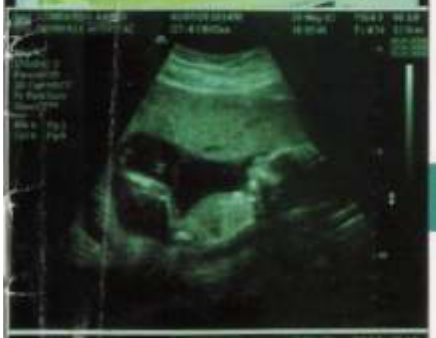

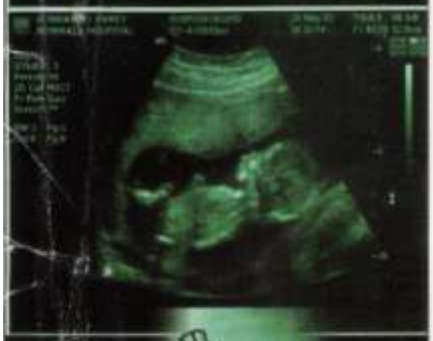

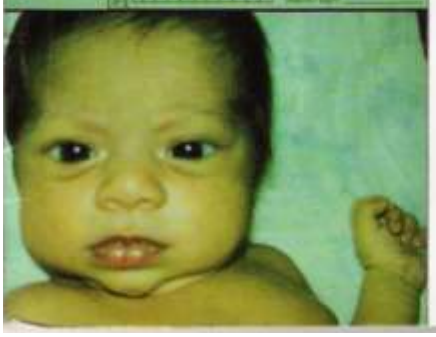

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PRENATAL DIAGNOSIS OF DUCHENNE MUSCULAR DYSTROPHY

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INTRODUCTION -

Muscular Dystrophies (MD) are a group of heterogenous inherited disorders characterized by progressive muscle wasting, weakness and degeneration, frequently accompanied by cardiomyopathy or mental retardation (1). Clinical course of the disease is highly variable, ranging from severe congenital forms with rapid progression to milder forms with a later onset and a slower course. At least 30 different forms of muscular dystrophies have been identified to date. The most common muscular disorder, Duchenne Muscular Dystrophy (DMD) is estimated to affect 1 in 3500 newborn males worldwide.

DUCHENNE AND BECKER MUSCULAR DYSTROPHY

(Dystrophinopathies) Duchenne and Becker are allelic disorders. DMD is a disease of muscle that becomes evident in early childhood but BMD is milder with late onset. The diseases are inherited as an X-linked recessive trait and predominantly affect boys. Weakness is progressive and affects mainly the proximal limb musculature. Most patients with DMD are confined to a wheelchair by the age of 12 years and death occurs in the late teens due to respiratory and cardiac failure. BMD is clinically similar but mild disease, with onset in teenage or early 20's. DMD may occasionally present at birth as hypotonia, or later with failure to thrive or delay in learning to walk. Calf hypertrophy is characteristic. Gower's sign is an early diagnostic test. The DMD patients without clinically remarkable calf hypertrophy can be diagnosed by the presence of the valley sign or Pradhans sign (2). Cardiac changes often culminating in cardiac failure are at times a dramatic cause of death in patients of DMD. One third of DMD patients suffer moderate to severe non progressive form of mental retardation.

Both DMD and BMD are caused by mutations in the dystrophin gene. The gene is located on Xp21 locus, spans 2.5 Mb and consists of 79 exons. The gene is transcribed into a 14 kb mRNA. Large intragenic deletions, duplications and point mutations in the dystrophin gene have been detected in the patients (3). Testing for the dystrophin gene mutations has become a part of routine diagnostic evaluation of patients who present with progressive proximal muscle weakness and high serum creatine kinase levels. Deletions of one or more exons of the dystrophin gene account for 55-65% of cases of D/BMD, which tend to be clustered (hotspots) in two regions of the gene. The deletional hot spots are in the first 20 exons of the gene and the second deletional hot spot is between exons 42-52 (Fig 1).

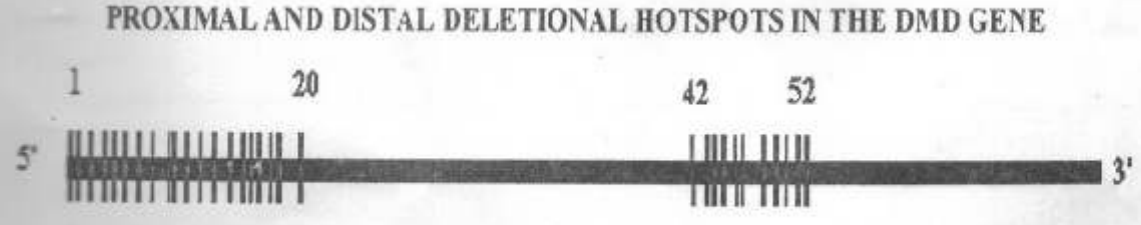

Fig 1 : Diagramatic representation of the DMD gene. The exons are represented as red lines. Note the variable sizes of the intervening intronic sequences.

