Chapter 7: Prenatal diagnosis of Sickle cell anaemia using Polymerase Chain reaction.

7.1: Introduction - Prenatal Diagnosis.

In the ancient days (1690 A.D.) the knowledge of developing fetus was obtained by performing postmortem of the gravid uterus. Later blighted ova and early aborted embryos had been studied. But these studies were mostly anatomical ones. Spontaneous abortions also provided the material for biochemical studies of fetal blood and tissues, revealing important physiological changes during development, while the living fetus, safe inside the mother's womb, has been palpated, listened to, X-rayed for much of the 20th century. But now for the last 15 years we can visualize the fetus by ultra-sonography, and the environment can be examined by amniocentesis. Under the guidance of ultrasound, samples of blood and fetal tissue can be taken for analysis. These things bring the modern laboratory technique for the direct diagnosis of living fetus.

Table 7.1.1 shows structural anomalies diagnosed by ultrasound examination. Table 7.1.2 shows disorders diagnosed by fetal blood sampling while table 7.1.3 shows diseases diagnosed by either amniotic fluid or chorionic villus sampling.
Table 7.1.1: Table showing structural anomalies diagnosed by ultrasound.

| 1. Craniospinal defects. |
| 2. Fetal tumours.        |
| 4. Urinary tract anomalies. |
| 5. Limb deformities.     |
| 6. Cardiac anomalies.    |

Table 7.1.2: Table showing disorders diagnosed by fetal blood sampling.

| 1. Hemoglobinopathies. |
| 2. Coagulation disorders. |
| 3. Metabolic disorders.  |
| 4. Fetal Infection.     |
| 5. Chromosomal anomalies. |
| 6. Duchenne Muscular Dystrophy. |
| 7. Severe combined Immunodeficiency. |

Table 7.1.3: Table showing disorders diagnosed by examination of amniotic fluid or chorionic villus biopsy.

| 1. Neural Tube Defects. |
| 2. Chromosomal anomalies. |
| 3. Metabolic disorders. |
| 4. Haemophilia.          |
| 5. Hemoglobinopathies.   |
7.2 Sickle cell anaemia - Prenatal diagnosis.

Prenatal diagnosis for sickle cell anaemia was first introduced in 1972. This marked the beginning of a technology for prenatal diagnosis of hemoglobinopathies. Detection of sickle hemoglobin became a practical reality, when methods for acquisition of fetal blood and for analysis of globin chain synthesis were developed. In the beginning fetoscopic blood sampling and the presence of beta S chains was used for diagnosis. However, a fetal loss of about 5% due to these invasive procedures has provided the impetus for the development of diagnostic approaches that use fetal DNA rather than fetal blood.

Examination of amniotic cell or chorionic villus DNA for a specific genetic trait offers a safe and general method for prenatal detection of genetic disease. Molecular analysis was first performed by Kan and his co-workers for alpha thalassemia when liquid hybridization methods permitted estimation of gene number. Subsequent techniques of restriction enzyme mapping of genes were applied to detect structural gene deletions in fetal DNA. Detection of hemoglobinopathies resulting from point mutations in the DNA, however, requires a more specific assay.

An indirect approach to identification of the sickle gene in DNA was developed by Kan and Dozy, on the basis of their finding that in American blacks, the gene was associated with a variant HpaI restriction site flanking the beta gene. In most normal human DNA digested with HpaI,
the beta globin gene occurred on a DNA fragment 7.6 kb long. Polymorphisms at this site in West African populations resulted in the beta gene on fragments 7.6 and 13.0 kb long. It was found that 13.0 kb fragment was linked to the beta S gene (figure 7.2.1). Though safe and useful, the approach requires family study to establish linkage arrangement. Often, however, linkage analysis cannot distinguish normal and mutant genes within a family. This technique is applicable in only 70% African black cases. In case of sickle cell gene present in India, such linkage is not observed.

