Genetic Diagnosis, Prevention and Molecular Pathophysiology of Duchenne Muscular Dystrophy and Non-invasive Diagnosis of Familial Neuromuscular Disorders

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By

SAKTHIVEL MURUGAN SM

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Dr. B.R.LAKSHMI

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PILANI - RAJASTHAN

CERTIFICATE

This is to certify that the thesis entitled "***Genetic Diagnosis, Prevention and Molecular Pathophysiology of Duchenne Muscular Dystrophy and Non-invasive Diagnosis of Familial Neuromuscular Disorders***" and submitted by Mr. Sakthivel Murugan SM, ID. No. 2007PHXF435 for award of Ph.D. Degree of the Institute embodies original work done by him under my supervision.

Signature of the Supervisor:

Name in Block Capital letters : **Dr. B.R.Lakshmi**

Designation : **Head,**
Molecular Diagnostic Facility,
Sundaram Medical Foundation,
4th Avenue, Shanti Colony,
Anna Nagar, Chennai - 600 006.
Tamil Nadu. India

Date:

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ABBREVIATIONS

NMDs	**NeuroMuscular Disorders**
ALS	**Amylotrophic Lateral Sclerosis**
DMD	**Duchenne Muscular Dystrophy**
SMA	**Spinal Muscular Atrophy**
MD	**Muscular Dystrophy**
BMD	**Becker Muscular Dystrophy**
CMD	**Congenital Muscular Dystrophy**
TMD	**Tibial MD**
LGMD	**Limb Girdle MD**
EDMD	**Emery–Dreifuss MD**
ECM	**Extra Cellular Matrix**
DFB	**Dystrophin Family Binding site**
CC	**Coiled Coil**
NMJ	**NeuroMuscular Junction**
DAPC	**Dystrophin-Associated Protein Complex**
AQP4	**Aquaporin-4**
NOS	**Nitric Oxide Synthase**
MLPA	**Multiplex Ligation-dependent Probe Amplification**
CGH	**Comparative Genomic Hybridisation**
MAPH	**Multiplex Amplifiable Probe Hybridization**
NGRL	**National Genetics Research Laboratory, Manchester**
DQs	**Dosage Quotients**
DHPLC	**Denaturing High Performance Liquid Chromatography**
FISH	**Flourescnet InSitu Hybridisation**
SSCP	**Single Strand Conformation Polymorphisim**
FM-CSCE	**Fluorescence Multiplex – Conformation Sensitive Capillary Elecrophoresis**
PTT	**Protein Truncation Test**
HRM	**High-Resolution Melting**
hrMCA	**High-Resolution Melting Curve Analysis**
DGGE	**Denaturing Gradient Gel-Electrophoresis**

SSCA	**Single-Strand Conformation Analysis**
MCC	**Maternal Cell Contamination**
PGD	**Preimplantation Genetic Diagnosis**
CPK	**Creatine PhosphoKinase**
IHC	**ImmunoHistoChemistry**
STR	**Short Tandem Repeat**
QmfPCR	**Quantitative Multiplex Fluorescence PCR**
MZ	**MonoZygotic**
AIS	**Androgen Insensitivity Syndrome**
XCI	**X Chromosome Inactivation**
ICM	**Inner Cell Mass**
UPD	**UniParental Disomy**
TS	**Turner's Syndrome**
ALS	**Amyotrophic Lateral Sclerosis**
EMG	**Electromyography**
NGS	**Next Generation Sequencing**
SNP	**Single Nucleotide Polymorphism**
LOH	**Loss Of Heterozygosity**
GTC	**Genotyping Console**
CNVs	**Copy Number Variations**
MR	**Mental Retardation**
ASD	**Autism Spectrum Disorders**
DD	**Developmental Delay**
MCA	**Multiple Congenital Anomalies**
CGH	**Comparative Genomic Hybridization**
BAC	**Bacterial Artificial Chromosome**
PAC	**P1–Derived Artificial Chromosome**
DGS/VCFS	**DiGeorge Syndrome/VeloCardioFacial Syndrome**
AML	**Acute Myeloid Leukemia**
ITD	**Internal Tandem Duplication**
FAP	**Non-Familial Adenomatous Polyposis**
PD	**Parkinson Disease**

HBD-2	**Human Beta-Defensin – 2**
CCL3L1	**CC Chemokine Ligand 3-Like 1**
MAPD	**Median Absolute Pairwise Difference**
QC	**Quality Control**
DGV	**Database Genomic Variance**

CHAPTER 1 – INTRODUCTION AND REVIEW OF LITERATURE

INTRODUCTION

Neuromuscular disorders (NMDs) are a group of genetic and acquired disorders which affect the peripheral nervous system and muscle. Their principle effect therefore is on the ability to perform voluntary movements, with resulting significant incapacity including, at the most extreme, complete paralysis. Many neuromuscular disorders were recognised in the nineteenth century, particularly by the great French neurologists (Emery, 1998). Neuromuscular disorders include some of the most devastating diseases that afflict mankind including amylotrophic lateral sclerosis (ALS), congenital muscular dystrophies and myopathies like Duchenne muscular Dystrophy (DMD) and spinal muscular atrophy (SMA).

Neuromuscular disorders range in onset from *in utero* (Ravenscroft et al., 2011) to old age, but to a large extent affect infants, children and teenagers. Some neuromuscular disorders are acquired, such as botulism, idiopathic inflammatory myopathies (dermatomyositis, inclusion body myositis and polymyositis), Lambert-Eaton syndrome, myasthenia gravis, pharmaceutical induced myopathies such as steroid or statin myopathy, snake bite, and tetanus, but the vast majority are genetic or have genetic susceptibility (Emery, 2002) including statin myopathy (Krivosic-Horber et al., 2004; Link et al., 2008; Supala-Berger et al., 2009).

Muscular dystrophies (MD) constitute a part of neuromuscular disorders and encompass over 30 different inherited diseases, all involving progressive weakness and degeneration of skeletal muscle. Muscular Dystrophies (MDs) can be devastating diseases with weakened skeletal muscles leading to loss of ambulation, difficulties in breathing and eating, in most cases, premature death. Key features of dystrophic muscle include central nuclei, small regenerating fibres and accumulation of connective tissue and fatty tissue (Figure 1.1). MDs do not usually involve the central nervous system or peripheral nerves (although

some do), (Belpaire-Dethiou MC et al., 1999) despite the close anatomical relationship between skeletal muscle and nerves, they can affect the heart and other organs.

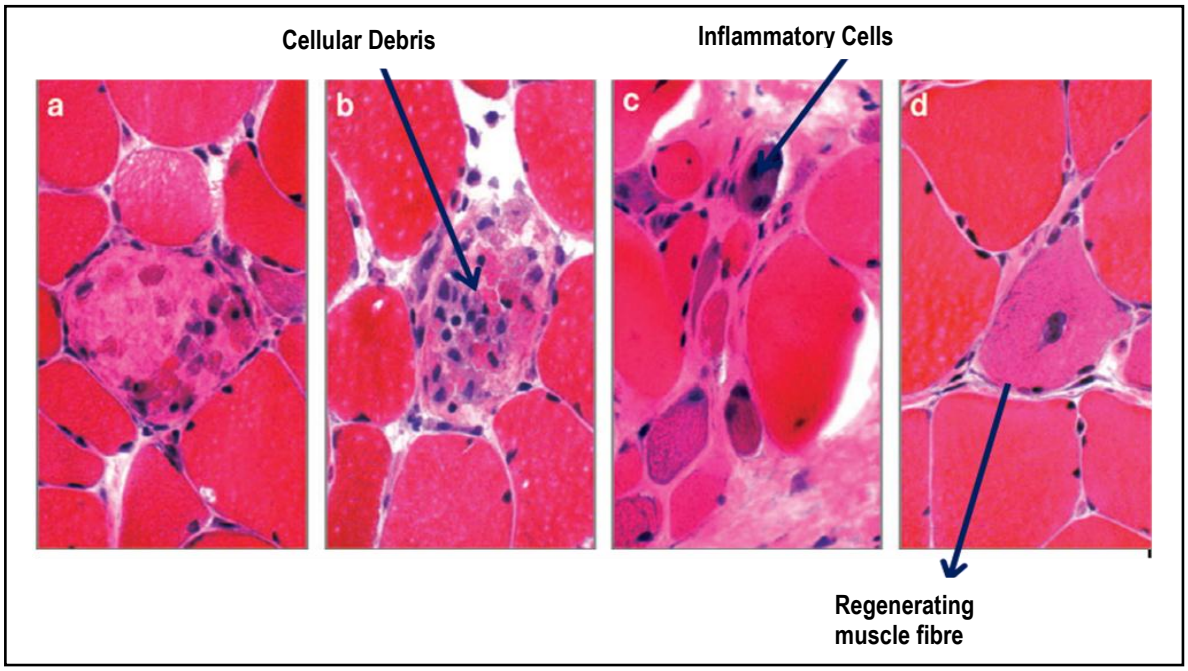

Figure 1.1: Myopathic muscle histology. *Figure shows H&E staining of necrotic fibers. Figures a and b show that the outline of the original fiber is still detectable. The fibers are filled with cellular debris and inflammatory cells. Satellite cells cannot be reliably distinguished from mononuclear inflammatory cells. Figures c and d show examples of regenerating myofibers with more blue-purple, basophilic cytoplasm, and enlarged activated nucleus. Some of these myofiber nuclei are internalized and do not occupy the normal subsarcolemmal location. (Adapted from McNally and Pytel, 2007)*

1.1 CLASSIFICATION OF MUSCULAR DYSTROPHIES

Traditionally the MDs have been grouped according to their clinical and pathological manifestations (Table 1.1 for the more common types of MD). The greatest advances in muscular dystrophies in the last 25 years have been in the identification of many of the genes mutated in the disorders. Figure 1.2 shows the muscles affected in the common types of muscular dystrophies.

Table 1.1: Common types of Muscular Dystrophies

S. No	Muscular Dystrophies	Features
1	Becker muscular dystrophy Inheritance – X-linked Recessive	Similar to Duchenne muscular dystrophy, however, phenotypically variable. Generally milder and follows a less severe course than DMD.
2	Congenital muscular dystrophy (CMD) Inheritance – Autosomal recessive	Clinically and molecularly heterogeneous, onset of symptoms (including hypotonia, muscle weakness and joint contractures) at birth or within the first 6 months of life.
3	Duchenne muscular dystrophy Inheritance - X-linked Recessive	Most common muscular dystrophy with rapidly progressive skeletal and cardiac muscle weakness. Most patients are diagnosed before 6 years of age, are wheelchair- bound by 12 years of age and rarely live beyond 30 years of age.
4	Emery–dreifuss muscular dystrophy (EDMD) Inheritance – Autosomal dominant	Clinically characterized by slowly progressive muscle weakness and wasting with tendon contractures, arrhythmia, humero-peroneal distribution and/or cardiomyopathy. EDMD can be autosomal dominant (mutations in lamin A and C) or X-linked (emerin deficiency).
5	Facioscapulohumeral muscular dystrophy (FSHD) Inheritance – Autosomal dominant	A progressive and often asymmetric muscular dystrophy, preferentially affecting the muscles of the face, shoulders and upper arms. It is associated with high-frequency hearing loss and retinal vascular abnormalities.
6	Fukuyama congenital muscular dystrophy (FCMD) Inheritance – Autosomal recessive	A severe, autosomal recessive CMD (most prevalent in Japan) that manifests from early infancy with generalized muscle weakness and hypotonia. Severe mental retardation and ophthalmologic abnormalities are also characteristic.
7	Limb girdle muscular dystrophy (LGMD) Inheritance – Autosomal dominant and recessive	A heterogenous disorder with predominant involvement of the shoulder girdle and pelvic muscles. Age of onset (childhood to adulthood), severity (severe to very mild) and inheritance (dominant and recessive) varies greatly.
8	Myotonic dystrophy (DM) Inheritance – Autosomal dominant	The most common adult muscular dystrophy. A severe, slowly progressive multisystem disease with generalized muscle weakness and wasting, nervous system effects, cardiac conduction defects, cataracts and myotonia (delayed relaxation after muscle contraction).
9	Oculopharyngeal muscular dystrophy (OPMD) Inheritance- Autosomal dominant	A late-onset disorder with swallowing difficulties, eyelid drooping and proximal limb weakness. Pathologically characterized by the presence of clusters of tubular filaments forming nuclear inclusions in skeletal muscle fibres.
10	Tibial muscular dystrophy (TMD) Inheritance – Autosomal dominant	Usually a milder, adult-onset disease involving the distal muscles of the body, such as the anterior compartment of the legs.
11	Walker–warburg syndrome (WWS) Inheritance – Autosomal recessive	A CMD associated with characteristic brain and eye malformations, namely retinal and cerebellar malformations and type II lissencephaly. Due to multi-organ involvement, patients rarely live more than a year.

Genetically, MDs can be inherited in a dominant or recessive manner or, in many cases, caused by *de novo* mutations, which are therefore sporadic. As the genetic causes have become apparent, MDs have been named to reflect the defective gene, for example, 'laminopathies', 'titinopathies', 'dystrophinopathies' and 'dysferlinopathies', with the same defective gene possibly causing more than one disease phenotype; these gene describing titles sometimes group clinically distinct diseases together. For example, mutations in the gene that encodes the giant sarcomeric protein titin can cause both Tibial MD (TMD) and the more severe limb girdle MD (LGMD2J), depending on whether the patient is heterozygous for the mutation (TMD) or homozygous for the mutation (LGMD2J) (Hackman et al., 2002).

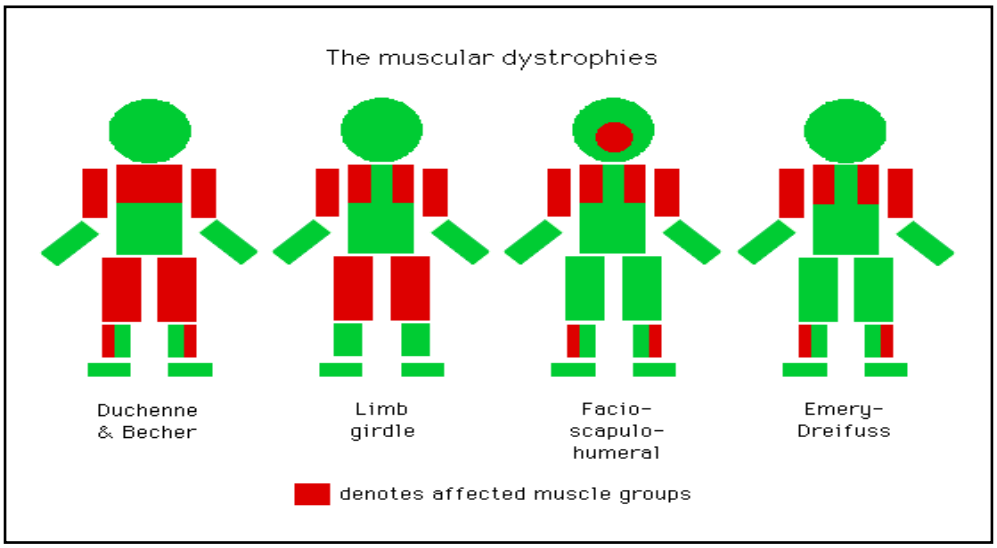

Figure 1.2: Muscles affected in some common muscular dystrophies. *Figure shows the muscle groups afftected in some common MDs. It can be observed that in DMD the proximal muscles are the most affected (Adapted from Emery, 1998)*

Moreover, some mutated genes not only cause more than one clinically distinct MD, but can also cause diseases that do not primarily affect skeletal muscle. Mutations in the *LMNA* gene, which encodes the nuclear proteins lamin A and lamin C can cause MDs (Bonne et al., 1999; Muchir et al., 2000) as well as other diseases such as the premature ageing disease Hutchinson–Gilford progeria syndrome (Eriksson et al., 2003). To further complicate the situation, more than

one gene can produce the same MD phenotype; for example, Emery–Dreifuss MD can be caused by autosomal dominant mutations in the *LMNA* gene (Bonne et al., 1999) or X-linked mutations in the gene encoding another nuclear protein, emerin (Bione et al., 1994).