MUTATION DETECTION:

Due to large intagenic deletions in approximately 60% of patients with DMD/BMD, DNA based mutation analysis is carried out by multiplex PCR, using 18-24 sets of primers. Approximately 30-40% of the cases are not due to gene deletion or duplication. In such cases a point mutation can be suspected. Single Strand Conformational Polymorphism (SSCP) analysis followed by automated sequencing has been successful in screening for point mutations. However, point mutation analysis is very tedious and expensive. The phenotype of cases with deletion of single exons does not differ significantly from those with deletion of multiple exons. Dystrophin staining with monoclonal antibody may be useful in the differential diagnosis of patients with other dystrophinopathies.

Mutations in the DMD gene are responsible for DMD. However in rare instances, a disruption of the dystroglycan-dystrophin link as in γ SG mutations may also cause a Duchenne-like phenotype.

PRENATAL DIAGNOSIS OF DMD/BMD -

Since there is no cure or effective treatment for progressive muscular dystrophy, prevention of the disease is important and strongly depends on carrier-status information. Prenatal diagnosis can be achieved by DNA studies on chorionic villi sample at around 10-11 weeks of gestation or from cultured amniotic fluid cells at around 16-18 weeks gestation.

DNA test- In case of deletion in index patient, prenatal diagnosis is straight forward. DNA is extracted form about 20 – 25 mg of dissected fetal cells. The tissue is washed twice in phosphate buffered saline and then homogenized in liquid nitrogen. DNA is then extracted using a standard column extraction method. In case amniotic fluid is used for the diagnosis, the cells are cultured for 72 hours before harvesting. DNA is then extracted using the standard method.

Before proceeding to mutation detection, it is first necessary to determine the presence of maternal contamination. Maternal contamination can lead to wrong results and hence if the tissue shows evidence of maternal contamination, it should be discarded. Maternal contamination can be tested by using highly polymorphic Variable Number of Tandem Repeat (VNTR) markers like Apolipoprotein B (ApoB) or D1S80 which is a VNTR present on chromosome 1. The gel photograph of maternal contamination is shown in Fig 2.

The next step is to determine sex of the fetus since it is very rare for a female to manifest the symptoms of DMD. This is done by finding out the presence of Y chromosome-specific sequences like amleogenin or SRY. The gel photograph of the amleogenin marker is shown in Fig 3.

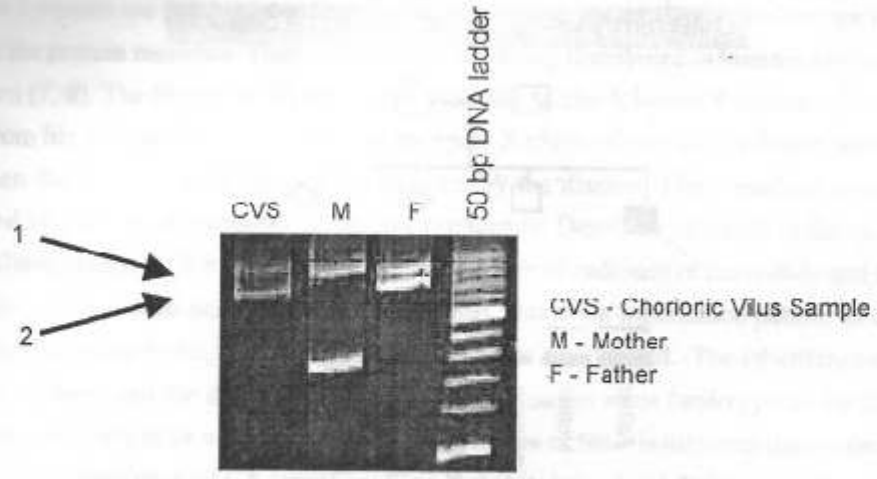

Fig 2 – Gel photograph showing an absence of maternal contamination. The marker to the left of the ladder is the ApoB VNTR marker. Note that the chorionic villi sample (CVS) shows one allele each from mother (M) and father (F). The upper allele (1) is from the mother and the lower allele(2) is from the father.