Direct identification of mutant gene in DNA is possible by virtue of the specificity of restriction enzymes. A single nucleotide change in an enzyme's cleavage site is readily detected if the appropriate enzyme is used. In this method first DNA is digested with a suitable restriction enzyme and the fragments are resolved on agarose gel. The separated fragments are then transferred to a support, either nitrocellulose or nylon membrane by Southern blotting technique and then hybridized with radioactive labelled probes and fragment size is determined. Now, instead of radioactive labelling different nonradioactive chemical labelling as available, still its sensitivity is not as good as radioactive probing. In 1978 Nienhuis suggested that the sickle mutation (glutamic acid to valine in position 6 of the beta chain or CCTGAGG to CCTGTTGG in the DNA) might be detected directly with the enzyme MnlI, which recognizes the sequence GAGG. This sequence is present 10 times in beta
Fig 7.2.1: Figure showing polymorphisms of Hpa I recognition site on the 3' side of the beta globin gene and prenatal diagnosis using Hpa I polymorphic site.
globin gene, and the small fragments that result are not suitable for restriction mapping. Subsequently Geever in 1981 demonstrated that a different enzyme Ddel, which recognizes the sequence CTNAG (where N is any nucleotide), could be used to distinguish normal from sickle genes. Chang and Kan have reported the successful application of this assay to the prenatal diagnosis of sickle cell anaemia. However, this method has technical limitations that have hampered its wide use in clinical situations.

Ddel cleavage sites are frequent in beta globin gene and its immediate flanking region. The A to T mutation within codon 6 of the beta S gene alters a normal Ddel site. Thus, in normal DNA digested with Ddel enzyme, DNA fragments 0.17 and 0.20 kilobases long are generated by sites immediately upstream and downstream from this position, whereas a 0.37 kilobases fragment is generated from the beta S gene because of loss of one Ddel site. Modifications of techniques for restriction enzyme mapping are required to identify these small DNA fragments, and they considerably reduce the sensitivity of the method. Recognizing these difficulties, Chang and Kan advocated its use only in the absence of DNA polymorphism suitable for linkage analysis. Experience of Stuart et al with this method indicate that many laboratories skilled in restriction enzyme mapping procedures found it exceedingly difficult to apply clinically. Stuart et al and Chang et al reported another assay in 1982, for sickle mutation in DNA that is free of the limitations imposed by
the methods in use. It differs from the method of Geever et al in the selection of the restriction enzyme, MstII, that cleaves DNA, on an average, less frequently than DdeI. The fragments, 1.15 and 1.35 kilobases generated from beta A and beta S genes, respectively, were ideally suited for restriction enzyme mapping (Fig 7.2.2.). But use of these conventional methods of restriction mapping analysis was limited due to,

1. Large amount of genomic DNA required for analysis,
2. Requires radioactive probes,
3. Result takes minimum 1 week,
4. Procedures are tedious and laborious.

In 1985, Saiki et al performed polymerase chain reaction and used it for the diagnosis of sickle cell anaemia. Amplification of beta globin DNA sequences that carry the sickle mutation, was carried out using Klenow fragment of E. coli DNA polymerase. This provided sufficient quantity of DNA for analysis. Next, a short radiolabeled synthetic DNA sequence homologous to normal beta A globin sequences was hybridised to the amplified target sequences. The hybrid duplexes are then digested sequentially with two restriction endonucleases. The presence of beta A or beta S sequences in the amplified target DNA from the patient determines whether the beta A hybridization probe anneals perfectly or with a single nucleotide mismatch. This difference affects the restriction enzyme digestion of the DNA and the size of the resulting radiolabeled digestion products, which can be
Fig 7.2.2: Figure showing recognition sites for Mst II restriction enzyme used in antenatal diagnosis of sickle cell disease.
distinguished by electrophoresis followed by autoradiography. This method was sufficiently sensitive and rapid that the prenatal diagnosis can be made within one day. This method used Klenow fragment of DNA polymerase I which is thermodabile and radioactive probes which are hazardous.

7.3 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was invented by Saiki and originally applied by a group in Human Genetics Department at Cetus for amplification of human beta globin DNA and for the prenatal diagnosis of sickle cell anaemia. The method, (PCR), first described in 1985 by scientists at Cetus Corp. (Division of Perkin Elmer) was a technique that enabled them to isolate and obtain substantial quantity of defined DNA sequences. Employing PCR in concert with newly refined DNA hybridization techniques provided clinical diagnostics with a distinctly powerful and precise tool. Although a relatively new technique, PCR amplification has already found extensive application in the diagnosis of genetic disorders, the detection of nucleic acid sequences of pathogenic organisms in clinical samples, the genetic identification of forensic samples, and the analysis of mutations in activated oncogenes.