Dystrophin was the first mutant protein shown to cause MD. Mutations of the dystrophin gene, the largest gene in the human genome, cause the most common MD, Duchenne MD (DMD), as well as the milder phenotype of Becker MD (Koenig et al., 1988). Differing mutations within the dystrophin gene determine whether a patient shows a DMD or BMD phenotype. DMD results from an absence of dystrophin or expression of a non-functional protein, whereas BMD has been associated with reduction of wild-type dystrophin or expression of a partially functional protein.

Although DMD was first described in the 1830s, it was not until 1975 that electron microscopy and biochemical analyses indicated that patients with DMD had a defect in the plasma membrane (sarcolemma) of muscle fibres (Mokri and Engel, 1975). The gene was located to Xp21 using linkage analysis with restriction fragment length polymorphisms (Davies et al., 1983). The causative gene was identified in 1987 and encodes the dystrophin protein (Koenig et al., 1988) (so-called because its deficiency causes dystrophy), which is localized at the sarcoplasmic surface of the sarcolemma. This was one of the first genes to be identified by positional cloning and its discovery led to the identification of other genes involved in MDs.

Historically, defects in structural proteins have been predominantly associated with MD. However, the understanding now is that the underlying mechanisms not only involve loss of structural proteins but also defective enzymes, disruption of sarcolemma-repair mechanisms and the loss of signalling molecules. Judging from the location of mutant proteins does not seem to dictate whether a mutant protein can cause MD. Proteins that are involved in post-translational modifications can also cause MD, as can expansions or deletions of nucleotide-repeat sequences that are not necessarily in protein-coding regions of the genome.

1.2 DUCHENNE MUSCULAR DYSTROPHY

Duchennetype muscular dystrophy (also known as Meryon's disease) is the commonest form of muscular dystrophy. The disease was first described by the Neapolitan physicians Giovanni Semmola in 1834 and Gaetano Conte in 1836 (Emery 2002). However, DMD is named after the French neurologist Guillaume Benjamin Amand Duchenne (1806–1875), who, in the 1861 edition of his book "*Paraplegie hypertrophique de l'enfance de cause cerebrale*", described and detailed the case of a boy who had this condition. A year later, he presented photos of his patient in his "*Album de photographies pathologiques*." In 1868 he gave an account of 13 other affected children. Duchenne was the first who did a biopsy to obtain tissue from a living patient for microscopic examination. (http://en.wikipedia.org/wiki/Duchenne_muscular_dystrophy#History)

DMD is inherited as an X linked recessive trait and therefore predominantly affect boys. (Figure 1.3) Its global incidence has been estimated as approximately 1 in 3500 live male births (Emery, 1998). It is a serious condition with progressive muscle wasting and weakness which causes most boys to start using wheelchairs by age 12 and to die in their 20s. Up to a third of boys with Duchenne dystrophy have some degree of intellectual impairment, and in severe cases special schooling may have to be considered. Becker muscular dystrophy is clinically similar but milder, with onset in the teenage years or early 20s. Loss of the ability to walk may occur later and many individuals with Becker dystrophy survive into middle age and beyond.

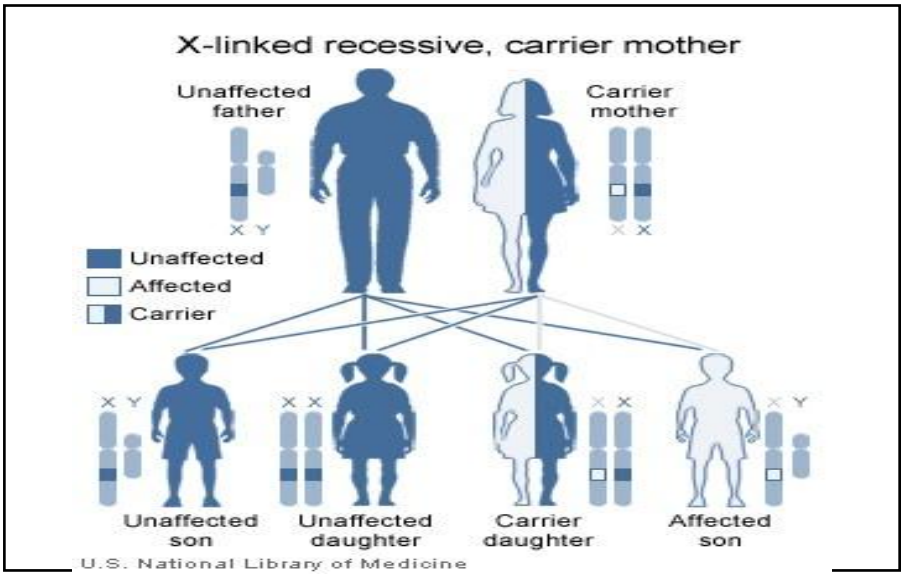

Figure 1.3: Inheritance pattern in Duchenne muscular dystrophy *Figure shows a typical X-linked inheritance. DMD is inherited as an X-linked recessive trait. Only males are affected and females are genereally carriers. (Adapted from* http://ghr.nlm.nih.gov/ *- Genetic Home Reference)*

Much research over many years had failed to identify the basic biochemical defect in DMD. However, in 1982 it was the first gene associated with a disease to be localized using chromosomally defined DNA markers (Murray et al., 1982). Shortly thereafter the gene itself was isolated, (Kunkel et al., 1985; Ray et al., 1985) cloned, and sequenced. In 1987 its protein product was identified and termed dystrophin (Hoffman et al., 1987).

1.2.1 Clinical Progression of Duchenne and Becker Muscular Dystrophies:

Typically, DMD patients are clinically normal at birth, although serum levels of the muscle isoform of creatine kinase are elevated. The first symptoms of DMD are generally observed between the ages of 2 and 5 years (Dubowitz, 1978; Jennekens et al., 1991), with the child presenting with a waddling gait or difficulty in climbing stairs. There is often a delay in the achievement of motor milestones, including a delay in walking, unsteadiness, and difficulty in running. Subsequently, the onset of pseudohypertrophy of the calf muscles, proximal limb muscle weakness, and Gowers' sign (the use of the child's arms to climb up his body when going from a lying to standing position) suggest DMD (Gowers, 1879). Eventually, decreased lower limb muscle strength and joint contractures

result in wheelchair dependence, usually by the age of 12 (Emery, 1998). Weakness of the arms occurs later along with progressive kyphoscoliosis. Most patients die in their early twenties as a result of respiratory complications due to intercostal muscle weakness and respiratory infection. Death can also be the result of cardiac dysfunction with cardiomyopathy and/or cardiac conduction abnormalities observed in some patients.

In individuals affected by BMD, the clinical course is similar to that of DMD, although the onset of symptoms and the rate of progression are delayed. More than 90% of patients are still alive in their twenties, with some patients remaining mobile until old age (Emery, 1998). There is a continuous clinical spectrum between a mildly affected BMD patient and a severely affected DMD patient. BMD and DMD patients also present with mild cognitive impairment, indicating that brain function is also abnormal in these disorders (Blake and Kroger, 2000; Mehler, 2000)

1.2.2 Histological Features:

Normal skeletal muscle consists of muscle fibers that are evenly spaced, angular, and of a relatively uniform size. Muscle, being a syncytium, is multinucleated with nuclei located at the periphery of the fiber. Fetal DMD muscle is histologically normal except for occasional eosinophilic hypercontracted fibers (Emery, 1977; Bertorini et al., 1984; Lotz and Engel, 1987). Necrotic or degenerating muscle fibers are characteristically seen in all postnatal DMD muscle biopsies even before muscle weakness is clinically observed. Degenerating fibers are often seen in clusters (grouped necrosis), and studies of longitudinal and serial transverse muscle sections show this process is often confined to segments of the muscle fiber (Schmalbruch, 1984). These necrotic fibers are subject to phagocytosis, and muscle biopsies from DMD patients reveal the presence of inflammatory cells at perimysial and endomysial sites (Arahata and Engel, 1984; Arahata and Engel, 1986). These cells are predominantly macrophages and $CD4^+$ lymphocytes (McDouall et al., 1990). A seondary sign of muscle fiber necrosis, at least in the early stages of the dystrophinopathies, is the active regeneration of muscle to replace or repair lost or damaged fibers (Schmalbruch, 1984). Early regenerating fibers are recognized by virtue of their small diameter, basophilic RNA-rich cytoplasm, and large, centrally placed

myonuclei (Bell and Conen, 1968; Bradley et al., 1972; Schmalbruch, 1984). Eventually, the regenerative capacity of the muscles is lost and muscle fibers are gradually replaced by adipose and fibrous connective tissue, giving rise to the clinical appearance of pseudohypertrophy followed by atrophy (Emery, 1998). The combination of progressive fibrosis and muscle fiber loss results in muscle wasting and ultimately muscle weakness.

1.2.3 Dystrophin Gene:

The identification of the DMD gene on the X chromosome was the first triumph of positional cloning and opened up a new era in DMD research (Monaco et al., 1986; Koenig et al., 1987) The gene was localized to Xp21 by studies of rare female DMD patients with balanced X: autosome translocations with the translocation breakpoint in Xp21 (Boyd and Buckle, 1986). This localization was confirmed using DNA markers (Davies et al., 1983), and the disease was shown to be allelic with a milder disease of similar clinical course, BMD (Kingston et al., 1983). The gene was eventually identified by taking advantage of a patient with a large deletion who suffered from four X-linked phenotypes including DMD (Francke et al., 1985). The DMD gene is the largest described, spanning ~2.5 Mb of genomic sequence (Figure 1.4) (Coffey et al., 1992; Monaco et al., 1992) and is composed of 79 exons (Coffey et al., 1992; Monaco et al., 1992; Roberts et al., 1993). The dystrophin gene remains the largest gene associated with a disease that has been identified.

The full-length 14-kb mRNA transcribed from the DMD locus was found to be predominantly expressed in skeletal and cardiac muscle with smaller amounts in brain and covered a large genomic region (Monaco et al., 1986; Koenig et al., 1987). It takes more than 24 hours to be transcribed, and it consists of at least 85 exons (including its different isoforms) with introns making up 98% of the gene. The protein product encoded by this transcript was named dystrophin since the lack of it causes dystrophy (Koenig et al., 1987).

1.2.4 Tissue-Specific Promoters:

Expression of the full-length dystrophin transcript is controlled by three independently regulated promoters. The brain (B), muscle (M), and Purkinje (P) promoters consist of unique first exons spliced to a common set of 78 exons

(Figure 1.4) (Nudel et al., 1989; Chelly et al., 1990; Boyce et al., 1991; Makover et al., 1991) The names of these promoters reflect the major site of dystrophin expression. The B promoter drives expression primarily in cortical neurons and the hippocampus of the brain (Barnea et al., 1990; Chelly et al., 1990; Gorecki et al., 1992), while the P promoter is expressed in the cerebellar Purkinje cells and also skeletal muscle (Holder et al., 1996). The M promoter results in high levels of expression in skeletal muscles and cardiomyocytes and also at low levels in some glial cells in the brain (Barnea et al., 1990). These three promoters are situated within a large genomic interval of ~400kb (Figure 1.4) (Boyce et al., 1991).

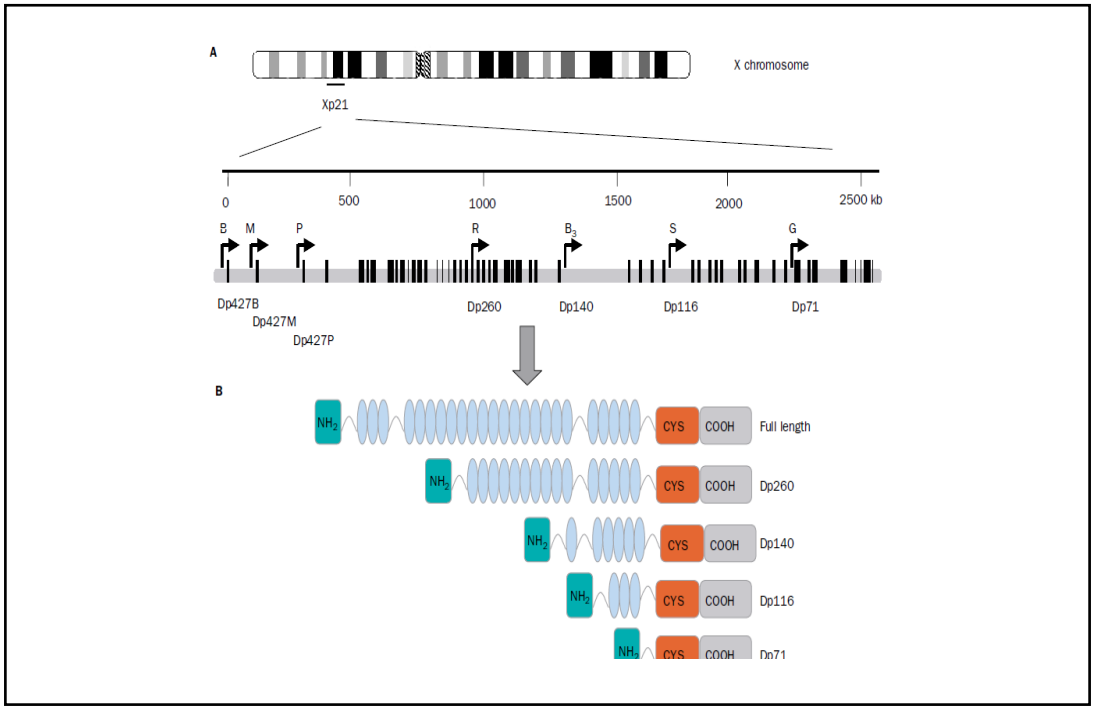

Figure 1.4. Genomic organization of the dystrophin gene and its isoforms. *Figure shows dystrophin gene isoforms. A: Genomic organisation of the dystrophin gene, located in Xp21. The black vertical lines represent the 79 exons of the dystrophin gene distributed over about 2·5 million bases. The arrows indicate the various promoters: in particular are brain (B), muscle (M), and Purkinje (P) promoters; R, B3, S, and G represent the Dp260 (retinal), Dp140 (brain3), Dp116 (Schwann cells), and Dp71 (general) promoters. B: The domain composition of the various dystrophin proteins is indicated. The amino-terminal domain (in blue) is followed by the spectrin like domain (light blue), the cysteine*

rich (in orange), and the carboxy-terminal domain (in grey). (Adapted from Muntoni et al., 2003)

1.2.5 Dystrophin Isoforms and Splice Variants

The DMD gene also has at least four internal promoters that give rise to shorter dystrophin transcripts that encode truncated COOH-terminal isoforms. These internal promoters can be referred to as retinal (R), brain-3 (B3), Schwann cell (S), and general (G). Each of these promoters utilizes a unique first exon that splices in to exons 30, 45, 56, and 63, respectively, (Blake et al., 1996; Benson et al., 2001) to generate protein products of 260 kDa (Dp260) (134a), 140 kDa (Dp140) (295), 116 kDa (Dp116) (72), and 71 kDa (Dp71) (43, 241,291). (Table 1.2)

Table 1.2: **Isoforms of Dystrophin, promoter's location and tissue expression**.

Isoform Name	Synonym	Synonym	Protein length	Amino acids	mRNA	Promoter location	Tissue of Expression
Dp260	Retinal dystrophin	R-dystrophin	260 kDa			Intron 29	Retina
	Dp260-1	R-1		2344	9773 bp		Retina
	Dp260-2	R-2		2341	9916 bp		Retina
Dp140			140 kDa	1225	7410 bp	Intron 44	CNS, kidney
	Dp140b			1243	7378 bp		Kidney
	Dp140ab			1230	7339 bp		Cerebellum, kid
	Dp140c			1115	7050 bp		Cerebellum
	Dp140bc			1133	7048 bp		Cerebellum, kid
Dp116	Apo-dystrophin 2	S-dystrophin	116 kDa	956	5623 bp	Intron 55	Schwann cells
Dp71	Apo-dystrophin 1	G-dystrophin	71 kDa	617	4623 bp	Intron 62	Ubiquitous
	Dp71b		72.2 kDa	635	4591 bp		Ubiquitous
	Dp71a		68.9 kDa	604	4584 bp		Ubiquitous
	Dp71ab		70.8 kDa	622	4552 bp		Ubiquitous
Dp40	Apo-dystrophin 3		40 kDa	340	2.2 kb		Ubiquitous

Dp71 is detected in most non muscle tissues including brain, kidney, liver, and lung (Blake et al., 1992; Hugnot et al., 1992; Lederfein et al., 1992; Schofield et al., 1994; Howard et al., 1998; Howard et al., 1999; Sarig et al., 1999) while the remaining short isoforms are primarily expressed in the central and peripheral nervous system (Byers et al., 1993; Schofield et al., 1994; D'Souza et al., 1995; Lidov et al., 1995). Dp140 has also been implicated in the development of the kidney (Durbeej et al., 1997). These COOH-terminal isoforms contain the necessary binding sites for a number of dystrophin-associated proteins, and although the molecular and cellular function of these isoforms has not been elucidated, they are thought to be involved in the stabilization and function of non- muscle dystrophin-like protein complexes. Alternative splicing at the 3'-end of the dystrophin gene generates an even greater number of isoforms (Feener et al., 1989; Bies et al., 1992). These splice variants not only affect full-length

dystrophin but are also found in the shorter isoforms such as Dp71. This differential splicing may regulate the binding of dystrophin to dystrophin-associated proteins at membranes (Crawford et al., 2000) of cells where they are expressed.