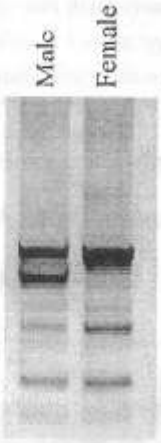

Fig 3 – Gel photograph of the amleogenin marker. Note the extra dark band seen in the male

In case the fetus is female, no further testing is done. If fetus is definitely male, it is subjected to mutation analysis based on type of mutation in the index case.

Using multiplex PCR, if a deletion is present in an affected individual (proband), it becomes a marker for the family which can be used for prenatal diagnosis (PND) in that particular family. The DNA extracted from the tissue is subjected to the same mutation analysis as the proband and the diagnosis is easily made (4, 5). Fig 4 shows a DMD family with an unaffected and affected male child and two CVS samples for prenatal diagnosis.

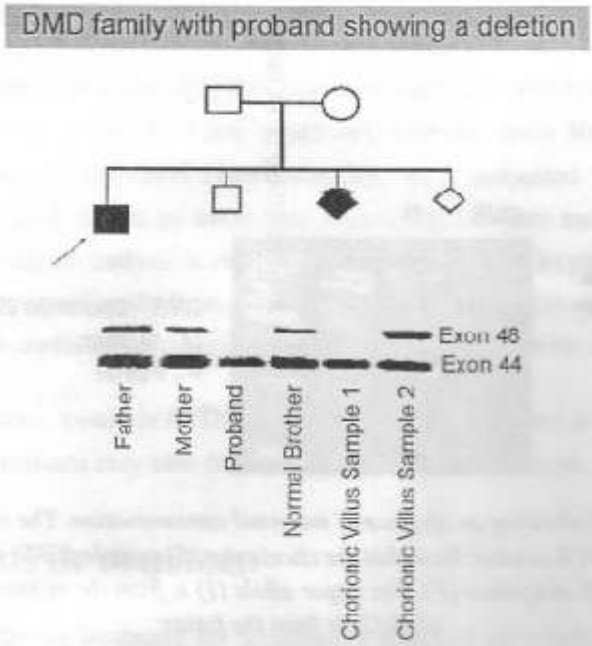

Fig 4 – Gel picture of a DMD family with two chorionic villi samples for analysis. The CVS sample1 on the left shows a deletion similar to the proband. The CVS sample 2 on the right shows an unaffected fetus

The shortcoming of the multiplex PCR is that only a few exons are tested. This shortcoming has been overcome by recently introduced Multiple Ligation Dependant Probe Amplification (MPLA) method. MLPA DMD kits are commercially available (MRC Holland) and probes allow a scanning of all the 79 exons of the DMD gene (6) (Fig 5).

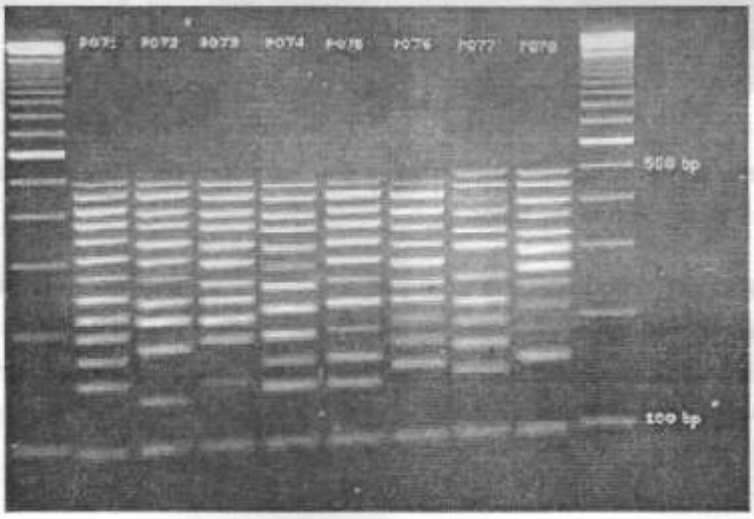

Fig 5 – Gel picture of the Multiple Ligation Dependant Probe Amplification. Note the large number of exons that have been screened in one PCR reaction. The gel shows a complex rearrangement detected in the DMD gene (A duplication of exons 52–55 and 63–67, and a triplication of exons 68–79).