Polymerase Chain Reaction is used to amplify a segment of DNA that lies between two regions of known sequence. As shown in the figure 7.3.1 and 7.3.2 two oligonucleotides are used as primers for a series of synthetic reactions that are catalyzed by a DNA polymerase. These oligonucleotides
Fig 7.3.1: Figure showing typical PCR of a DNA using primers P1 and P2. Only two cycles are shown.
Fig 7.3.2: Figure showing PCR reaction after n cycles.
typically have different sequences and are complementary to the sequences that (a) lie on opposite strands of the template DNA and (b) flank the segment of DNA that is to be amplified. The template DNA is first denatured by heating in presence of large molar excess of each of the two oligonucleotides and the four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerase. The cycle of denaturation, annealing, and DNA synthesis is then repeated many times. Because the product of one round of amplification serve as templates for the next, each successive cycle essentially doubles the amount of the desired DNA product.

The major product of this exponential reaction is a segment of double stranded DNA whose termini are defined by the 5' termini of the oligonucleotide primers and whose length is defined by the distance between the primers.

The original protocols for polymerase chain (94,95,96) reactions, used the Klenow fragment of E.coli DNA polymerase I to catalyze the extension of the annealed oligonucleotide primers. However this enzyme is inactivated at the temperatures that are required to denature the DNA, each round of synthesis required the addition of fresh aliquot of enzyme. Although these reactions generally worked well for the amplification of smaller fragments of DNA (<200 bp), the results with larger templates were disappointing.
Frequently the yield was poor, and the products were often heterogeneous in size, presumably because 'mispriming' occurred at the temperatures used for catalytic extension of the oligonucleotides. In addition, some of the products were 'shuffled' and consisted of mosaics of different alleles of the target sequences.

These problems were solved with the introduction of a thermostable DNA polymerase purified from the thermophilic bacterium Thermus aquaticus (Taq DNA polymerase). This enzyme which can survive extended incubation at 95°C, is not inactivated by the heat denaturation step and does not need to be replaced at every round of the amplification cycle. In addition, because annealing and extension of oligonucleotides can be carried out at elevated temperatures, mispriming is greatly reduced. This results in substantial improvements in the specificity, yield of the amplification reaction and in the size of the amplified product. Although, extremely efficient, exponential amplification of target sequences is not an unlimited process. Under normal reaction conditions, the amount of Taq DNA polymerase becomes limiting after 25-30 cycles of amplification (i.e., after an amplification level of approximately 10^6 has been attained). If further amplification is required, a sample of the amplified DNA can be diluted 1000 to 10,000 fold and used as the template for further rounds of synthesis in a fresh polymerase chain reaction. By this method, amplification levels of 10^9 to
can be achieved during the course of 60 sequential cycles of denaturation, annealing, and primer extension. This allows the detection by Southern hybridisation of a single copy of the target sequence in the presence of a 10 fold excess of irrelevant DNA.

The prime attribute of PCR in human diagnostics is that specific DNA sequences can be amplified from among the thousands of different genes in a human DNA sample. Specificity is accomplished by employing a pair of primers to define the boundaries of the intended target sequence. These primers are approximately 20 nucleotides in length. The amplification is accomplished within hours by placing the reaction tube in an instrument that cycles through the temperature changes. Number of commercial instruments are available that are capable of changing temperatures precisely within seconds. In just a few hours, specific DNA is produced in quantity and purity, that in the past required weeks of labour to obtain. After target DNA is amplified there are several methods that can be employed to detect its presence; DNA hybridization technology is already successfully employed in clinical laboratories. The amplification of target DNA and the technology used to detect the amplified product form the foundation for the development of sensitive and specific diagnostic tests kits to be used in medical laboratories.