1.2.6 The Dystrophin Protein

Dystrophin is 427-kDa cytoskeletal protein that is a member of the α-spectrin/α-actinin protein family (Koenig et al., 1988).This family is characterized by a NH_2 - terminal actin binding domain followed by a variable number of repeating units known as spectrin-like repeats. Dystrophin can be organized into four separate regions based on sequence homologies and protein-binding capabilities (Figure 1.5). These are the actin-binding domain at the NH_2 terminus, the central rod domain, the cysteine-rich domain, and the COOH-terminal domain. The NH_2 terminus and a region in the rod domain of dystrophin bind directly, but do not cross-link cytoskeletal actin (Winder et al., 1995; Rybakova et al., 1996). The rod domain is composed of 24 repeating units that are similar to the triple helical repeats of spectrin. This repeating unit accounts for the majority of the dystrophin protein and is thought to give the molecule a flexible rod-like structure similar to α-spectrin. These α-helical coiled-coil repeats are interrupted by four proline-rich hinge regions (Koenig and Kunkel, 1990). At the end of the 24th repeat is the fourth hinge region that is immediately followed by the WW domain. The WW domain is a recently described protein-binding module found in several signaling and regulatory molecules (Bork and Sudol, 1994). The WW domain binds to proline-rich substrates in an analogous manner to the src homology-3 (SH3) domain (Macias et al., 1996). Although a specific ligand for the WW domain of dystrophin has not been determined, this region mediates the interaction between β-dystroglycan and dystrophin, since the cytoplasmic domain of β-dystroglycan is proline rich. However, the entire WW domain of dystrophin does not appear to be required for the interaction with dystroglycan because Dp71, a dystrophin isoform that contains only part of the WW domain, is reported to bind to β-dystroglycan (Rosa et al., 1996). Interestingly, transgenic mice over expressing Dp71 in dystrophin-deficient muscle restore β-dystroglycan and the DPC at the membrane but do not prevent muscle degeneration (Cox et al., 1994; Greenberg et al., 1994).

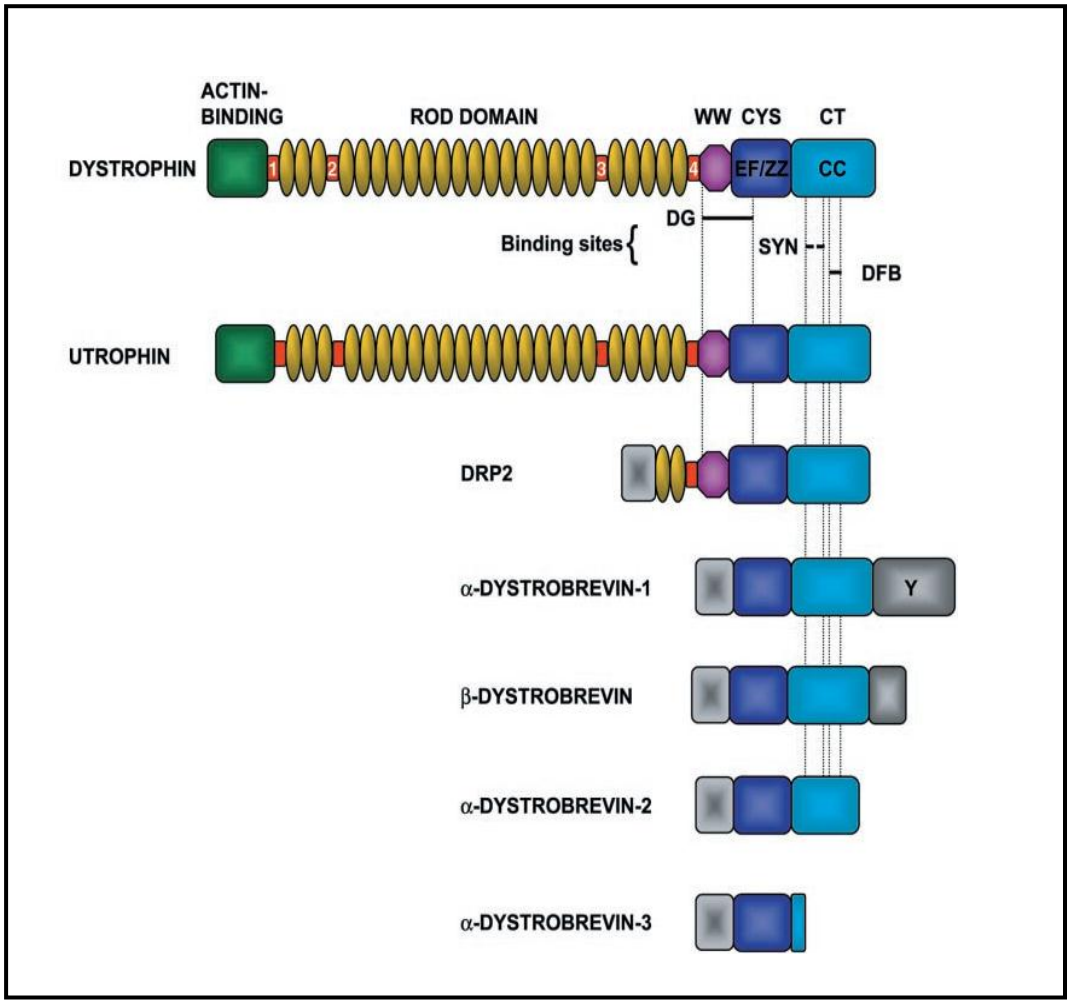

Figure 1.5: Schematic diagram showing the organization of the human Duchenne muscular dystrophy gene and related protein family. *The identifiable domains in the cysteine-rich (CR) region and COOH terminus (CYS) of dystrophin are identified. These are the WW domain, the EF hands, the ZZ domain, and the paired coiled-coil (CC). The four proline-rich hinge regions are designated 1–4. The binding sites for β-dystroglycan (DG), syntrophin (SYN), and the dystrophin family binding site (DFB) are shown for each protein (dotted lines). The organization of the utrophin protein shows that it is very similar to dystrophin, whereas the DRP2 and the dystrobrevins proteins only have sequence similarity to the COOH-terminal regions of dystrophin as shown. Three β-dystrobrevin isoforms are expressed in muscle representing successive COOH-terminal truncations. The β-dystrobrevin- 1 isoform has additional COOH-terminal sequence that contains the sites for tyrosine phosphorylation. β-Dystrobrevin is not expressed in*

muscle and is most similar to β-dystrobrevin-1 but lacks the sites for tyrosine phosphorylation (Adapted from Blake et al., 2002).

The WW domain separates the rod domain from the cysteine-rich and COOH-terminal domains. The cysteine rich domain contains two EF-hand motifs that are similar to those in α-actinin and that could bind intracellular Ca^{2+} (Koenig et al., 1988). The ZZ domain is also part of the cysteine-rich domain and contains a number of conserved cysteine residues that are predicted to form the coordination sites for divalent metal cations such as Zn^{2+} (Ponting et al., 1996). The ZZ domain is similar to many types of zinc finger and is found both in nuclear and cytoplasmic proteins. The ZZ domain of dystrophin binds to calmodulin in a Ca^{2+}-dependent manner (Anderson et al., 1996). Thus the ZZ domain may represent a functional calmodulin-binding site and may have implications for calmodulin binding to other dystrophin-related proteins.

The ZZ domain does not appear to be required for the interaction between dystrophin and β-dystroglycan (Rentschler et al., 1999).The COOH terminus of dystrophin contains two polypeptide stretches that are predicted to form α-helical coiled coils similar to those in the rod domain (Blake et al., 1995). Each coiled coil has a conserved repeating heptad $(a\text{-}g)_n$ similar to those found in leucine zippers, where leucine predominates at the "d" position (Lupas 1996; Burkhard et al., 2001). This domain has been named the CC (coiled coil), domain. Approximately 3–5% of proteins have coiled-coil regions. Coiled coils are well-characterized protein interaction domains. The CC region of dystrophin forms the binding site for dystrobrevin and may modulate the interaction between syntrophin and other dystrophin-associated proteins (Blake et al., 1995; Sadoulet-Puccio et al., 1997).

1.2.7 Binding partners of Dystrophin and their interactions

Despite being the first mutant protein shown to cause MD, the exact function of dystrophin has not yet been elucidated. Dystrophin anchors the sarcolemma to the actin cytoskeleton in the sarcoplasm and therefore has an important structural role during muscle contraction and muscle stretch. Dystrophin is expressed at the sarcolemma and is enriched at the costameres and sites of cell–cell contact, namely the myotendinous junction and the neuromuscular junction (NMJ).

Dystrophin is thought to be an elastic and flexible protein owing to triple helical repeats located in its rod domain (Grum et al., 1999) and therefore dystrophin probably protects the muscle cell from the stresses caused by the force created during muscle contraction.

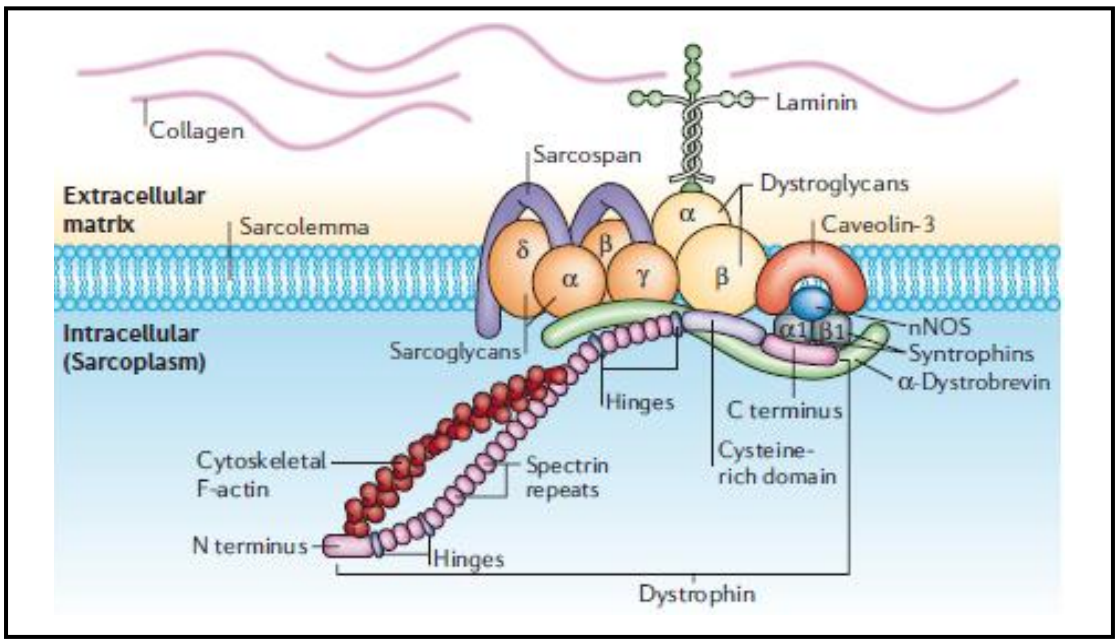

Figure 1.6: Dystrophin Associated Glycoprotein complex. *Figure shows Dystrophin binding to the DAPC at the sarcolemma. The N-terminus binds to F-actin and the C-terminus to the Dystroglycan complex. (Adapted from Davies and Nowak, 2006)*

Muscle fibres that lack dystrophin are less stiff than normal fibres (Pasternak et al., 1995). The N terminus of dystrophin shares high homology with spectrins and α-actinins and therefore dystrophin belongs to the spectrin superfamily (Koenig et al., 1988). The N terminus and spectrin repeats of dystrophin bind to the cytoskeleton through filamentous (F)-actin (Hemmings et al., 1992; Rybakova et al., 1996). The ~1,200 amino acids that are found between these two regions of dystrophin provide an extended lateral connection between dystrophin and F-actin, with 1 dystrophin molecule binding to the equivalent of 24 actin monomers, which are present as polymerized actin (Rybakova and Ervasti, 1997) (Figure 1.6).

The actin that binds to dystrophin (γ-actin) is a different type of actin from that found in the thin filaments of sarcomeres (α-actin) (Rybakova et al., 2000). In the

absence of dystrophin, costameric actin (γ-actin) disappears, whereas the actin in the contractile apparatus (α-actin) is retained (Rybakova et al., 2000).

Dystrophin also binds to dystroglycan through the cysteine-rich domain and to α-dystrobrevin through the C-terminal domain (Figure 1.6), dystroglycan and α-dystrobrevin are involved in various MDs. The C terminus of dystrophin binds to the dystrophin-associated protein complex (DAPC), a group of proteins that can be purified as a macromolecular structure from muscle-fibre membranes that have been solubilized by detergents (Ervasti et al., 1990; Yoshida and Ozawa, 1990). The DAPC consists of cytoplasmic, transmembrane and extracellular proteins, and therefore provides a strong mechanical link and mediates interactions between the intracellular cytoskeleton and the ECM (Rando, 2001) (Figure 1.6). It is thought that mutant protein members of the DAPC cause a loss of sarcolemmal integrity and therefore render muscle fibres more vulnerable to damage (Petrof et al., 1993). In the absence of dystrophin, the DAPC becomes destabilized, leading to diminished levels of the other DAPC proteins (Ervasti et al., 1990; Yoshida and Ozawa, 1990). Therefore, the protective role of dystrophin and the other members of the DAPC anchoring the sarcolemma to the internal actin cytoskeleton and to the ECM is lost. The increasingly fragile sarcolemma is subjected to mechanical injury, which results in progressive muscle-fibre damage and sarcolemmal leakage. Regeneration gradually fails as the pool of endogenous satellite cells ceases to compensate for the damaged muscle fibres. The extent of necrosis and membrane weakness is exacerbated by physical exercise, but is improved by muscle immobilization (Davies and Nowak, 2006).

1.2.8 Mutations in DMD

The frequency of DMD coupled with a high new mutation rate (1×10^{-4} genes/generation) has led to the characterization of hundreds of independent mutations. Mutations that cause DMD generally result in the absence, or much reduced levels, of dystrophin protein while BMD patients generally make some partially functional protein. There is some correlation between mutations in the DMD gene and the resulting phenotype. The study of such mutations has revealed the importance of a number of the structural domains of dystrophin and facilitated the design of dystrophin "mini-genes" for gene therapy approaches (Acsadi et al., 1991). Approximately 65% of DMD and BMD patients have gross

deletions of the DMD gene (Monaco et al., 1985; Koenig et al., 1989). Duplications occur in roughly 6 to 10% of males with either DMD or BMD. The remaining mutations are small deletions, insertions, point mutations, or splicing mutations, most of which introduce premature stop codons (Mendell et al., 2001; Prior and Bridgeman, 2005). Unlike the large deletions that cluster in just two regions of the dystrophin gene, small deletions and point mutations appear to be evenly distributed throughout. To date, 501 deletions, 84 duplications, and 989 point mutations have been documented in the dystrophin gene (Leiden muscular dystrophy database; www.dmd.nl).