In cases where no deletion is detected in the proband, linkage studies based on simple tandem repeat (STR) polymorphism can be carried out. Several polymorphic repeat markers containg variable number of

dinucleotide (CA) repeats are present in and around the dystrophin genes. There markers are in intronic region and do not affect the protein sequence. These markers are randomly distributed in humans and inherited in simple Mendelian pattern (7, 8). The linkage analysis is done basically to check which X chromosome the proband has been inherited from his mother. If the foetus shows the same X chromosome which is also inherited by proband in the family, then the foetus is also likely to be affected by the disease. These markers can be amplified by PCR and resolved by acrylamide sequencing gel electrophoresis. Detection of bands in the gel can be done by silver staining. Alternatively, PCR can be carried out in presence of radioactive nucleotide and resolved product bands can be detected after autoradiography on X-ray films. Based on inheritance pattern of alleles in parents and proband (plus unaffected sibling if available), haplotypes are constructed. The inheritance of the haplotypes is analysed in the proband and the fetus. If the fetus has inherited the same haplotype as the DMD child in the family, the fetus is also likely to be affected. In case the haplotype of fetus is different than in proband, then fetus is unaffected. The only limitation of CA repeat analysis is that it depends on linkage which might be subject to errors due to recombination. The DMD locus is so large that markers lying at the two extremes themselves show a recombination frequency of 0.12. It is, therefore, advisable to use at least 6 markers for linkage analysis. It may be added that linkage test can only be used if there is a clear history of the disease in the family. Due to high incidence of new mutations, it is not advisable to go for linkage analysis in sporadic cases. A family showing CA repeat analysis for the DMD gene is shown in Fig 6.

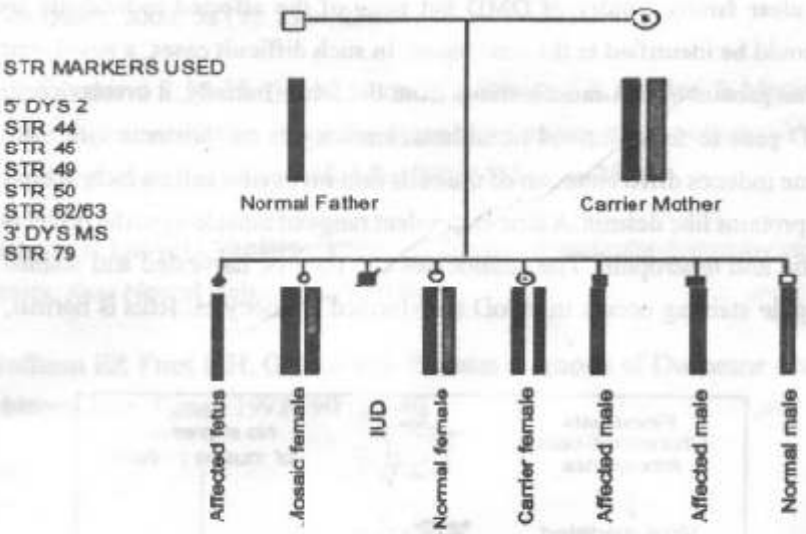

Fig 6 – STR analysis of a DMD family. Mutation analysis showed no deletion. Note that the two affected males have inherited the same X chromosome from the mother. The carrier female has also inherited the same X chromosome from the mother. The normal female has inherited the normal X chromosome from the mother

Immunohistochemistry – Prenatal diagnosis of DMD can also be carried out by immunostaining for the dystrophin protein in the fetal muscles (9) (Fig 7). Although dystrophhin only comprises about 0.2% of the skeletal muscles, immunostaining for the protein can be done and the protein shows up as a peripheral rim of stain. Poly and monoclonal antibodies against different epitopes of dystrophin are used. Immunostaining is done in cases where molecular methods fail to provide a definitive diagnosis (10). In such cases, in utero fetal muscle biopsy may serve as a diagnostic option. The fetal gluteal muscle is usually chosen for the biopsy and it is obtained by an ultrasound guided biopsy. Rarely maternal contamination may occur due to a transient opening up of the needle and it may lead to a diagnostic dilemma.

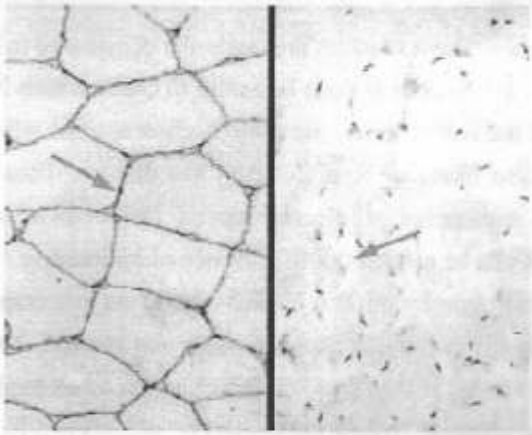

Fig 7 – Immunostain for the dystrophin protein. The microphotograph (A) shows a normal muscle with a peripheral staining of dystrophin (arrow). The microphotograph (B) shows the muscle of a patient with DMD where dystrophin staining is absent on the sarcolemmal membrane (arrow).