**Diagnostic Applications of PCR:** Scientists are just beginning to understand the attributes and opportunities of PCR and to evaluate its potential applications in medical
diagnostics. Following are the areas of human diagnostics where PCR is currently being applied.

i) Infectious diseases: PCR provides reliable detection where pathogens conventionally have been too scarce to be detected by antigen tests, or difficult, impossible, and dangerous to grow. Detection of HIV DNA in AIDS patients is an example of one application of PCR in this category that has been documented over the last four years.

Some infectious disease states require indirect testing methods (i.e., antibody levels of cytomegalovirus 'CMV' or cold agglutinin testing for Mycoplasma pneumoniae) as the most diagnostically reliable alternative. These methods lack utility when looking for acute infection, testing neonates, or specifically diagnosing a clinical problem. With PCR the analyte is the actual DNA of the suspected pathogen, and since that DNA can be amplified, organisms at very low concentrations can be detected. This is similar to microbiological culture techniques, with the added advantage that the organism does not have to be viable and the specimen can be much smaller.

A rapid diagnostic method is developed by Shankar et al from AIIMS, Delhi; where they have amplified a 240 bp product of a MPB 64 coding protein since this protein is specific for M. Tuberculosis. Tuberculosis meningitis (TBM), the most dangerous form of extrapulmonary tuberculosis, difficult to differentiate from pyogenic meningitis, occurs in 7-12% of tuberculous patients in developing countries. Despite the
availability of effective chemotherapy, the mortality and morbidity remain high. Delay in diagnosis is directly related to poor outcome. Bacteriological methods are inadequate for diagnosis of TBM because there are too few organisms in the CSF for consistent demonstration by direct smear, and cultural identification takes 6-8 weeks. Hence early diagnosis is going to help for TBM patients.

Potentially, PCR can be used to test antibiotic susceptibility of organism by looking for the presence of genes conferring resistance. Being able to identify and define the susceptibility of an infectious organism within hours is one of the valuable possibilities with PCR.

ii) Genetic Diseases: In the domain of genetic disorders, PCR is currently most useful in detecting those genetic mutations for which no other test is conclusive. For example, some diseases are defined at the gene level, yet products of the mutant gene are not known or difficult to detect and DNA analysis provides the only answer. Other available DNA analysis tests are time consuming, require more quantity of DNA than the PCR, many times require radioactive probe. Prenatal testing early in gestation can be accomplished with the use of chorionic villus samples or amniocentesis. Among the genetic diseases currently being prenatally investigated for potential PCR diagnostics are the hemoglobinopathies, hemoglobin S, hemoglobin C, and disorders such as cystic fibrosis, haemophilia, Duchen Muscular Dystrophy.
Utilisation of PCR is having an impact on the diagnosis of cystic fibrosis. This autosomal recessive disorder affects approximately 1 in 2000 Caucasian newborns. Classification of over 60 heterogeneous mutations of the gene resulting in an affected protein has allowed identification of carrier state. It has been estimated that 85% of Caucasian and Northern European carriers can now be identified through DNA analysis. There are ten common mutations present in thalassemia that are common in Indian subcontinent and are easily detected by PCR with ARMS primers.

iii) Cancer diagnostics: Oncologist may be able to define mutations associated with cancer where the expression of that gene has not been observed or detected. Sometimes the mutation is expressed in the mRNA assembly; and in these cases, examination of the genetic message is the best diagnostic tool. In large solid tumors and blast crises in leukemias, obtaining enough material of DNA diagnosis may not be a problem. But in situations where the patients are in remission, or biopsies are small, PCR provides a distinct advantage. In these circumstances, PCR can give the clinician information to monitor patients in remission with the ability to detect relapse before it is clinically evident. Since less sample is needed and PCR has the ability to detect one malignant cell in thousands, early detection of some cancers may be facilitated.

PCR is also used to find and detect genetic sequences that may associate viral pathogens with malignancy. Two
examples of this are human papilloma virus (HPV) for cervical cancer and human T-leukocyte virus (HTLV)-I for acute T cell leukemia.

iv) Transplantation: Scientific knowledge of the human leukocyte (HLA) antigens of the major histocompatibility complex (MHC) has exploded in the past decade due to the ability to analyze DNA. The HLA class II antigens are key factors in inducing and assisting an immune response and as such have been implicated in transplant rejection and graft versus host disease. With DNA analysis, scientists have been able to define areas of differentiation in the DR, DQ, and DP genes that serological and biological assays could not distinguish. PCR has facilitated this analysis and has also providing the basis for retrospective as well as prospective studies on thousands of transplant events. This work is vital in differentiating relevant mismatches from nonrelevant ones and will lead to more effective use of transplantation medicine and better prediction of the outcome for the patient.