1.2.9 The Reading Frame rule for DMD gene deletions

After the characterization of many such mutations, it became apparent that the size and position of the deletion within the DMD gene often did not correlate with the clinical phenotype observed. This observation can be largely explained by the reading frame theory of Monaco et al (Monaco et al., 1988). This argues that if a deletion leads to the expression of an internally truncated transcript without shifting the normal open reading frame, then a smaller, but functional version of dystrophin could be produced. This scenario would be consistent with a BMD phenotype. If, on the other hand, the deletion creates a translational frame shift, then premature termination of translation will result in the synthesis of a truncated protein. (Figure 1.7) This latter scenario is often associated with extremely low levels of dystrophin expression due to mRNA or protein instability and results in a DMD phenotype. With the use of this reading frame theory and the knowledge of exon structure of the DMD gene, it has been possible in many cases to predict whether a young male is likely to develop BMD or DMD (Koenig et al., 1989). However, there are exceptions to this reading frame rule (Malhotra et al., 1988; Baumbach et al., 1989; Winnard et al., 1993), and there are cases in which complete dystrophin deficiency may be associated with a relatively benign phenotype (Hattori et al., 1999).

The vast majority of large deletions detected in BMD and DMD cluster around two mutation “hot spots” (Koenig et al., 1989; Koenig and Kunkel, 1990), although the reasons for this are unclear. It is possible, however, that the chromatin structure in Xp21 influences the occurrence of deletion or recombinant hotspots. Deletion cluster region I spans exons 45–53 (Beggs et al., 1990) and

removes part of the rod domain, while deletion cluster region II spans exons 2–20 and removes some or all of the actin-binding sites together with part of the rod domain (Liechti-Gallati et al., 1989). Most of the breakpoints occurring in cluster region II occur in the large introns 1 and 7. Most of these large deletions can be detected using a simple multiplex PCR test that screens the exons most commonly deleted and allows accurate genetic counseling in the majority of affected families via DNA-based diagnostics (Beggs and Kunkel, 1990; Chamberlain, 1992).

One-third of DMD cases are caused by very small deletions and point mutations, most of which introduce premature stop codons (Lenk et al., 1993; Roberts et al., 1994). Unlike the large deletions that cluster in two regions of the DMD gene, small deletions and point mutations appear to be evenly distributed throughout the gene (Roberts et al., 1994; Gardner et al., 1995; Prior et al., 1995). Although it might be predicted that such mutations would give rise to normal amounts of truncated protein, usually very little or no protein is detected, indicating that the corresponding transcripts or the truncated proteins are unstable (Hoffman et al., 1987).This has disappointing implications for the functional dissection of the dystrophin protein, since many mutations do not generate any information regarding the importance of a particular domain. Despite this setback, a small number of useful mutations have been identified that generate a mutated or truncated protein and convey information regarding the functional importance of the different dystrophin domains. At the NH_2 terminus of dystrophin, the importance of the actin-binding domain was demonstrated by the identification of missense mutation (Arg for Leu-54) that resulted in a DMD phenotype associated with reduced amounts of protein (Prior et al., 1995). DMD patients have been described with in-frame deletions of exons 3–25 and produce normal amounts of truncated protein (Vainzof et al., 1993).

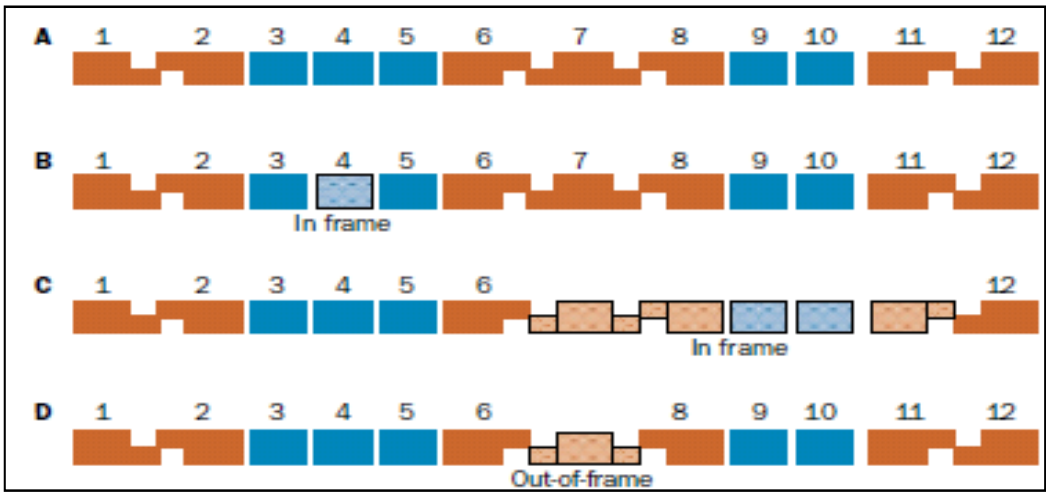

Figure 1.7: Effects of DMD exon deletions on the open reading frame. *Figure shows the effects of different genomic deletions on the reading frame of the dystrophin gene (A). The removal of exon 4 (B) and of exon 7-11 (C) maintains the open-reading frame. The deletion of exon 7 leads to the loss of the open-reading frame (D). (Adapted from Muntoni et al., 2003)*

The rod domain of dystrophin has been found to accommodate large in-frame deletions without serious clinical consequences. The most notable example was the discovery of a patient with an in-frame deletion of 46% of the dystrophin coding sequence which resulted in only a mild case of BMD (deletions of exons 17–48) (England et al., 1990). This observation suggests that the rod domain acts as a spacer between the actin binding domain and the cysteine-rich and COOH-terminal domains of dystrophin, and truncation of this region merely shortens the bridge between these two functional regions without adversely affecting the function of the protein. Indeed, this deletion has been the basis of a dystrophin mini-gene that was incorporated into expression plasmids as well as retroviral and adenoviral vectors for transfer to muscle fibers *in vivo* (Acsadi et al., 1991; Dunckley et al., 1993; Ragot et al., 1993). Furthermore, this mini-dystrophin was able to restore the normal muscle phenotype in transgenic *mdx* mice (Phelps et al., 1995; Wells et al., 1995). Other large deletions of the rod domain have also been observed in BMD patients (Love et al., 1991; Winnard et al., 1993). Although few missense mutations have been described in DMD patients, two informative substitutions have been identified in the cysteine-rich domain. The substitution of a conserved cysteine residue with a tyrosine at position 3340 results in reduced but detectable levels of dystrophin. This mutation alters one of the coordinating residues in the ZZ domain that is thought to interfere with the

binding of the dystrophin-associated protein β-dystroglycan (Lenk et al., 1996). Another reported substitution of an aspartate residue to a histidine residue at position 3335 is also thought to affect the β-dystroglycan binding site, and although there was normal localization and amounts of dystrophin detected, a severe phenotype resulted (Goldberg et al., 1998). Interestingly, the cysteine-rich domain is never deleted in BMD patients, suggesting that this domain is critical for dystrophin function (Raats et al., 2000).

A small number of cases have been reported in which an abnormally truncated protein that is deleted for the COOH terminus is synthesized and localized at the sarcolemma. A DMD patient was found to have a deletion that removed almost the entire cysteine-rich and COOH-terminal domain (Hoffman et al., 1991; Bies et al., 1992). The abnormal protein was normally localized but resulted in a severe clinical phenotype. Another DMD patient has been reported to be deleted for everything 3' of exon 50 but again generates a truncated protein that is localized to the sarcolemma (Helliwell et al., 1992). These examples illustrate the functional importance of the cysteine-rich and COOH-terminal domains of dystrophin that presumably reflects their interactions with other dystrophin-associated proteins.

1.3 PATHOPHYSIOLOGY OF DMD

Although the responsible gene and its product, dystrophin, have been characterized for more than 15 years, and a mouse model (*mdx*) has been developed and extensively studied, comprehensive understanding of the mechanism leading from the absence of dystrophin to the muscular degeneration is still lacking. In patients with DMD, muscle biopsy characteristically demonstrates necrotic or degenerating muscle fibers, often observed in clusters. (Figure 1.8) These necrotic fibers are surrounded by macrophages and $CD4^+$ lymphocytes. Small immature centrally nucleated fibers are also observed, reflecting muscle regeneration from myoblasts (Schmalbruch, 1984; McDouall et al., 1990) that results in a balance between necrotic and regenerative processes in the early phase of the disease. Later, the regenerative capacity of the muscles appears to be exhausted and muscle fibers are gradually replaced by connective and adipose tissue. Therefore the manifestations of DMD are considered to result from imbalance between muscle fiber necrosis and myoblast regeneration, the

primary pathologic feature being necrosis, although animal evidence suggests that regenerative capacity per se may decrease with age (Bockhold et al., 1998).

The full length human dystrophin protein is composed of 3685 amino acid residues with a molecular weight of 427 kD and is a subsarcolemmal component of the cytoskeleton (Cohn and Campbell, 2000). Dystrophin shows structural homology with spectrin and α-actinin and contains four distinct domains. Dystrophin binds F-actin filaments at its amino-terminal domain and parts of the helical-rod domain. The C-terminus and a cysteine-rich domain interact with integral membrane proteins, including sarcoglycan, dystroglycans, syntrophin, and dystrobrevin, which are assembled together to form the DAPC (Figure 1.5). The DAPC provides a crucial structural and signaling link between the ECM and the intracellular actin cystoskeleton across the sarcolemma (Cohn and Campbell, 2000; Rando, 2001).

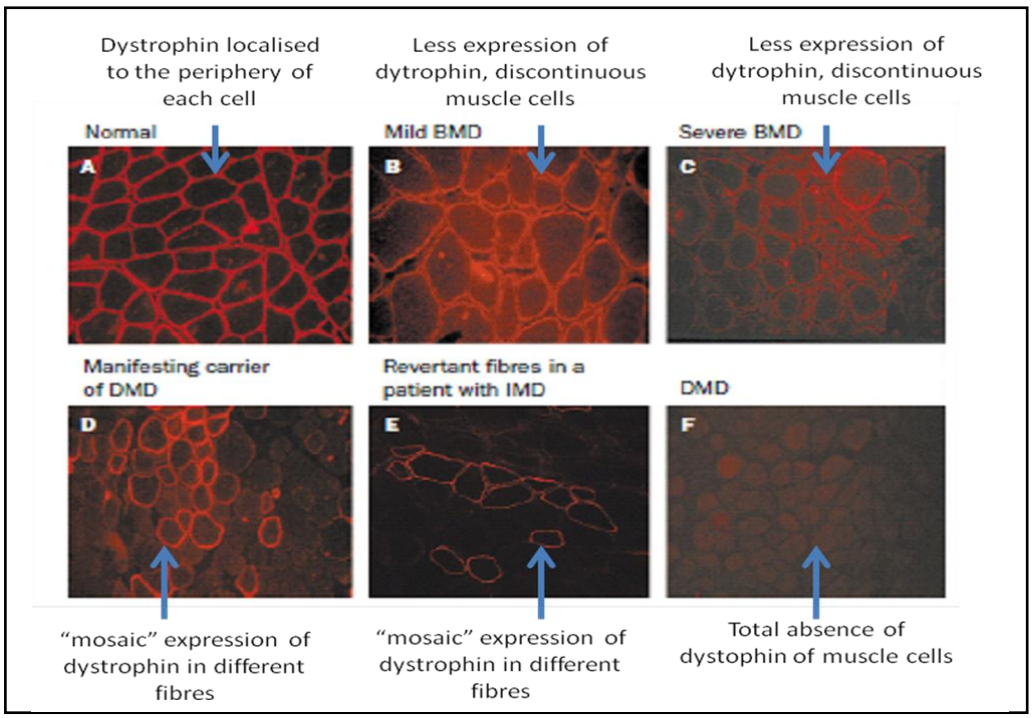

Figure 1.8: Immunocytochemical findings in normal and patients' muscle. *Figure 1.8 shows immunocytochemical findings of normal and dystrophic muscles. Normal muscle (A) shows dystrophin is localised at the periphery of each muscle fibre; In patients with BMD (B and C), there is low protein expression in the muscles that is commonly discontinuous, in a manifesting carrier of DMD (D) a "mosaic" expression of dystrophin in different fibres is seen, an apparently similar pattern characterizes patients with IMD (E) or in a*

patient with an intermediate between DMD and BMD phenotype, who show relatively abundant revertant fibres. In patients with DMD (F) the protein expression is absent from muscle tissue. (Adapted from Muntoni et al., 2003)

Deficiency of dystrophin expression affects formation of the DAPC and causes a disruption of the molecular bridge (Blake et al., 2002).These effects have at least two downstream consequences: first, the cell membrane is more fragile and can be mechanically damaged during eccentric muscle contraction; and second, membrane proteins, especially mechano-sensitive ion channels are dysregulated (Vandebrouck et al,. 2002; Iwata et al., 2003; Kumar et al., 2004). In both DMD patients and a DMD animal model, intracellular calcium levels are elevated. In cultured DMD and *mdx* myotubes, leak channel activity, which affects the calcium permeability of the sarcolemma, is increased (Porter et al., 2002; Porter et al., 2003). Enhanced calcium influx through calcium/stretch- activated channels is observable in young *mdx* diaphragm muscles isolated before the onset of significant pathology. The mechanisms underlying the dysregulated calcium homeostasis remain somewhat controversial. One possibility is that muscle activity results in microlesions of the dystrophic membrane, allowing abnormal Ca^{2+} influx and overloading (Porter et al., 2003).The calcium overloading then results in the activation of Ca^{2+} dependent proteases and contributes to muscle fiber degeneration. The aberrant hyperactivation of signaling cascades also promotes an inflammatory response (Blake et al., 2002) by elevating expression of inflammatory mediators and chemo-attractants in dystrophin-deficient muscles prior to the onset of major disease symptoms (Porter et al., 2002; Porter et al., 2003).

Other cellular factors may also be involved in the pathophysiology of DMD. Aquaporin-4 (AQP4) is a protein enriched in the sarcolemma of normal skeletal muscle and may participate in accommodating the rapid changes in cell volume and hydrostatic forces that occur during contraction. Absence of AQP4 in dystrophin-deficient muscles suggests a possible association with the pathophysiological process (Wakayama et al., 2002).The impaired expression of another molecule, the muscle membrane-associated neuronal nitric oxide synthase (NOS), is also detected in Duchenne patients, and expression of a nitric

oxide synthase transgene can ameliorate muscular dystrophy in *mdx* mice (Wehling et al., 2001).

The pathophysiologic changes following the loss of dystrophin are still speculative. Several hypotheses coexist: Dystrophin is considered a key structural element in the muscle fiber, and the primary function of the DAPC is to stabilize plasma membrane, although a role of signaling is still possible. Mechanically induced damage seems particularly harmful to dystrophin-deficient fibers. Eccentric contractions put a high stress on fragilized membranes and provoke microlesions that could eventually lead to massive calcium entry, loss of calcium homeostasis, activation of Ca^{2+} -dependent proteases, and finally to cell death. Altered regeneration, inflammation, apoptosis, impaired vascular adaptation, and fibrosis are probably secondary events that take part in the muscle dystrophic degeneration (Figure 1.9).

It is unpredictable when gene therapy strategies will be clinically available; a successful strategy remains to be discovered. Improved therapeutics to reduce secondary features of the disease will be of great importance too. A better understanding of these mechanisms could prove useful for producing new adjuvant treatments.

