yielded bands of good visibility upon digestion and gel electrophoresis, and the results were easily obtained. However, there was a drawback in using the DMD internal control. This was due to the fact that the bands obtained by the samples along with the DMD internal control, i.e. the 175 bp, 189 bp, & 198 bp bands interfered with each other and made it impossible to safely give a result without the risk of misinterpretation. Thus, the use of the DMD control was discontinued after a few initial trials.

As was mentioned earlier, the system 2 was optimized in its PCR amplification. However, even with the modified conditions, some samples did not give clear bands upon digestion. This led to the repetition of the said samples with PCR and subsequent digestions. Strong and clear bands were a crucial factor for system 2 as the digested bands were of relatively smaller sizes. (84 bp, 56 bp, 31 bp, 30 bp, 28 bp & 18 bp)

Due to the failure in obtaining digestion results for some of the samples to detect the A1298C allele, the outcome of the analysis had to be limited for the samples which gave acceptable results.

Results for restriction digestion of fragments for C677T allele detection

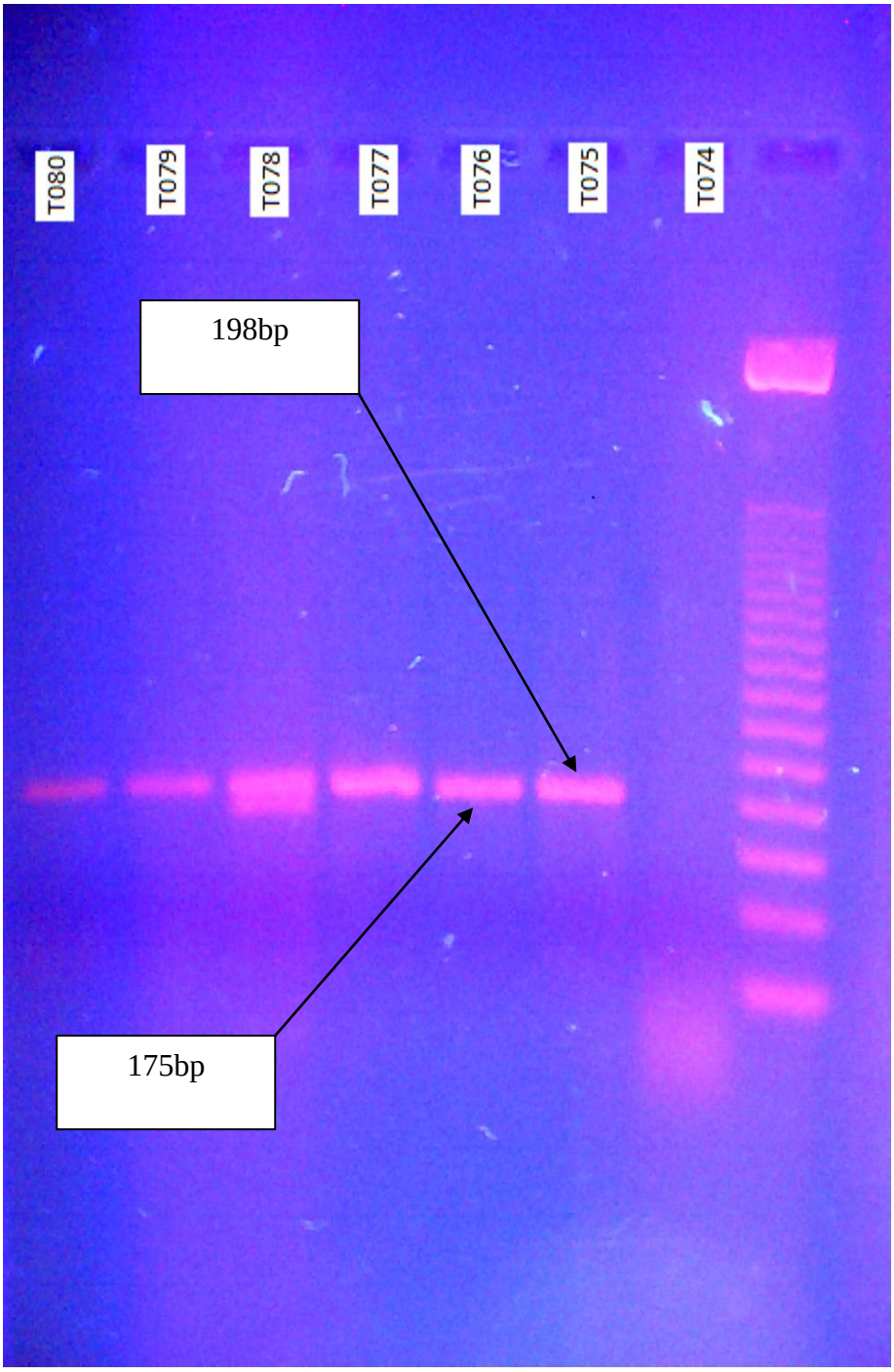

Figure 3.4. Both homozygous wild type and heterozygous mutant genotypes (with a 50 bp ladder)