In addition to detecting mutations that are implicated in transplantation rejection, an additional reason for investigation in this area is the implication of certain class II types and their linkage to disease. Predisposition to organic disease as well as susceptibility to certain infectious and immunological processes can be linked to the differences in these genes. Such kind of linkages are found effective in diabetes and hypertension. PCR
provides clinicians with the tools to conduct large epidemiological studies to investigate this relationship.

v) Forensic sciences - PCR in the forensic laboratory DNA fingerprinting, which uses the considerable allelic variation caused by differences in the number of repeat regions in human minisatellite DNA, has many applications. In forensic science it has been used to identify or exclude suspects by analysis of blood, semen, or other biological material left at the scene of the crime. Until recently this technology was limited because it usually required between 50 nanogram and 1 microgram of undegraded DNA. It has now been shown that hypervariable regions can be amplified by PCR using primers on either side of the minisatellite. Alleles of between 5 and 10 kb have been produced and co-amplification of six minisatellite loci permits the construction of a fingerprint. This technique is now so sensitive that only a single cell is necessary to achieve results, thus overcoming the limitation of sample size. This means that instead of venous puncture to obtain a sample suspects need only be subjected to a fingerprick or a mouthwash in order to obtain sufficient material for a test.
Use of PCR for diagnosis of hemoglobinopathies -

Following are the different methods by which abnormal hemoglobins and thalassemias can be identified. These methods are now being routinely applied for prenatal diagnosis of hemoglobinopathies.

a) PCR and electrophoresis
b) PCR and restriction digestion
c) PCR and hybridisation with allele specific oligomers
d) PCR by Amplification Refractory Mutation System
e) PCR and sequencing of the PCR product

a) PCR and electrophoresis - Small deletions of 0.1kb to 2kb are easily and quickly detected by PCR. For this primers are selected such that they flank the deleted region and further that the amplified product spans the entire deletion. After electrophoresis the product of PCR which is shorter by the deleted length is analysed. A 619 bp deletion causing thalassemia in Indian population is very easily detected by this method.

b) PCR and restriction digestion - This is useful when there is a point mutation giving rise to either abnormal hemoglobin or thalassemia and restriction site is either created or lost due to mutation. Sickle cell hemoglobin is a very good example of this where restriction site of MstII is lost due to change in 6th codon from CTC to CAC. This is illustrated later in this chapter.

RFLP analysis also can be performed by this technique. Primers for PCR are chosen in such a way that polymorphic
After PCR, restriction digestion is performed with the polymorphic restriction enzyme and RFLP is determined.

c) PCR and hybridisation with allele specific oligomers (Dot blot hybridization): This technique is useful to detect the possible disease causing mutation from a heterogenous molecular basis. This is particularly applicable to beta thalassemia where number of different mutations in beta globin gene cause the disease. Allele specific oligomers (ASO) may be either radioactive or non-radioactive.

DNA from the region of interest is amplified by PCR, and is simply blotted on nylon membrane and probed with appropriate oligonucleotides. This 'dot-blot technology' is combined with non-radioactively labelled probes (like biotin, digoxigenin), which provides a very rapid and simple laboratory approach to the diagnosis of thalassemia. This technique is particularly useful when restriction enzyme is not available to differentiate a mutation.

d) Amplification Refractory Mutation System (ARMS)—
The mutations that are characterized by a PCR method based on allele specific priming is known as Amplification refractory mutation system. The basis of this system is that oligonucleotide with a mismatched 3' end will not function as amplimers in the PCR under appropriate specific conditions. Thus an oligonucleotide primer is designed such that its 3' end is complementary to the sequence of the mutation that is being screened for, so that when the PCR is performed under
stringent conditions the presence of an amplified product will suggest the presence of mutation while its absence will suggest the presence of normal DNA sequence at the site of the mutation.