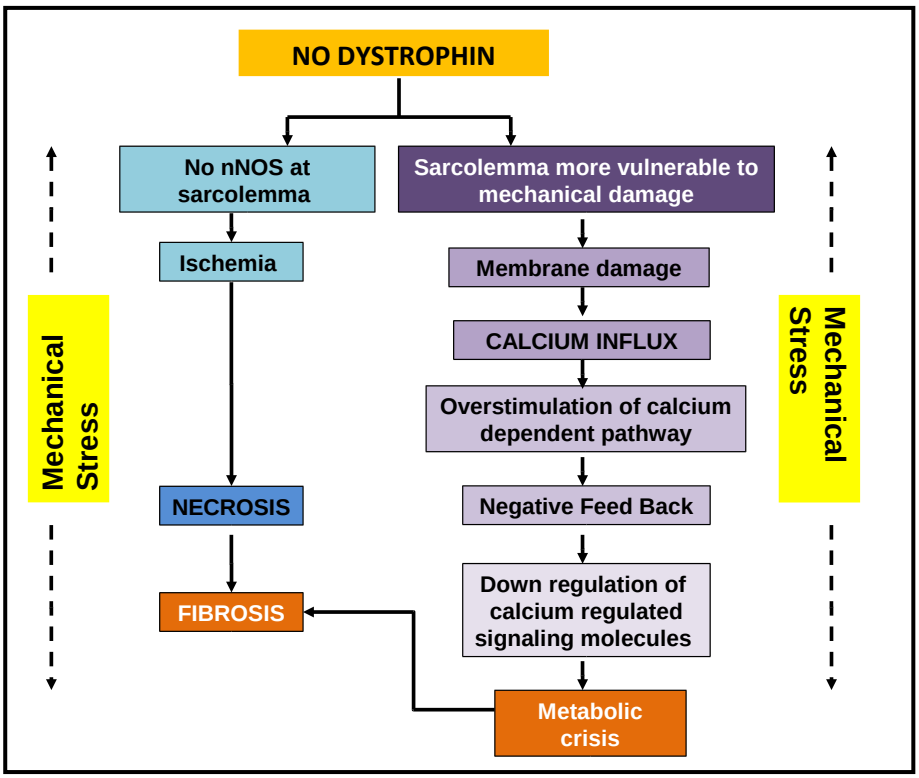

Figure 1.9: Hypothetical flowchart of DMD pathology. *DMD pathology is suspected to be due to both mechanical instability induced by membrane damage, leading to metabolic crisis and NIS induced ischemia and necrosis.*

1.4 DIAGNOSIS OF DMD

The aim of care around diagnosis is to provide an accurate and prompt diagnosis, allowing initiation of appropriate interventions, continuing support and education, and minimising the length and impact of a potentially protracted diagnostic process. Diagnosis should be done by a neuromuscular specialist who can assess the child clinically and can rapidly access and interpret appropriate investigations in the context of the clinical presentation. Family follow-up and support after diagnosis will often be augmented by support from geneticists and genetic counsellors.

1.4.1 When to suspect DMD

Suspicion of the diagnosis of DMD should be considered irrespective of family history and is usually triggered in one of three ways:

(1) Most commonly, the observation of abnormal muscle function in a male child.

(2) The detection of an increase in serum creatine kinase tested for unrelated indications or

(3) After the discovery of increased transaminases (aspartate aminotransferase and alanine aminotransferase, which are produced by muscle as well as liver cells).

The diagnosis of DMD should thus be considered before liver biopsy in any male child with increased transaminases. Initial symptoms might include delayed walking, frequent falls, or difficulty with running and climbing stairs. Although DMD is typically diagnosed at around 5 years of age, the diagnosis might be suspected much earlier because of delays in attainment of developmental milestones, such as independent walking or language, such delays have been documented prospectively by following patients with DMD identified by newborn screening.

Common early symptoms in Duchenne muscular dystrophy

- Calf hypertrophy
- Difficulty in climbing stairs
- Difficulty to stand up from supine position

- Difficulty in walking
- Frequent falls
- Gower's sign

The presence of Gower's sign (Figure 1.10) in a male child should trigger the diagnostic investigation of DMD, especially if the child also has a waddling gait. Toe walking might be present but is not additionally helpful in deciding whether to suspect DMD. In the presence of a positive family history of DMD, there should be a low threshold for testing creatine kinase, although this will be influenced by the age of the child. In a child less than 5 years of age, suspicion of DMD probably cannot be excluded completely by a normal muscle examination. However, with increasing age, a normal muscle examination renders the chance of a child having DMD progressively less likely. A boy older than 10 years of age with normal muscle function is thus highly unlikely to have DMD.

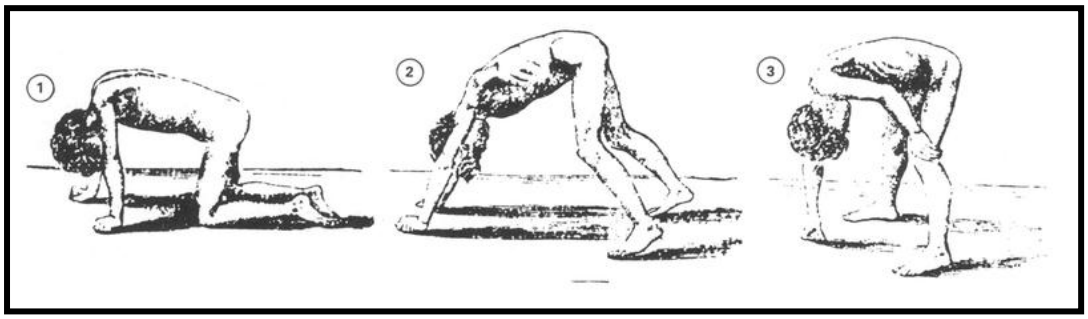

Figure 1.10: Gower's sign in Duchenne muscular dystrophy. *This highlights the difficulty while the child gets up from supine position. (Adapted from* http://pt.wikipedia.org/wiki/Ficheiro:Gower's_Sign.png*)*

1.4.2 Confirmation of the diagnosis

The route to confirming the diagnosis depends on local availability of rapid and reliable testing, which must be interpreted alongside the clinical presentation owing to the range of severity possible with dystrophin mutations. Testing for a DMD mutation in a blood sample is always necessary even if DMD is first confirmed by the absence of dystrophin protein expression on muscle biopsy. The results of genetic testing provide the clinical information required for genetic counselling, prenatal diagnosis, and consideration for future mutation-specific

therapies. Different types of mutations in DMD can be the genetic basis for DMD.

1.5 MOLECULAR DIAGNOSIS OF DMD

1.5.1 Diagnosis in male patients

Affected males suspected to have a dystrophinopathy based on high serum creatine kinase (CK) levels and/or muscle biopsy, are referred for a molecular confirmation of the clinical diagnosis. Molecular confirmation of a dystrophinopathy is achieved by demonstrating the presence of a clearly pathogenic variant in the DMD gene. Absence of a DMD mutation would reduce the likelihood of a patient having a dystrophinopathy, with the reduction being dependent on the sensitivity of the mutation screening procedure(s) used. It is currently not possible to refute a diagnosis of a dystrophinopathy based on the results of genetic testing, since no mutation detection protocol which is currently available can demonstrate 100% sensitivity.

From the patients' and relatives' perspectives, the speed with which a diagnosis can be made is extremely important in order to minimize anxiety and to reduce the risk of recurrence of the disease in the family. Therefore, in some instances a muscle biopsy and dystrophin analysis by immunohistochemistry might be needed to establish a quick and definite diagnosis. Appropriate genetic counselling in the family is dependent on the knowledge of the diagnosis in the patient. This should be borne in mind when determining the appropriate procedures and methods to follow, and the approach may vary in different centres depending on the availability of different tests and facilities, and economic factors. A number of methods have been tried for the diagnosis of DMD gene mutations (Table 1.3)

Table 1.3: List of different methods used in the diagnosis of mutations in Duchenne muscular dystrophy.

Method	Mutations type detected	References
Southern blot hybridization using cDNA probes	Deletions and Duplications	Darras et al., 1987
mPCR	Deletions	Chamberlain et al., 1988
mPCR	Deletions	Beggs et al., 1990
Quantitative multiplex PCR	Deletions, Duplications	Yau et al., 1986
PTT	Point mutations, small insertions and deletions	Roest et al., 1993
SSCP	Point mutations, small insertions and deletions	Mendell et al., 2001
dHPLC	Point mutations, small insertions and deletions	Bennett et al., 2001
Full gene sequencing	Point mutations, small insertions and deletions	Flanigan et al., 2003
FM-CSCE	Point mutations, small insertions and deletions	Ashton et al., 2008
array CGH	Deletions, Duplications	Bovolenta et al., 2008, Hegde et al., 2008
HRM	Point mutations, small insertions and deletions	Almomani et al., 2009

1.5.2 Testing for deletions and duplications

Since whole exon deletions are the predominant type of mutation in the DMD gene (~65%), an initial screen which detects the majority of deletions should be the minimum level of diagnostic test offered. A number of methods have been described, with the following being the most popular choices currently in use, Multiplex PCR, to amplify the exons known to be most commonly deleted. The two PCR multiplex sets of Chamberlain et al. (Chamberlain et al., 1988) and Beggs et al. (Beggs et al., 1990), or recent improvements on these made by a

number of centres, collectively enable the detection of about 98% of all DMD deletions.

These two assays do not characterize the end points of all deletions, since they do not test all exons. Where the end point(s) of a deletion is (are) not determined by the multiplex PCR system being used, it is beneficial to use additional PCR assays to characterise the extent of the deletion, whenever possible. Details of PCR primers for all DMD exons can be found at www.dmd.nl. Quantitative assays of all exons offer an improvement in mutation detection rate, since they will detect all whole exon deletions, and additionally whole exon duplications (~10% of DMD mutations). Further advantages are that these assays characterise the end points of most rearrangements (at the exon level of resolution), and can also be used for carrier testing of females.

Of the quantitative methods available, multiplex ligation-dependent probe amplification (MLPA) (Schouten et al., 2002) is currently the most widely used method. Quantitative multiplex PCR of selected exons (Yau et al., 1996) and Southern blot hybridization using cDNA probes (Darras et al., 1987) have both been widely used in the past, but have been superseded in many labs by the convenience and commercial availability of MLPA. A recently developed quantitative approach to assay the DMD gene with high resolution is array CGH (comparative genomic hybridisation) (Bovolenta et al., 2008; del Gaudio et al., 2008; Hegde et al., 2008). This method uses thousands of oligonucleotides to interrogate copy number across the entire 2.2 MB genomic region of the DMD gene including all exons and introns, and thereby maps rearrangement breakpoints to relatively narrow intervals depending on the spacing of the oligonucleotides at the breakpoints. It also can detect loss or gain of sequences at intronic breakpoints associated with some inversions and complex rearrangements, thereby offering a slightly higher mutation detection rate than MLPA and other exon-based tests. If a method identifies an apparent single exon deletion or duplication based on the absence or increased amplification, of a single PCR amplification, or hybridisation, that result must be confirmed using an alternative assay. This different assay will verify whether the initial result could have been caused by a sequence variant (e.g. SNP), preventing hybridisation of a primer, probe, etc., or for duplications if the result was an anomaly. This can be achieved using the same method, for example with different primers to amplify

an exon which looks deleted with multiplex PCR, or using a different method to assay that exon. High density array CGH has a further advantage here over most other methods which generate only a single result per exon, since most deletions or duplications are likely to be detected by several oligonucleotides on the array. This eliminates the possibility of a false positive result due to the presence of a SNP in a single probe or primer.

If a duplication of a single or multiple exons is identified it is important to test all exons for the possibility of additional exons being duplicated, since a number of apparently non-continuous duplications have been reported (White et al., 2006; Bovolenta et al., 2008). When interpreting the deletion or duplication result if the predicted severity is discrepant with the observed clinical phenotype, it may be useful to repeat the tests on a second sample and/or to carry out additional laboratory tests using different methods, or offering more detail, in order to look for an explanation to the discrepancy. This additional work is not essential, and may not be possible in all centres.

1.5.3 DMD gene copy number analysis

Although ~95% of deletions can be detected in males using multiplex PCR, other methods must be used to determine duplications, as well as the carrier status of females. The most commonly applied methods are quantitative multiplex PCR and quantitative Southern blotting. The drawback of quantitative multiplex PCR is that often not all exons are examined, meaning that small and rare mutations are missed. Southern blotting is usually applied to determine the boundaries of the mutation, which is essential for distinguishing DMD from BMD, i.e. frame disrupting from open reading frame changes. Using high-quality Southern blots it is possible to perform a quantitative analysis and detect duplications. However, this technique is time consuming, it is difficult to exactly determine the duplication boundaries, it can be difficult to detect duplications in females and triplications will be missed.

Multiplex Amplifiable Probe Hybridization (MAPH) and MLPA, developed by a group in MRC, Holland were DNA based methods which were very successful in picking deletions and duplications of whole exons throughout the entire 79 exons of the DMD gene. MAPH is based on a quantitative PCR of short DNA probes

recovered after hybridisation to immobilized genomic DNA. Each probe represents a single exon, which is amplified and cloned into a specific vector. By reamplification using primers from the multiple cloning sites it is possible to co-amplify all probes in one reaction using a single pair of primers.

1.5.4 Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA, another similar method to MAPH, was developed by the same group based on Multiplex Ligation-dependent Probe Amplification, (Schouten et al., 2002). The advantage of MLPA compared to MAPH is that a lower amount of input DNA is required and that MLPA is a one-tube assay. MLPA is a multiplex PCR method detecting abnormal copy numbers of up to 50 different genomic DNA or RNA sequences, which is able to distinguish sequences differing in only one nucleotide. The MLPA technique is easy to use and can be performed in many laboratories, as it only requires a thermocycler and capillary electrophoresis equipment. Up to 96 samples can be handled simultaneously, with results being available within 24 hours.

1.5.4.1 Principle of MLPA

The principle of MLPA is that it is not the target sequences that are amplified, but MLPA probes hybridise to the target sequence. In contrast to a standard multiplex PCR, a single pair PCR primers is used for MLPA amplification. The resulting amplification products of a SALSA MLPA kits range between 130 and 480 nt in length and can be analysed by capillary electrophoresis. Comparing the peak pattern obtained to that of reference samples indicates which sequences show aberrant copy numbers. The MLPA technique uses specially designed probes to amplify the target DNA. (Figure 1.11) Each MLPA probe consists of two oligonucleotides, one synthetic and one M13-derived single-stranded DNA fragment. For each probe there is a target specific sequence that can be ligated when correctly hybridized to its target. All probes have the same PCR primer sequences at their ends. The non-hybridizing stuffer sequence of each probe has a different length and sequence enabling separation by electrophoresis.

The principle of MLPA is based on the identification of target sequences by hybridization of pairs of MLPA probes that bind to adjacent sequences and can then be joined by a ligation reaction. In order to make one copy of each target

sequence, specific MLPA probes are added to a nucleic acid sample for each of the sequences of interest. The sequences are then simultaneously amplified with the use of only one primer pair, resulting in a mixture of amplification products, in which each PCR product of each MLPA probe has a unique length. One PCR primer is fluorescently or isotopically labelled so that the MLPA reaction products can be visualized when electrophoresed on a capillary sequencer or a gel. Resulting chromatograms show size-separated fragments ranging from 130 to 490 bp. The peak area or peak height of each amplification product reflects the relative copy number of that target sequence. Comparison of the electrophoresis profile of the tested sample to that obtained with a control sample enables the detection of deletions or duplications of genomic regions of interest.

1.5.4.2 MLPA® Probe Chemistry

MLPA® probes consist of two oligonucleotides, each containing a PCR primer sequence and a sequence complementary to the target, known as the hybridization sequence. The two probes hybridize immediately adjacent to each other. When the probes correctly hybridize to the target sequence they are ligated by a thermo stable ligase enzyme. The PCR primers exponentially amplify the ligated probes. One of the primers is labeled with a fluorescent dye to visualize the amplification product of the probe. (Figure 1.11) MLPA® based detection assays can be run in a single tube as non-ligated probes do not need to be removed. Sequence type electrophoresis is used to separate the resulting PCR products. Each MLPA® probe length is designed such that it can be easily identified when the amplification product of the PCR is run through a gel. The difference in size is achieved with the help of the stuffer sequence.

The MLPA reaction can be divided in five major steps: 1) DNA denaturation and hybridisation of MLPA probes 2) ligation reaction 3) PCR reaction 4) separation of amplification products by electrophoresis and 5) data analysis (Figure 1.12). During the first step, the DNA is denatured and incubated overnight with a mixture of MLPA probes. MLPA probes consist of two separate oligonucleotides, each containing one of the PCR primer sequences. The two probe oligonucleotides hybridise to immediately adjacent target sequences. Only when the two probe oligonucleotides are both hybridised to their adjacent targets can they be ligated during the ligation reaction. Because only ligated probes will

be exponentially amplified during the subsequent PCR reaction, the number of probe ligation products is a measure for the number of target sequences in the sample. The amplification products are separated using capillary electrophoresis. Probe oligonucleotides that are not ligated only contain one primer sequence. As a consequence, they cannot be amplified exponentially and will not generate a signal. The removal of unbound probes is therefore unnecessary in MLPA and thus makes the MLPA method easy to perform.