Results for restriction digestion of fragments for A1298C allele detection

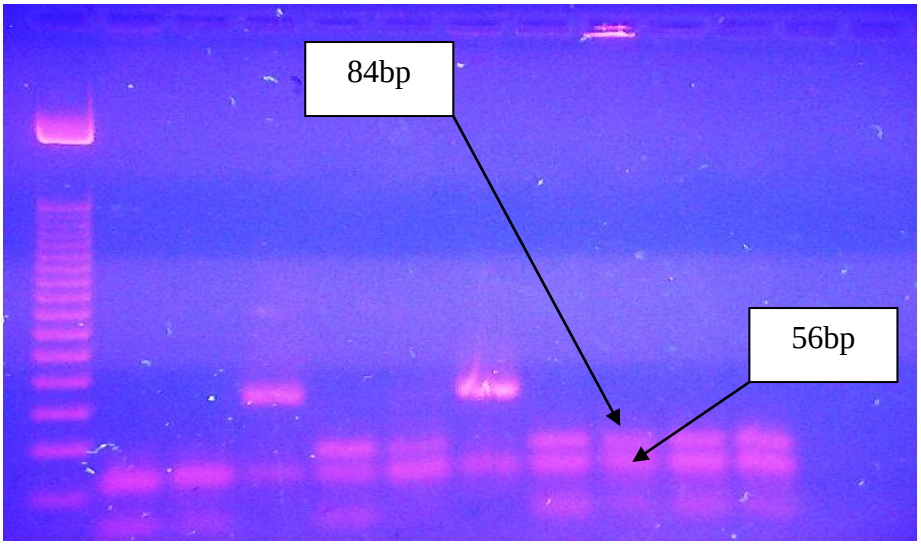

Figure 3.5. Positive results for both homozygous wild type and heterozygous mutants with a 50 bp ladder.

The results obtained by the RFLP assay were used as a guide to give a final result. Based on this, one could determine if an individual is at a high risk or normal genetic status. The following tables give the genetic combinations expected and the result each combination would give. This was followed in giving the final overall result at the end of the study.

INTERPRETATION OF RESULTS

The following tables give the combinations of genotypes which enable to determine if a person is normal and unaffected or is at risk. The final overall result was given based on the given genotype combinations. It is interesting to note that the homozygous wild type condition at the 677 position puts the individual at NO RISK regardless of the genotype condition at the 1298 position. Therefore, this enables a final conclusive result to be given even without knowing the 1298 condition, provided the 677 position was mutation free.

NORMAL & UNAFFECTED

Table 3.1. Genotype combinations which render the individual normal and unaffected

C677T	A1298C
HOMOZYGOUS WILD TYPE	HOMOZYGOUS WILD TYPE
HOMOZYGOUS WILD TYPE	HETEROZYGOUS MUTANT

HOMOZYGOUS WILD TYPE	HOMOZYGOUS MUTANT
HETEROZYGOUS MUTANT	HOMOZYGOUS WILD TYPE

AT RISK

Table 3.2. Genotype combinations which render the individual at risk

C677T	A1298C
HETEROZYGOUS MUTANT	HETEROZYGOUS MUTANT
HETEROZYGOUS MUTANT	HOMOZYGOUS MUTANT
HOMOZYGOUS MUTANT	HOMOZYGOUS WILD TYPE
HOMOZYGOUS MUTANT	HETEROZYGOUS MUTANT
HOMOZYGOUS MUTANT	HOMOZYGOUS MUTANT

ASSESSMENT OF RISK FACTOR OF HYPERHOMOCYSTEINEMIA BASED ON GENETIC ANALYSIS.

RESULTS FOR C677T MUTATION DETECTION

All 79 samples analyzed to detect the 677 genetic condition yielded results.

The results thus obtained by the RFLP assay of the samples were tabulated. The overall result is as follows. 89.9% of individuals were NORMAL (HOMOZYGOUS WILD TYPE), 3.8% of individuals were HETEROZYGOUS MUTANTS and 6.3% of individuals were HOMOZYGOUS MUTANTS. The data obtained for the analysis was used to calculate the allele and genotype number and the corresponding frequencies.