MYOD TRANSFORMATION IN AMNIONCYTES –

Sometimes, there is clear family history of DMD but none of the affected individuals are surviving and no intragenic mutation could be identified in the fetal tissue. In such difficult cases, a novel strategy is based on the induction of dystrophin protein in non-muscle tissue from the fetus. Initially, it involves retroviral or adenoviral delivery of the MyoD gene to fetus-derived fibroblasts, amniocytes or chorionic villi cells. Expression of the introduced MyoD gene induces differentiation of the cells into myogenic cells which, within a few days, start to express early muscle proteins like desmin. A time-dependent range of muscle-specific proteins then get expressed, including myosin, titin and dystrophin. The amniocytes can then be harvested and stained with antibodies to dystrophin. If dystrophin staining occurs in myoD transformed amniocytes, fetus is normal, otherwise likely to be affected (Fig 8).

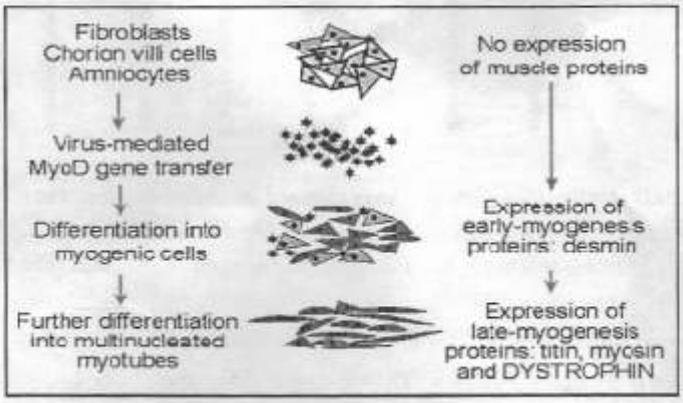

Fig 8 – Diagrammatic representation of MyoD differentiation and expression of the Dystrophin protein.

Conclusion – DMD is a relentlessly progressive disorder which leads to death in the late teens or early adulthood. It is essential that prenatal diagnosis be done so that the affected fetus maybe aborted early. Newer and better diagnostic tests are now available for definitive prenatal diagnosis of Duchenne Muscular Dystrophy .

REFERENCES –

1. Sciandra F, Bozzi M, Bianchi M, Pavoni E, Giardina B, Brancaccio A. Dystroglycan and the muscular dystrophies related to the dystrophin-glycoprotein complex. Ann 1st Super Sanita 2003; 39: 173 – 181.

2. Pradhan S. New clinical sign in Duchenne muscular dystrophy. Pediatr Neurol 1994; 11: 298-300.

3. Mukherjee M, Mittal B. Muscular dystrophies. Ind J Pediatrics,2004, 71, 161-168

4. Mukherjee M, Pandey GS, Keshri A, Phadke S, Mittal B. Prenatal diagnosis of genetic disorders by molecular methods. Perinatology 2004, 6, 221-230.

5. Panigrahi I, Mittal B. Carrier detection and prenatal diagnosis in Duchenne/Becker muscular dystrophy. *Indian Pediatrics* 2001; 38: 631- 639.

6. Schwartz M, Dune M. Multiple Ligation Dependant Probe Amplification is superior for detecting deletions / duplications in Duchenne muscular dystrophy. Clin Genet 2004; 67: 189 – 191.

7. Chaturvedi LS, Mittal RD, Srivastava S, Mukherjee M, Mittal B. Analysis of dinucleotide repeat loci dystrophin gene for carrier deletion, germline mosaicisim and de novo mutation in Duchenne muscular dystrophy. *Clin Genet* 2000. 58 (3): 234 - 236.

8. Chaturvedi LS, Srivastava S, Mukherjee M, Mittal RD, Phadke S R, Pradhan S, Mittal B. Carrier detection in non-deletional Duchenne/Becker muscular dystrophy families using polymorphic dinucleotide (CA) repeat loci of dystrophin gene. *Indian J Med Res* 2001; 113: 19 – 25.

9. Martin JJ, Ceuterick C, Lubke U, Van Broeckhoven C. Duchenne muscular dystrophy immunohistochemistry of foetal muscles. Acta Neurol Belg. 1993; 93:130-8.

10. Kuller JA, Hoffman EP, Fries MH, Golbus MS. Prenatal diagnosis of Duchenne muscular dystrophy by fetal muscle biopsy. Hum Genet. 1992 ; 90 : 34-40.