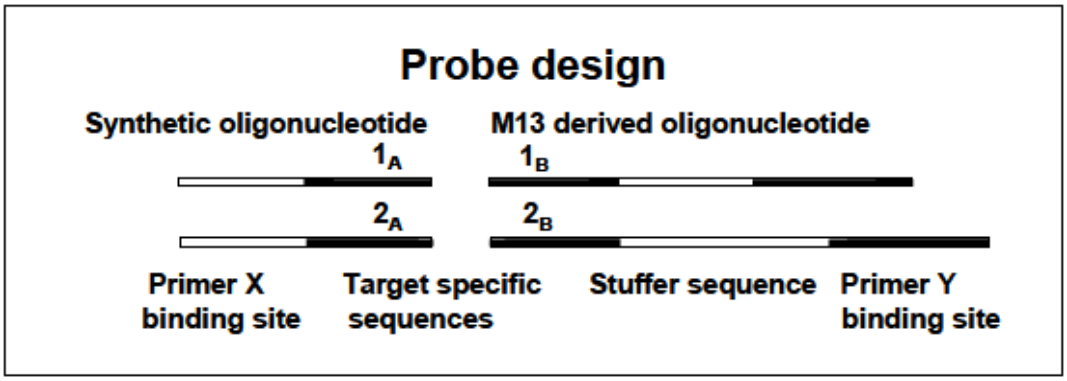

Figure 1.11: **MLPA probe design.** *Each MLPA probe consists of two oligonucleotides, one synthetic and one M13-derived single-stranded DNA fragment. For each probe there is a target specific sequence that can be ligated when correctly hybridized to its target. The left fragment of the probe has the forward primer X binding site, and target specific sequences. The right fragment has the target specific sequences , the stuffer sequence and the primer Y binding site. Once hybridized to its target sequence the probe as such is amplified and size separated (Adapted from* www.mlpa.com*).*

1.5.4.3 Analysis of MLPA data

Since normalisation of the electrophoresis results is essential for obtaining useful MLPA data, data analysis forms a crucial step of the MLPA procedure. Data analysis can be done when the data has passed the raw data and peak pattern evaluation. Data analysis can be done using one of the following softwares;

1. Coffalyser, MRC-Holland
2. National Genetics Research Laboratory, Manchester (NGRL) spreadsheet
3. Softgenics
4. JSI Medical systems etc.

The NGRL and Coffalyser softwares are free softwares and are created on Microsoft Excel.

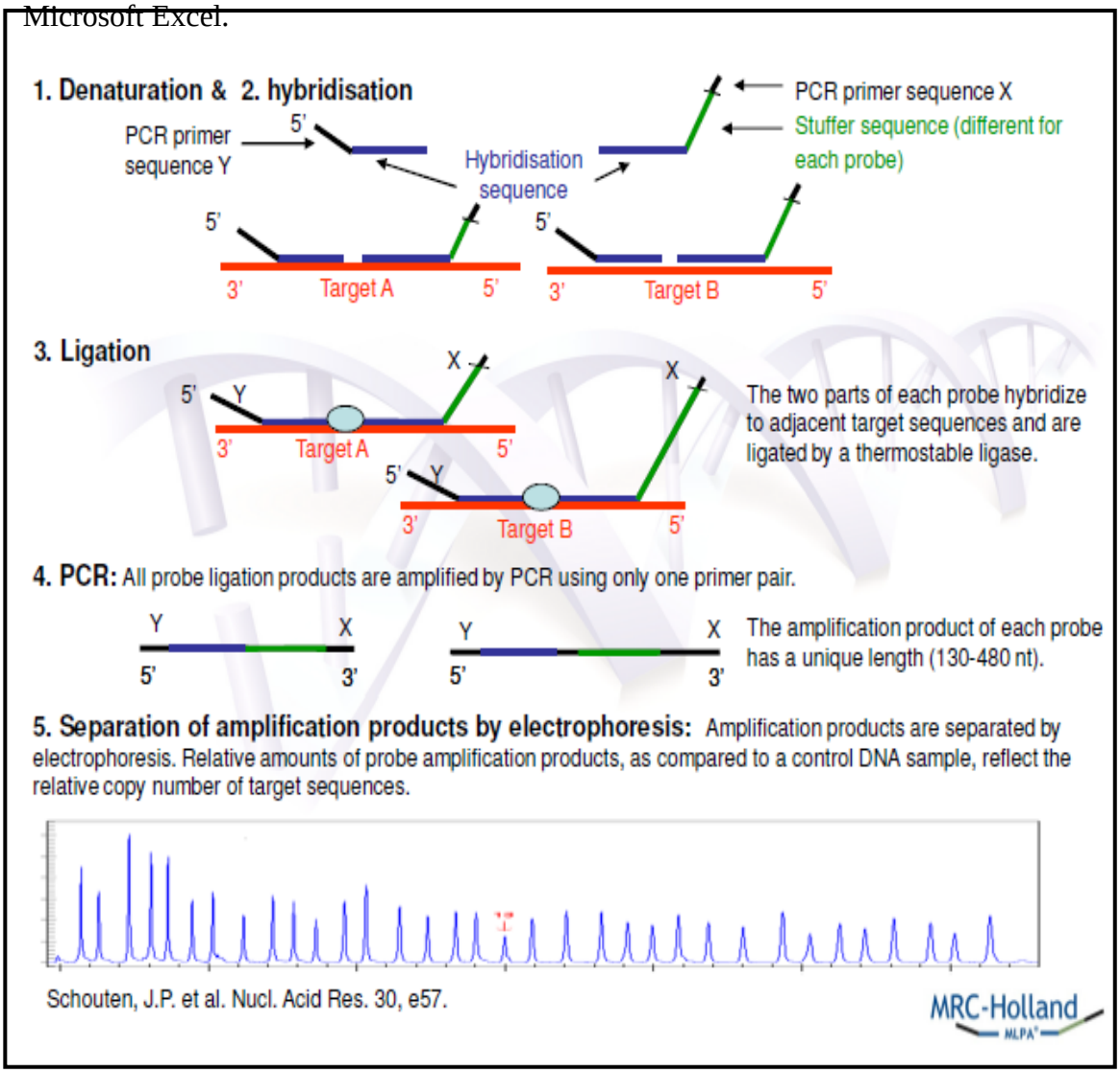

Figure 1.12: Outline of MLPA technique and steps involved. *Genomic DNA is hybridized with specialized probe targeted to the DMD gene exons, following which PCR is done to amplify the probes and separated by capillary/gel electrophoresis. (Adapted from* www.mlpa.com*)*

Input data for the spreadsheets may be either peak heights or peak areas. However, peak heights are recommended since comparisons between the peak heights and peak areas as measures of peak intensity has shown that the variance of peak area measurements are consistently higher than those for peak heights. This may be due to peak smoothing or the arbitrary cut-off of peaks that occurs in

fragment analysis programs. Peak heights appear to be a simpler and therefore more consistent measure than peak area.

The three differences representing the three competing hypotheses are then converted into probabilities of deviation using the t-statistic. The precise probability for each amplimer is thus determined by two factors (i) the underlying variability in the batch of five normal controls for that particular ligation product and (ii) the size of the difference between the test sample for that ligation product and the control samples. Finally the relative likelihood of each of the three competing hypotheses is calculated for each ligation product as an odds ratio to indicate which hypothesis is more likely. For instance if the observed deviation from the normal hypothesis of the test sample is predicted to occur in 10% cases and the deviation from the deleted hypothesis is predicted to occur in 0.1% of cases then the relative odds of the normal to deleted hypotheses is 100:1 in favour of the normal hypothesis.

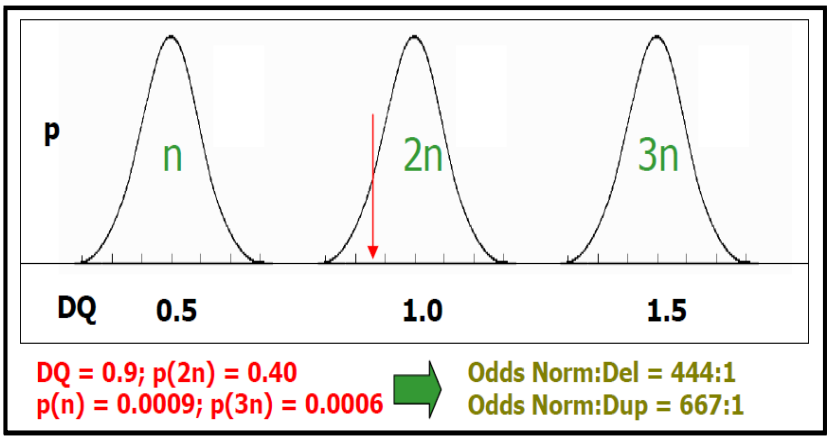

Figure 1.13 Method used for calculating relative likelihoods. *Three curves represent the relative probability distribution of dosage quotient for a given ligation product for each of the hypotheses, n – deleted, 2n – normal, 3n – duplicated. The probability distribution is calculated in practice by the t-statistic. In the illustrated example the measured DQ of 0.9 equates to a probability of this being a normal result of 0.40, a probability of being a deleted result of 0.0009 and a probability of being a duplicated result of 0.0006. Dividing the Normal probability by the Deleted probability yields an odds ratio of 444:1 and dividing the Normal by the Duplicated probability yields an odds ratio of 667:1 (Adapted from* www.mlpa.com*).*

1.5.4.4 Interpretation of data

The actual settings in the NGRL worksheets that have been set for the conditional formats may vary depending on the spreadsheet but typical ranges are as follows:-

Normal DQ 0.85 – 1.15

Deleted DQ 0.35 – 0.65

Duplicated DQ 1.35 – 1.65

Equivocal DQ 0.65-0.85 & 1.15-1.35

1.5.4.5 Advantages of MLPA

Using MLPA for copy number detection offers many advantages over other techniques. First of all, methods which were primarily developed for detecting point mutations, such as sequencing and DHPLC, generally fail to detect copy numbers changes. Southern blot analysis, on the other hand, will reveal many aberrations but will not always detect small deletions and is not ideal as a routine technique. Although well-characterized deletions and amplifications can be detected by PCR, the exact breakpoint site of most deletions is unknown. Furthermore, when comparing MLPA to FISH, MLPA not only has the advantage of being a multiplex technique, but also one in which very small (50-70 nt) sequences are targeted, enabling MLPA to identify the frequent, single gene aberrations which are too small to be detected by FISH. Moreover, MLPA can be used on purified DNA. Finally, as compared to array CGH, MLPA is a low cost and technically uncomplicated method. Although MLPA is not suitable for genome-wide research screening, it is a good alternative to array-based techniques for many routine applications.

1.5.5 Detection of small rearrangements and point mutations in DMD gene

1.5.5.1 Testing for other mutation types

The common methods which are used for detection of exon deletions and duplications contribute to approximately 75% all DMD gene mutations. The other 25% constitute point mutations and small rearrangements like small insertions and deletions. Due to the large size of the DMD gene and the large number of exons, screening them for point mutations is very cumbersome and is done only after confirming the diagnosis with muscle immunohistochemistry.

If no deletion or duplication has been found, the clinical diagnosis cannot be confirmed nor excluded. If the clinical features, family history, and/or results of muscle biopsy suggest a dystrophinopathy, further tests should be offered to search for a pathogenic mutation.

Numerous methods have been applied to scan the DMD gene for nucleotide changes, including: Single Strand Conformational Polymorphisms (SSCP) (Mendell, Buzin et al. 2001), denaturing High Performance Liquid Chromatography (dHPLC) (Bennett et al., 2001), Fluorescnce Mulitplex-Conformation Sensitive Capillary Electrophoresis (FM-CSCE) (Ashton et al., 2008), Protein Truncation Test (PTT) (Roest et al., 1993), High Resolution Melting (HRM) (Almomani et al., 2009). These pre-screens aim to offer a lower cost alternative to sequencing all the 79 DMD exons, however, since the cost of sequencing has reduced dramatically over recent years it may be more appropriate to sequence the full gene now (Flanigan et al., 2003). Sequencing can be performed on RT-PCR derived cDNA from muscle RNA, or on genomic DNA. If a pathogenic variant is identified in cDNA, it should be followed by sequencing of the appropriate region(s) in genomic DNA to confirm the result, and characterise the variant at the nucleotide level to allow future DNA-based testing in relatives. Note that for some variants identified in cDNA it may not be straightforward or even possible to characterise the sequence change at the genomic level, e.g. deep intronic mutations.

Complex rearrangements, or variants located deep into the large introns of the gene will not be detected using standard methods of DNA-based mutation screening, and RNA-based methods offer a reasonable likelihood of being able to detect them. These mutations appear to be of low frequency (approximately 2% of DMD mutations).

RNA-based point mutation screening is considered as the most powerful technique to screen for deleterious, non-exon-deletion / duplication changes in the DMD-gene. By amplifying the entire DMD coding region from an RNA template, all deleterious truncating mutations will be resolved, including those affecting RNA-splicing. The Protein Truncation Test (PTT), an RNA-based screening method, has been proven to be very effective. However, PTT is not the simplest method to implement and an RNA sample, preferably from a muscle biopsy, is not always available. PTT on lymphocyte RNA is possible, but more

difficult to perform (Tuffery-Giraud, 2004). An alternative is to use RNA obtained after MyoD-induced in vitro muscle differentiation. The cDNA fragments obtained after RT-PCR can also be used for sequencing to determine the mutations present (Hamed 2006)

- High-resolution Melting Curve Analysis (hrMCA) for DNA-based point mutation screening: hrMCA is simple, cheap and very sensitive (>98%) and applied as a pre-sequencing tool, resolving those fragments that contain variants, it is very cost-effective.

- Denaturing Gradient Gel-Electrophoresis (DGGE) (Hofstra 2004), having a close to 100% sensitivity, is once implemented a very effective technique. However, DGGE is laborious, and it uses several PCR and electrophoresis conditions and is difficult to automate.

- Direct sequencing or SCAIP (Single Condition Amplification/Internal Primer) is a straightforward and effective method but it is rather costly (>79 separate exon fragments to analyse) (Flanigan, 2003).

- **Single-Strand Conformation Analysis (SSCA)** / DOVAM (detection of virtually all mutations, (Mendell, 2001 / Buzin, 2005) is simple, cheap and effective but laborious (e.g. demanding electrophoresis of all (>79) exon fragments each using several electrophoretic conditions).

- **Denaturing High Performance liquid Chromatography (DHPLC):** Characteristics for DHPLC (Bennett, 2001) are similar to those for SSCA. However, DHPLC is easier to automate but requires specific specialized equipment.

Compared to DGGE, SSCA and DHPLC are considered as good but more laborious alternatives. Direct sequencing is very powerful, but also more costly. With few exceptions, mostly only the protein coding regions of the DMD gene are analysed. Studies analysing other regions (promoters, 5'UTR and 3'UTR) have so far not revealed many changes (e.g. Tubiello, 1995, Flanigan, 2003).

Next generation sequencing approaches also offer improved likelihood of being able to detect the full spectrum of DMD mutations, since the whole gene can be sequenced, including the introns. However, additional RNA-based studies may be required to evaluate the pathogenic effects of some of the many intronic variants likely to be detected. In rare instances, the occurrence of more than one DMD mutation in a family has been reported (Mostacciuolo et al., 1994). So, in extended pedigrees with more than one affected male, it may be wise to test all patients.

1.5.6 Carrier diagnosis and prevention in DMD

Due to the lack of efficient rehabilitation and treatment of progressive muscular dystrophy, counseling and prenatal diagnosis are options that medical geneticists can offer today, and their decision depends on information of the carrier status. Indeed, in the families with a single affected male, a crucial point is to determine whether the disease is derived from a ‘‘de novo’’ mutation or from a genetic defect inherited from a carrier mother. Figure 1.14 shows a common pedigree of a X-linked disorder, for example DMD, hemophilia etc. As shown in the pedigree, females are mostly carriers and they generally don’t show clinical symptoms. Sixty percent of the cases are inherited and hence carrier analysis is necessary to help preventing the disorder.

1.5.6.2 Cadidates for carrier analysis

- Practically, if the mother of an affected boy (*proband*) has another affected relative, she is an obligate carrier.
- If there is an affected brother or one affected son-possible carrier.
- But in most families there is only one affected patient.
- Therefore, female relatives of affected males are candidates for carrier assessment.