Table 3.3. The allele and genotype numbers with the corresponding frequencies for the C677T allele

Allele/ Genotype	Allele/ Genotype number	Frequency
C	145	91.8%
T	13	8.2%
CC- HOMOZYGOUS WILD TYPE	71	89.9%
CT- HETEROZYGOUS MUTANT	3	3.8%
TT- HOMOZYGOUS MUTANT	5	6.3%

RESULTS FOR A1298C MUTATION DETECTION

For the analysis of the 1298 genotype, however, not all samples yielded results. Only 55 samples gave distinguishable bands upon electrophoresis.

The overall result obtained was as follows.

41.8% of individuals were NORMAL (HOMOZYGOUS WILD TYPE)

32.7% of individuals were HETEROZYGOUS MUTANTS

25.5% of individuals were HOMOZYGOUS MUTANTS

The allele number and genotype number, along with the respective frequencies were calculated as follows.

Table 3.4. The allele and genotype numbers with the corresponding frequencies for the A1298C allele

Allele / Genotype	Allele/ Genotype number	Frequency
A	64	58.2%
C	46	41.8%
AA- HOMOZYGOUS WILD TYPE	23	41.8%
AC - HETEROZYGOUS MUTANT	18	32.7%
CC - HOMOZYGOUS MUTANT	14	25.5%

Of 79 individuals tested, there was observed a C677T allele frequency of 3.8%. A frequency of 6.3% was observed for individuals homozygous for the mutation. As for the A1298C condition, only a total sample number of 55 were tested. Of these, 32.7% corresponded to the allele frequency whereas 25.5% were homozygous mutants. It should be mentioned that we encountered NO samples positive for the combined heterozygosity of C677T and A1298C conditions.

By the combined analysis of results for both C677T and A1298C genotype conditions, an individual is declared to be NORMAL AND UNAFFECTED or to be AT RISK. The guidelines followed in arriving at this conclusion were elaborated upon previously.

It should be mentioned that even though the study did not succeed in analyzing all samples which were PCR amplified to detect the 1298 genotype condition, a conclusive result was given, as the 677 genotype in it's homozygous wild type condition, renders the individual at no risk, regardless of the genetic condition at the 1298 position. Similarly, the homozygous mutant of C677T renders the individual at risk and does not depend on the 1298 position genotype.

Thus, the final overall result obtained was for 77 out of 79 samples.

Of the 77 samples tested for C677T and A1298C mutation conditions,

93.5% OF INDIVIDUALS ARE AT NO RISK OF HYPERHOMOCYSTEINEMIA

6.5% OF INDIVIDUALS ARE AT RISK OF HYPERHOMOCYSTEINEMIA

4. DISCUSSION AND CONCLUSIONS

Homocysteine is an important compound in clinical biochemistry, as its elevated levels lead to various diseases as proven by studies. These include congenital malformations, adult cardiovascular disease, stroke, coagulation abnormalities, etc. Hyperhomocysteinemia has been associated with mutations of the MTHFR gene, mainly the C677T and A1298C mutations. These mutations result in a faulty gene product that fails to lower the concentration of Homocysteine in the blood.

Studies regarding the relationship between elevated levels of Homocysteine & related disease conditions and mutation conditions of the MTHFR gene have been performed at various locations around the world for the benefit of the medical community and in order to gain a better insight into the risks faced by individuals suffering from hyperhomocysteinemia.

The facts

According to the World Health Organization, the American Heart Association and the Alzheimer's Society of UK,

- At least one in two people die due to potentially preventable diseases. At least one in two individuals has elevated levels of Homocysteine in the blood.
- High Homocysteine levels increase the risk of heart attack by 50%

Globally, 16.7 million people die annually due to cardiovascular disorders.

- 2.5 million people die annually from heart attacks and strokes, and the disorders affect 32 million.

40% of deaths caused by stroke were attributed to high Homocysteine levels.

- Homocysteine levels above 14 umol/l increase the risk of developing Alzheimer's disease by 150%

Nearly 18 million suffer globally due to dementia, to which Alzheimer's disease contributes greatly. These facts show the impact on human health by the elevated concentration of a single amino acid. Thus it is clear that studying diseases related to hyperhomocysteinemia along with the genetic conditions of the individuals involved is important in medicine. Measuring of the Homocysteine levels in the blood alone is insufficient as it cannot determine if a person is at risk due to a faulty MTHFR gene.

Therefore, a combined biochemical and molecular biological assay could be performed in order to determine the cause for a disease suspected to be linked to hyperhomocysteinemia. However, the molecular biological assay would have a much greater potential in diagnosing a probable impairment of a person to control the blood Homocysteine concentration. This would allow the individual to take necessary precautions.