This technique is useful for prenatal diagnosis of beta thalassemia in Indian subcontinent and presently being used at our centre.

e) PCR and sequencing of the PCR product - This technique is employed when exact molecular basis for a given disease is not known. Without PCR, sequencing requires cloning of the genomic DNA in a suitable vector, it's transformation in to a host, amplification in the host and then sequencing reaction. But with PCR there is an advantage that it amplifies part of DNA without cloning and transformation. This is applicable particularly in those thalassemic cases where the mutation is unknown.

Future - Preimplantation diagnosis.

Another technical advance, in prenatal diagnosis of genetic disease is the possibility of combining in vitro fertilization. The advent of PCR together with the development of methods for biopsyng pre-embryos raised the possibility that it will be possible to combine in vitro fertilization with detection of genetic disease. Since it is now possible to amplify DNA from a single cell for genetic diagnosis.
7.4 : Prenatal diagnosis of sickle cell anaemia using PCR and MstII digestion

7.4.1 : Principle

During this procedure we have used two oligonucleotide (19mers) as primers to amplify beta globin region. This region is from -90 bases from beginning, through exon 1 and up to 117 nucleotides in IVS 1. MstII restriction enzyme has a site at 6th codon which is lost due to the Sickle cell mutation. In this case, the PCR product will be of 299bp. If normal, the product is digested with MstII restriction enzyme to two fragments of 192bp and 107bp. (Fig 7.4.1.)

Following two primers were used.

P1: GGG CTG GGC ATA AAA GTC A
P2: AAT AGA CCA ATA GGC A6A G

7.4.2 : Material and Method-

A family from Scheduled Caste community was chosen. After complete clinical examination blood samples were collected from all three members of the family. On the basis of hemoglobin electrophoresis and sickling test both the parents were carrier for sickle cell hemoglobin. Their first child was a patient of sickle cell anaemia. After initial screening 10 ml of venous blood was taken from all the three family members and was processed for DNA isolation and analysis. PCR was done on isolated DNA samples of all family members with DNA from normal control. Genetic counselling was done and parents were given full information about the disease, and were advised to visit Genetic Clinic as soon as
PRENATAL DIAGNOSIS OF SICKLE CELL ANAEMIA
USING PCR AND Mst II DIGESTION

β GLOBIN GENE

\[ \begin{align*}
5' & \quad \text{P 1} \quad \text{IVS I} \quad \text{P 2} \\
-90 & \quad 299 \text{ bp PRODUCT} \\
\hline
A & \quad \text{Mst II DIGEST} \\
\beta & \quad 107 \text{ bp} \quad 192 \text{ bp} \\
S & \quad 299 \text{ bp} \\
\beta &
\end{align*} \]

P1 AND P2 ARE THE TOW PRIMERS USED FOR AMPLIFICATION

5' P1: GGGCTGGGCATAAAAGTCA
P2: AATAGACCAATAGGCAGAG

Fig 7.4.1: Figure showing amplification of beta globin gene region with the Mst II restriction site and fragments generated after digestion.
Fig 7.4.2: Photograph showing 2% agarose gel electrophoresis pattern of amplified and MstII digested DNA from normal (AA), sickle cell trait (AS), and sickle cell anaemia (SS). Lane 1: PCR untreated product, Lane 2: Amplified MstII digested DNA of (AA), Lane 3: Amplified MstII digested DNA of (AS), Lane 4: Amplified MstII digested DNA of (SS), Lane 5: Amplified MstII digested DNA of Amniotic fluid, Lane 6: Marker DNA (pBR 322 Hind I digest)
the next pregnancy is confirmed. In spite of our counselling parents turn up late in the 14th week of pregnancy hence instead of CVB amniocentesis was done in the 16th week of gestation under ultrasonic guidance. About 20ml of amniotic fluid was withdrawn and foetal cells were collected by centrifugation for subsequent DNA isolation and analysis. Methods for DNA isolation and PCR are explained in chapter 3.5 and 3.6.