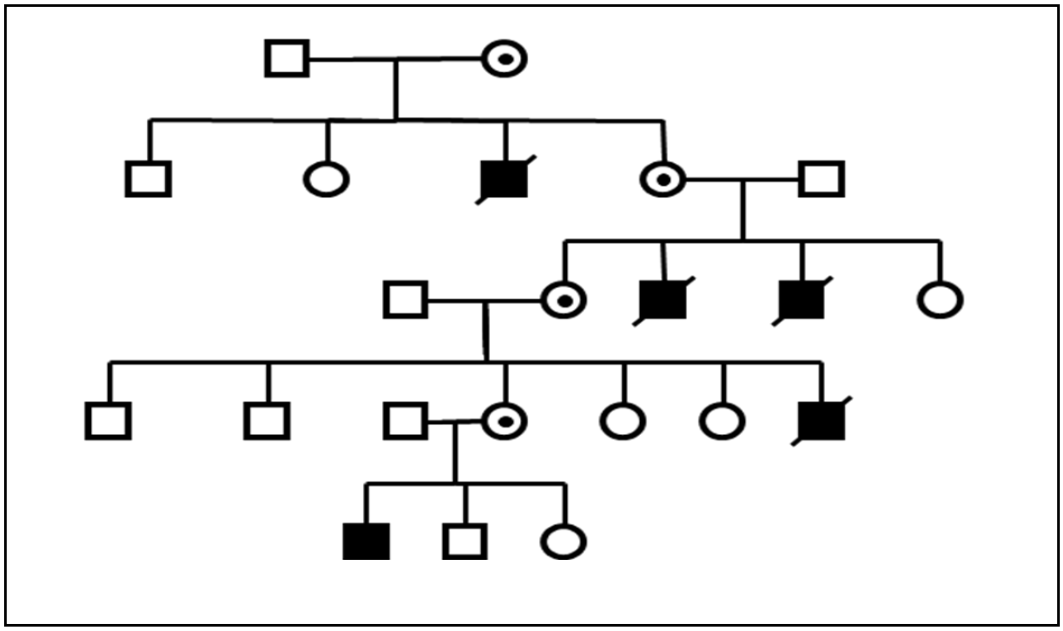

Figure 1.14: A common pedigree of X-linked recessive disorder. *Only males are affected and females are carriers. The X chromosome carrying the disease-causing mutation can be tracked through the family. Note: Shaded squares = affected males: dots in circles = carrier females. The disease is transmitted from mother to the son and daughters of affected males have 25% chance of being carriers. (Adapted* www.geneticseducation.nhs.uk*)*

1.5.6.2 Carrier diagnosis for a known familial mutation

Carrier diagnosis of female relatives at risk of being heterozygotes for a known mutation can be conducted by most of the methods that have been used to identify the mutation in the index case of the family. Whenever possible, a sample from the index case (or a known carrier) should be run as a control sample or (at least) a written report on the mutation of the index patient should be available in order to avoid data transmission problems. Clearly, the method chosen to test female relatives must be capable of detecting the mutation in the heterozygous state, i.e. masked by the presence of the corresponding normal allele. Deletions therefore require either a quantitative method (such as MLPA or array CGH), or a qualitative method, such as pulsed field or field inversion gel electrophoresis (den Dunnen et al., 1989). If the mutation of the index case cannot be found in genomic DNA from his mother, the frequency of germinal mosaics still confers a significant recurrence risk for future children.

1.5.6.3 Carrier detection for an unknown proband mutation

If an affected male is not available to be tested, female relatives at risk of being carriers should be offered mutation testing, using any of the methods discussed above which are able to detect heterozygous mutations. Testing should start with the woman who has the highest prior carrier risk, usually the mother of an index case. An initial screen for deletions and duplications is a sensible first test, as in testing affected males. All the precautions associated with testing a male for deletions and duplications are equally applicable to testing females, such as the need to verify copy number changes involving only a single exon. If no mutation is found and before proceeding to more complex tests, it is recommended to review the available clinical information. With a strong clinical diagnosis and/or X-linkage, sequencing or other point mutation screening methods would be the ideal next step, but these may not be available in all laboratories. If these are not available in a laboratory, it is recommended that samples are forwarded to another laboratory which is able to conduct these tests. Haplotype analysis is an alternate method if key family members are available.

A haplotype is a combination of alleles (DNA sequences) at adjacent loci on the chromosome that are transmitted together. A haplotype may be one locus, several loci, or an entire chromosome depending on the number of recombination events that have occurred between a given set of loci. In a second meaning, haplotype is a set of single-nucleotide polymorphisms (SNPs) or a single collection of STR mutations on a single chromosome of a chromosome pair that are statistically associated. It is thought that these associations, and the identification of a few alleles of a haplotype block, can unambiguously identify all other polymorphic sites in its region. Such information is very valuable for investigating the genetics behind common diseases, and has been investigated in the human species by The International HapMap Project. (Den Dunnen JT et al., 1987)

1.5.6.4 Diagnosis of manifesting carriers

A small proportion of female carriers of DMD mutations exhibit clinical symptoms, some of which can be as severe as found in male patients. Norman

and Harper (Norman and Harper, 1989) concluded that 2.5% of heterozygotes have muscle symptoms but the incidence of cardiomyopathy in female carriers may be higher (Politano et al., 1996; Hoogerwaard et al., 1999). Up to two thirds of carriers have persistently elevated serum Creatine Kinase (CK) levels (Emery, 1989). Confirmation of diagnosis for these manifesting females follows the same principles as for affected males, and mutation screening follows the same principles as testing for carrier status of an unknown mutation. Clinical manifestation in females is believed to result from non-random X-inactivation. Chromosomal aberrations (45, X0; X-autosome translocations) should also be considered in fully manifesting female child.

1.5.7 Prenatal diagnosis

Prenatal diagnosis for DMD/BMD should only be carried out for male pregnancies. At present, it is not possible to predict whether a female heterozygote for a DMD mutation will manifest any signs of the disorder or not, and therefore it would be inappropriate to offer prenatal diagnosis for a female fetus. The familial mutation will preferably be known in advance of testing a male pregnancy, and should be confirmed before the prenatal test, or can be confirmed in parallel with testing the fetus by using the proband's DNA as a control. The same technique can be used to test for the mutation as was used to identify or verify the mutation in the proband. A check for maternal cell contamination (MCC) of the fetal DNA must be carried out, since its presence at a significant level may affect the interpretation of the fetal result. This would be a particular problem for example when testing a male fetus for a deletion using a non-quantitative PCR-based assay, since the presence of any maternal DNA will mask the presence of a deletion in an affected male fetus, resulting in a false negative diagnosis. A check for MCC can be done with markers from the dystrophin gene region or with any polymorphic marker set used routinely in the laboratory, e.g. for identity testing. Further details and recommendations for checking for maternal cell contamination in prenatal samples can be found in the CMGS Best Practice Guidelines (Schrijver et al., 2007).

1.5.8 Preimplantation genetic diagnosis (PGD)

PGD is a specialist test carried out in a limited number of centres. For PGD, the same analytical considerations apply as for prenatal testing, but the special requirements of a PGD setting need to be considered, as documented in the Best Practice Guidelines from the European Society of Human Reproduction and Embryology PGD Consortium (Thornhill et al., 2005). These tests will not be discussed further in this document.

1.6 POTENTIAL THERAPIES FOR DMD

Currently there is no cure for any of the MDs, with only palliative and symptomatic treatment available for patients. The identification of the causative gene for Duchenne MD and Becker MD nearly 20 years ago was accompanied by a surge of optimism that a therapy or cure would soon follow. Unfortunately, translating the knowledge of the causative gene and its mutation into genetic therapy for MDs has proven to be a difficult task. There have been studies of possible treatments that function at the molecular level (chimeroplasts, short fragment homologous recombination and antisense oligonucleotide exon skipping), the cellular level (delivery of myoblasts, stem cells, viruses and plasmids) and the pharmacological level (myostatin blockade, upregulation of an alternative gene, proteasome degradation prevention and stop-codon read-through using aminoglycoside antibiotics and ataluren/PTC 124) (Nowak and Davies, 2004; Chakkalakal et al., 2005).

Two promising therapeutic approaches are the delivery of a normal replacement for the defective gene using adeno-associated virus (AAV) vectors (Blankinship et al., 2006) and the use of antisense oligonucleotides to induce exon skipping of the mutation containing exon or to extend a deletion. This produces an in-frame transcript that is translated into a functional, albeit smaller, protein (Wilton and Fletcher, 2006). One of the main hurdles facing therapeutic approaches is effective delivery of the therapeutic product to skeletal muscle, which makes up 30–40% of the total human body mass. Fortunately, recent success with systemic delivery has shown significant promise. For example, weekly intravenous injections of a morpholino oligonucleotide into the dystrophin-null mouse model, *mdx*, produced body-wide expression of dystrophin (Alter et al., 2006). Also, a single injection of an AAV8 virus into hamsters produced sustained expression of

the previously missing δ-sarcoglycan protein in both skeletal muscle and heart for longer than 12 months (Zhu et al., 2005). A novel combination of both viral delivery and antisense technology was accomplished in *mdx* mice, in which a single tail-vein injection of an AAV vector expressing an antisense U1 small nuclear RNA led to successful body-wide expression of a modified dystrophin protein for at least 12 weeks (Denti et al., 2006).

1.6.1 Therapies under investigation

Aminoglycosides: Up to 15% of individuals with DMD exhibit the gene mutation known as a premature stop codon. Suppression of stop codons has been demonstrated with aminoglycoside treatment of cultured cells, the treatment creates misreading of RNA and thereby allows alternative amino acids to be inserted at the site of the mutated stop codon. In the *mdx* mouse, *in vivo* gentamicin therapy resulted in dystrophin expression at 10%-20% of that detected in normal muscle (Barton-Davis et al., 1999), a level that provided some degree of functional protection against contraction induced damage.

Aminoglycoside therapy has been suggested as an alternative to gene therapy but could be aimed only at individuals with premature stop codons. In a preliminary study in which gentamicin (7.5 mg/kg/day) was administered to four individuals for two weeks, full-length dystrophin did not appear in the muscles of the treated individuals (Wagner et al., 2001). Some authors, unable to reproduce the results previously published for the mouse model of DMD, have called for more preclinical investigation of this potential therapy (Dunant et al., 2003). In an *in vitro* study (Kimura et al., 2005), dystrophin expression was detected in myotubes of males with DMD using gentamicin; however, the treatment was more effective in persons with the nonsense mutation TGA than TAA or TAG.

PTC124 is a new, orally administered non-antibiotic drug that appears to promote ribosomal read-through of nonsense (stop) mutations. (Figure 1.15) Preclinical efficacy studies in *mdx* mice have yielded encouraging results (Welch et al., 2007). A Phase I multiple-dose safety trial is ongoing (Hirawat et al., 2007).

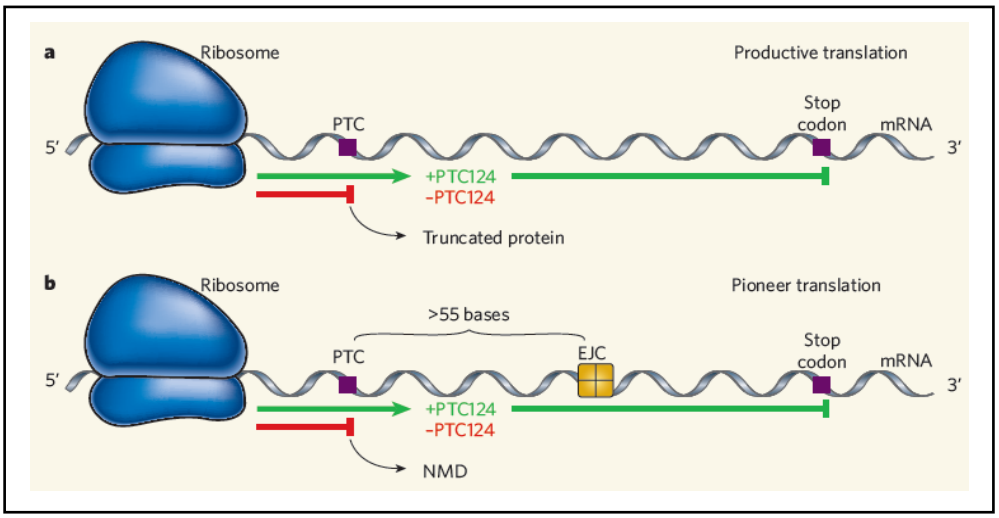

Figure 1.15: Action of PTC124 on termination codon. *PTC124 could directly suppress termination of 'productive' protein translation at a premature termination codon (PTC), leading to increased levels of functional full-length proteins. (Adapted from Anton Schmitz and Michael Famulok, 2007)*

Morpholino antisense oligonucleotides mediate exon skipping (Aartsma-Rus et al., 2006) and have improved the *mdx* mouse model of DMD (Wilton and Fletcher, 2005; Alter et al., 2006). (Figures 1.16 and 1.17)

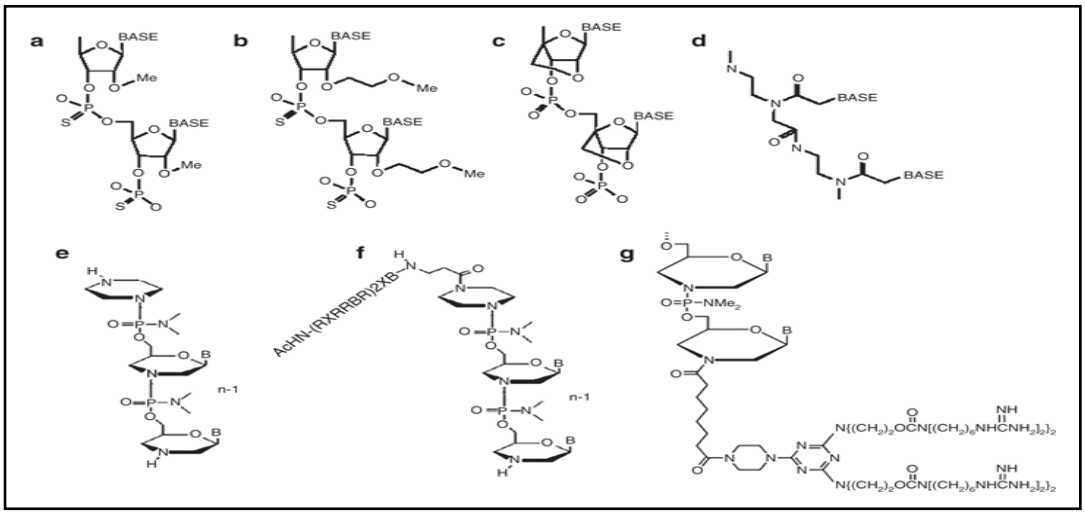

Figure 1.16 Chemistries of antisense oligomers. *(a) 2'-O-Methylphosphorothioate (2'OMePS AON); (b) 2'-O-methoxyethyl phosphorothioate; (c) locked nucleic acid (LNA); (d) peptide nucleic acid (PNA); (e) phosphorodiamidate morpholino oligomers (PMO); (f) AcHN-(RXRRBR)2XB*

peptide-tagged PMO (R, arginine, X, 6-aminohexanoic acid and B, ®- alanine) (PPMO); G, octa-guanidine PMO (Adapted from Lu et al., 2011).