From the study conducted, it is clear that the C677T mutation is the dominant mutation of the two. However, quite unexpected patterns emerged during the analysis of the said mutation condition, as we observed a greater number of individuals with the homozygous

mutation condition than those with the heterozygous mutation condition. (see the results table on previous pages). *In a normal study, with randomly acquired samples, generally the reverse would be expected*. However, since this was a study conducted using a relatively low number of samples (79), we could not come to a definite conclusion based on the observation. Furthermore, we were not notified on how the samples were collected, thus there is a possibility of this not being a random study.

The results from the study were then compared to results of other studies conducted elsewhere in the world. However, this did not give a clear pattern to follow, since, as mentioned earlier, the mutation conditions did not follow a constant distribution, but varied depending on the region, ethnic groups, age and even gender. Thus, our results merely gave us a pattern amongst the Sri Lankan population.The data from studies were obtained from the website of the National Office of Public Health Genomics which were made available through a HuGENET ^{R} review. According to their reports, there is a clear variation of the allele frequencies amongst the world population.

The C677T allele, being the more dominant, has been the focus of a larger number of studies with respect to the A1298C allele. The C677T allele was shown to be highest amongst Italians and also was seen to be abnormally high amongst white Hispanics. However, it is much lower in individuals from the region of Sub Saharan Africa. Following is a short overview of the findings of the said study.

Studies done on the C677T allele give evidence for the uneven allele distribution

Among whites from Europe, the allele frequency ranged from 22% in Norway to 44% in Italy. Britain and Ireland showed frequencies of 37% and 32% respectively, both countries having a high occurrence of NTDs. As expected, countries showing high allele frequencies also showed abnormally high occurrences of the homozygous condition. (ex. 18% in Italy).

Blacks from the Sub Saharan African region, on the other hand, showed very low allele frequencies of 7% in two major studies. One study involving four different tribes showed the total absence of the C677T homozygous condition.

African descendants in Brazil had a frequency of 5% and those of the U.S. had a 24% frequency. Studies conducted in Asia are very few; so much less data is available. However, a major Japanese study involving 3300 individuals showed an allele frequency of 34%.
Hispanic whites showed a relatively high frequency of 42% and the same frequency was seen in a group of Colombians. However, a major drawback in most studies has been the failure to report results based on age and gender. Nevertheless, a few studies have shown a low allele frequency among new born females than in males. A Japanese study showed the allele frequency to be lower in older people than among the young. The same pattern was observed in a Netherlands study, but was found to be true only amongst males.

Despite major studies conducted on the C677T allele, a lot less has been done on the A1298C allele. Studies in Canada and the Netherlands however, have shown the allele frequency to be nearly 9%. As for the compound heterozygous condition, the frequency was found to be 15% in Canada, 17% in the U.S. and 20% in the Dutch study.

What we observed from our study is as follows.

Considering the C677T allele, the frequency of 3.8% is extremely low when compared to other studies. The lowest frequency, reported from the region of Sub Saharan Africa is 7%. Even the frequency for the homozygous mutants is extremely low. Considering the A1298C

allele, the frequency obtained during the study which was 32.7% is extremely high when compared to those from Canada and the Netherlands, which were both reported as 9%.

The project conducted at GENETECH gave us satisfactory results, though being of a small scale. However, there was a major drawback as not being able to look into the medical conditions of the individuals. This was due to the samples belonging to the University of Sri Jayawardenapura, where the actual research was being done. The GENETECH research laboratory merely played the role of molecular biological diagnostics. However, armed with greater information, a much more conclusive outcome could have been expected.

Regarding the future prospects of research based on detection of C677T and A1298C mutations within the MTHFR gene and coming to a conclusion on the risk factor associated with the genetic condition of the individual, it can be safely said that a molecular biological research laboratory such as GENETECH could conduct a much widespread study regarding this matter. Since, according to research papers published, lot less data is available from the Asian region, Sri Lanka could perform a major study to throw light on the subject. In collaboration with a Biochemistry laboratory at one or more leading hospitals in the country and involving volunteers, a database could be set up, with studies related to genetic variations through geographical distribution, age, gender, etc. being taken into consideration. Thus, scientists both in Sri Lanka and abroad would have more data and background information to conduct further studies in the future.

The benefits gained by the medical community would also be large as they would have a clearer idea and much more information when dealing with a disorder suspected to be linked to hyperhomocysteinemia. A combined biochemical and molecular biological assay could be encouraged for such individuals under the guidance of a medical practitioner.

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