7.4.3 : Result

PCR amplified product after Mst II digestion, on 2% agarose gel electrophoresis showed that the AA DNA (normal) has two bands at 192 and 107 bp, as Mst II restriction site is present on both the alleles. In case of AS DNA (trait for sickle cell hemoglobin) three bands were seen; at 299, 192 and 107 bp, as one allele has lost restriction site due to sickle cell mutation while the other has Mst II site present. In case of SS DNA (sickle cell anaemia), agarose gel electrophoresis of Mst II digested DNA after amplification with primers P1 and P2, showed only one band at 299 bp, as both alleles has sickle mutation and the restriction site is lost. This is well explained in fig 7.4.1 and 7.4.2. This electrophoresis pattern of normal, trait and SS DNA was compared with the amplified and Mst II digested DNA from amniotic fluid cells, and it showed trait pattern. Hence it was advised the parents to continue the pregnancy. Four months after the delivery, blood sample of baby was collected and tested for phenotypic expression and found consistent
Fig 7.4.3: Figure showing the principle of chorion villus sampling to obtain trophoblast tissue for fetal DNA analysis.
with prenatal studies.

7.4.4: Discussion

Prevention is the best remedy for any genetic disorder. Prevention can best be achieved by

1) keeping the abnormal alleles apart (marriage counselling)

2) preventing birth of child with sickle cell disease. (Prenatal diagnosis)

In case of sickle cell anaemia, prenatal diagnosis can be achieved with following procedures,

a) Fetoscopic blood sampling and their analysis- This procedure requires best of technical expertise and experience to collect foetal blood from cord without maternal contamination. Even then there is 5% foetal loss. It requires special Coulter counter for separation of foetal and maternal cells on size exclusion basis. Foetal sample cannot be taken before 18th week of pregnancy and result is available in late second trimester, making it difficult for termination, if required.

b) Molecular analysis using linked polymorphism- This method is based on the finding that in American blacks, the HbS gene is associated with a variant Hpa I restriction site, flanking the beta gene. But such linkage is not observed in Indian population.

c) Direct identification of mutant gene- This can be performed with Dde I or Mst II restriction enzymes. In the conventional procedure by Southern Blotting, first genomic
DNA is digested with appropriate restriction enzyme followed by agarose gel electrophoresis. This DNA separated on agarose on the basis of fragment size is transferred to a nitrocellulose or nylone membrane. This transfer is essential as hybridization with genomic probes is not possible on the agarose gel and this transfer is known as Southern transfer. DNA transferred on the membrane is hybridized with radioactively labelled probe followed by autoradiography. It takes minimum one week to complete these procedures. These procedures are time consuming, laborious, and tedious. Moreover it requires radioactive probes which is hazardous.

Method used in this study i.e. PCR and Mst II digestion is easy, fast, convenient, reliable and nonhazardous. The average yield of DNA from 20 ml of amniotic fluid is 7 microgram, and the average yield from CVB is 4.6 to 7 microgram per villus (each villus containing 3 to 5 'bunches', each rather like the fronds of a coral) while minimum 10 microgram of DNA is required with conventional methods such as restriction digestion, Southern blotting and hybridisation with beta globin gene probe, while it can be performed on as less as 0.5 microgram of DNA, hence amniotic fluid cell culture is not required. If chorionic villus biopsy is performed diagnosis is available in 10-12 weeks of gestation making it easy to terminate if required and opted.

There are few centres in India which cater pernatal diagnosis for hemoglobinopathies. They are following fetoscopic blood sampling and analysis of the foetal blood
i.e. globin chain analysis. Taking into consideration high frequency of sickle cell hemoglobin amongst different population groups from state of Maharashtra, it was essential to establish prenatal diagnostic technique, using DNA analysis.

We made an attempt to establish this prenatal diagnostic procedure by DNA analysis from amniotic fluid and successfully carried out this and offered the first prenatal diagnosis. His method can be applied to CVB and diagnosis can be made in the first trimester.

Merits of our technique are as follow,

1) This is simple, rapid, inexpensive and reliable technique.

2) Diagnosis is available within 48 hours.

3) Almost all chemicals are manufactured and available in India (except restriction enzyme MstII and dNTPs) and cost per diagnosis is about 1000 rupees.

There are number of genetic and nongenetic diagnostic methodology, based on PCR. Establishment of PCR will facilitate use of this technique in other genetic (Thalassemia, Hemophilia, oncogenes) and non genetic (pathogene detection) disorders.