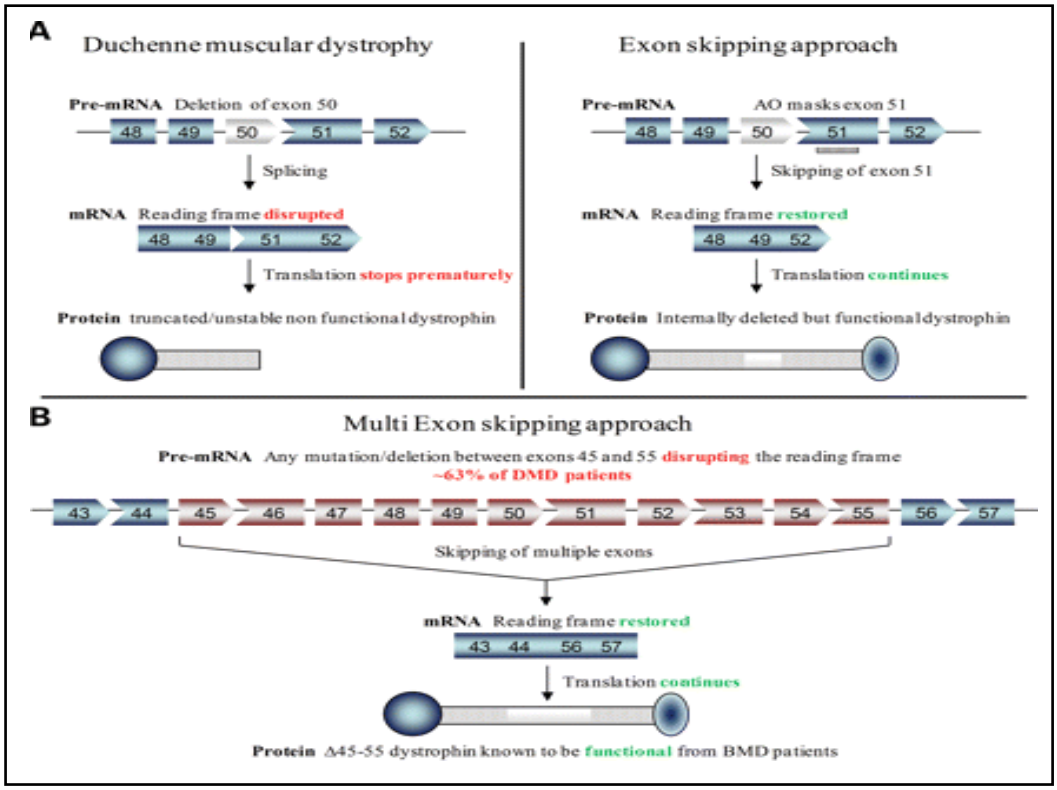

Figure 1.17: Antisense-mediated exon skipping rationale for DMD. *(A) Patients with DMD have mutations which disrupt the open reading frame of the dystrophin pre-mRNA. In this example, exon 50 is deleted, creating an out-of-frame mRNA and leading to the synthesis of a truncated non-functional or unstable dystrophin (left panel). An antisense oligonucleotide directed against exon 51 can induce effective skipping of exon 51 and restore the open reading frame, therefore generating an internally deleted but partly functional dystrophin (right panel). (**B**) Multi exon-skipping rationale for DMD. The optimal skipping of exons 45–55 leading to the del45–55 artificial dystrophin could transform the DMD phenotype into the asymptomatic or mild BMD phenotype. This multiple exon skipping could theoretically rescue up to 63% of DMD patients with a deletion. (Adapted from Aurelie Goyenvalle et al., 2011)*

Gene therapy: Experimental gene therapies are currently under investigation (Gregorevic and Chamberlain, 2003; Tidball and Spencer, 2003; van Deutekom and van Ommen, 2003; Nowak and Davies, 2004).
Gregorevic et al reported systemic administration of rAAV6 vectors resulting in successful delivery of *DMD* to affected muscles of dystrophin-deficient *mdx* mice (a mouse model for DMD).(Gregorevic et al., 2004)

Stem cell therapy is under investigation but remains experimental (Gussoni et al., 1997; Gussoni et al., 1999; Gussoni et al., 2002; Skuk et al., 2004).

1.6.2 Miscellaneous therapies

Creatine monohydrate has been studied as potential treatment in muscular dystrophies and neuromuscular disorders (Tarnopolsky and Martin, 1999; Walter et al., 2000; Louis et al., 2003). In a randomized, controlled, cross-over trial, 30 boys with DMD were given creatine (~0.1 g/kg/day) for four months and placebo for four months (Tarnopolsky et al., 2004). Treatment with creatine resulted in improved grip strength of the dominant hand and increased fat-free mass when compared to placebo; however, no functional improvement was noted. Given the limited data and modest benefit, treatment with creatine monohydrate cannot be recommended for treatment of DMD.

Cyclosporin was reported to improve clinical function in children with DMD who received the medication for eight weeks. Nevertheless, because of the rare reports of cyclosporin-induced myopathy in individuals receiving the medication for other reasons, the use of cyclosporin in treating DMD remains controversial (Sharma et al., 1993; Shin et al., 2012).

Histone deacetylase inhibitors have improved the *mdx* mouse by inducing the expression of the myostatin inhibitor follistatin (Minetti et al., 2006).

The strategies mentioned above are either not yet ready to be administered to the patient community or not therapies but just management strategies that prolong or delay the progression. The table 1.4 gives the list of therapeutic drugs and strategies in study now, and the stage of clinical trial.

Table 1.4: Ready-to-use, indirect, and dystrophin-restitution molecular therapy in clinical trials for DMD

Therapy	Completion	Phase	Status	ClinicalTrials.gov identifier and supporting references
READY-TO-USE/INDIRECT THERAPIES				
Randomized study of daily vs high-dose weekly prednisone therapy in DMD	December 2007	2/3	Completed	NCT00110669
Clinical trial of coenzyme Q10 and lisinopril in muscular dystrophies	December 2013	2/3	Recruiting	NCT01126697 (Folkers and Simonsen, 1995; Vandenburgh et al., 2009)
Tadalafil in BMD	December 2012	4	Recruiting	NCT01070511 (Asai et al., 2007)
iGF-1 therapy and muscle function in DMD	November 2011	1/2	Recruiting	NCT01207908
Sunphenon epigallocatechin-gallate in DMD	September 2012	2/3	Recruiting	NCT01183767
Long-term safety, tolerability, and efficacy of idebenone in DMD (DeLPHi extension)	February 2011	2	Active, not yet recruiting	NCT00758225
Extension study of ACe-031 in subjects with DMD	December 2012	2	Recruiting by invitation	NCT01239758 (Cadena et al., 2010)
Study of ACe-031 in subjects with DMD	February 2012	2	Recruiting	NCT01099761 (Cadena et al., 2010)
Phase iii study of idebenone in DMD	December 2011	3	Recruiting	NCT01027884
MOLECULAR THERAPIES				
Dystrophin restitution: stop codon read-through				
A Phase iia extension study of PTC124 in DMD	March 2011	2a	Terminated	NCT00759876 (Hamed, 2006; Hirawat et al., 2007; Welch et al., 2007; Welch et al., 2008)
A Phase iia study of ataluren (PTC124) in nonambulatory patients with nonsense-mutation-mediated D/BMD	June 2011	–	Suspended	NCT01009294 (Hamed, 2006; Hirawat et al., 2007; Welch et al., 2007; Welch et al., 2008)
Phase IIb study of PTC124 in D/BMD	December 2009	2/3	Completed	NCT00592553 (Hamed, 2006; Hirawat et al., 2007; Welch et al., 2007; Welch et al., 2008)
Phase IIb extension study of ataluren (PTC124) in D/BMD	December 2011	2/3	Terminated	NCT00847379 (Hamed, 2006; Hirawat et al., 2007; Welch et al., 2007; Welch et al.,

				2008)
Study of ataluren for previously treated patients with nmDBMD in the United States	July 2012	3	Recruiting	NCT01247207
Six month study of gentamicin in DMD with stop codons	July 2009	1	Completed	NCT00451074 (Malik et al., 2010)
DYSTROPHIN RESTITUTION: EXON SKIPPING				
A clinical study to assess the efficacy and safety of GSK2402968 in subjects with DMD	March 2011	3	Not yet recruiting	NCT01254019
Phase ii double-blind exploratory study of GSK2402968 in ambulant subjects with DMD	November 2011	2	Recruiting	NCT01153932
A double-blind, escalating dose, randomized, placebocontrolled study to assess the pharmacokinetics, safety, and tolerability of single subcutaneous injections of GSK2402968 in nonambulant subjects with DMD	January 2011	1	Recruiting	NCT01128855
Phase i/ii study of PRO044 in DMD	September 2011	1/2	Recruiting	NCT01037309 (van Deutekom et al., 2007)
Restoring dystrophin expression in DMD: a phase i/ii clinical trial using Avi-4658	March 2009	1/2	Completed	NCT00159250 (Kinali et al., 2009)
Dose-ranging study of Avi-4658 to induce dystrophin expression in selected DMD patients	June 2010	1/2	Ongoing	NCT00844597 (Kinali et al., 2009)

Although so much of work is going on in this field, not much has been achieved in terms of therapy or prevention that would reach the patient and its family directly. Most of the diagnostic and therapeutic strategies are either incomplete or too expensive to reach the patients and to alleviate the pain that the families undergo. The DMD gene was one of the early genes to be identified to cause a disease in the humans, but since then much has not been achieved owing to the size of the gene, the several isoforms that alter the clinical presentation, lack of complete knowledge on the functions of the dystrophin protein and pathophysiology of the disorder, overlap with several other similar muscular disorders in clinical symptoms and overlapping functions of the muscular

dystrophy proteins. However, with no therapies immediately available in the market and the dependency of the upcoming therapies on the mutation on the DMD gene, the aim of our study is to accurately identify the DMD gene mutations in patients affected by Duchenne and Becker muscular dystrophy. There are no standard methods for DMD gene mutation detection. In India most labs use mPCR as their primary mutation detection method, but the numbers of exons tested in the different labs vary from 16 to 25 exons. Moreover, diagnosis is not given for samples which does not show a deletion in the exons tested. This leads to only around 65% of the cases having their DMD gene mutations detected. Mutations detected by these methods also are just able to confirm the clinical diagnosis, but the complete diagnosis is not obtained since most often the borders of the deletion are not known. Exon borders of deletion are necessary

1) to understand the framedness of the deletion and
2) to get accurate diagnosis which is necessary to decide if the patient qualifies to be treated with a particular gene therapy strategy.

With no therapies in the corner, prevention becomes essential. Prevention can only be based on carrier diagnosis, genetic counseling and prenatal diagnosis. Most of the developed countries have ensured there are no familial cases in their countries. The common method in practice in India is quantitative mPCR where interpretation is subjective and not accurate. Accurate carrier diagnosis leads to accurate genetic counseling and effective prevention of familial disease.

The same will hold good for other neuromuscular disorders. Several genes and proteins have been identified known to cause several types of NMDs and their diagnosis is difficult mainly due to the number of genes and overlapping clinical symptoms. Muscle biopsy immunohistochemistry for identifying the deficient protein followed by direct gene sequencing is the method which is followed worldwide. However, this is both labor intensive and invasive causing severe discomfort for the patients and their families. Several groups are developing different strategies to overcome this hurdle and diagnose NMDs and the causative gene mutations.

Our study will focus on genetic diagnosis of DMD and other NMDs without the involvement of muscle biopsy, carrier diagnosis in DMD aimed at prevention and investigating the molecular pathophysiology.

CHAPTER 2: OBJECTIVES

Objective 1: Identification/Genetic diagnosis of Duchenne/Becker muscular dystrophy using a less invasive strategy of mPCR followed by MLPA.

Objective 1.1. To study the use of multiplex PCR for 30 DMD gene exons in identifying DMD gene deletions.

- To standardize mPCR for 30 exons in the hot spot regions of the DMD gene.
- To detect DMD gene deletions in patients with Duchenne/Becker muscular dystrophy.
- To identify the spectrum of DMD gene deletions in our study group.
- To select samples for downstream analysis and confirmation.

Objective 1.2. To perform MLPA for samples without a diagnosis in mPCR and those which unclear deletion borders to identify copy number changes throughout all 79 exons of the DMD gene.

- To standardize MLPA for DMD gene.
- To perform MLPA on D/BMD samples to look for exon deletions and duplications.
- To determine the mutation spectrum after mPCR and MLPA on D.BMD samples.
- To understand the usefulness of the protocol in DMD gene mutation detection.

Objective 2: To perform carrier diagnosis in D/BMD families aimed at prevention of the disorder through genetic counseling and prenatal diagnosis.

- To validate MLPA using other methods used for carrier diagnosis to understand its usefulness in carrier diagnosis.
- To perform MLPA on probable carriers in families with known proband DMD gene mutation.

- To study the rate of carriers in DMD and the usefulness of MLPA in diagnosing carriers.

Objective 3: Case study of a female with DMD to understand the diagnostic methodology and mechanism of disease.

- To clinically characterize the case with various tests to understand the clinical disease and to look for clinical symptoms of DMD.
- DMD gene mutation detection using mPCR and MLPA, to confirm clinical diagnosis.
- Further tests to understand the mechanism of the disease and interpretation.

Objective 4: To study genome wide copy number variations in DMD and comparing with controls to identify associated CNVs.

- To perform whole genome genotyping analysis to genotype SNPs and CNVs covering the whole genome.
- Further processing of data and extraction of CNVs for analysis.
- Analysis comparing CNVs of patients with controls to identify associated CNVs.

Objective 5: To study the use of genome wide SNP based homozygosity mapping in diagnosing neuromuscular disorders.

- To perform whole genome genotyping of SNPs in selected samples from families affected by hereditary muscular dystrophies.
- Perform homozygosity mapping to identify the risk locus.
- To identify candidate genes in the risk locus to arrive at a probable diagnosis.

CHAPTER 3 – METHODOLOGY

3.1 SAMPLES

The clinician sends the blood sample of the patient with duly filled in genetic request form (appendix 1) and the informed consent form (appendix 2) to our centre. The demographic data, clinical details, pedigree details and the requested tests are recorded by the clinician in the genetic request form. If the patient arrives at our centre with incomplete forms, we take in all necessary data like pedigree and family history details. Then informed consent is obtained from the patient or their relatives after explaining all the details, including the outcome of the test. After this 3 ml of Blood sample was collected in EDTA for further testing.

3.2 DNA EXTRACTION FROM BLOOD

DNA extraction from blood was done by salting out method described by (Miller et al., 1988), quantified and stored at –20°C until tested.

3.2.1 Reagents Required

1. 0.5 M EDTA stock pH 8.0

EDTA – 186.12g

Dissolved in distilled water and then volume was made upto 1L with distilled water.

2. ELB – Erythrocyte lysis buffer, pH 7.4

NH_4Cl – 8.29g

$KHCO_3$ – 1.0g

0.5 M EDTA stock – 400µl

Volume made upto 1L with distilled water.

3. 20% SDS (Sodium dodecyl sulphate)

200 g of SDS in 900ml distilled water.

Adjust pH to 7.2 with con.HCL

Volume made upto 1L with distilled water.

4. Proteinase K

10 mg/ml in TE buffer

5. 5M NaCl

Molecular weight – 58.5

58.5 g/L = 1M, 5x58.5= 292.5g/L=5M.

29.25g of NaCl is dissolved in distilled water and volume was made up to 100ml.

6. 70% ethanol

70 ml absolute alcohol + 30ml distilled water.

7. 1M Tris-EDTA (TE) Buffer pH 8.0

1M Tris – 5 ml

0.5M EDTA – 1 ml

Volume made upto 500 ml with distilled water. Adjust pH to 8.0.

Commercially available TE buffer (Invitrogen BioServices, USA) with reduced EDTA concentration was used for dissolving the DNA and quantitation.

Cell Lysis: For lysis of red blood cells, to 3 ml of blood 12 ml of ELB was added and stored on ice for 30 minutes followed by centrifugation at 3000 rpm for 10 minutes. The supernatant was carefully decanted without disturbing the pellet and it was mixed thoroughly and re-suspended with minimum volume of ELB and then mixed thoroughly with 10 ml of ELB for further lysis to occur. After centrifugation at 3000 rpm for 10 minutes the supernatant was discarded and the volume was made up to 3ml with ELB. To this 150 µl of 20% SDS (final concentration – 1%) and 100 µg proteinase K and was incubated at 37º C for 4 hours or overnight (or for rapid protocol at 56º C for 2 hours). The rapid protocol was followed for cell lysis.

3.2.2 Salting out: To 3.0 ml of sample, 0.3 ml of 5M NaCl and 3.0 ml of isopropyl alcohol was added and the tube was gently mixed till the solution appeared clear. Further the DNA was precipitated by slowly swirling the tube which was transferred carefully into a 1.5 ml vial containing 500 µl of freshly prepared 70% alcohol. This was mixed well, and allowed it to stand for 15 minutes and then centrifuged at 13,000g for 10 minutes. This procedure was repeated 3 times to ensure all salts are removed. The DNA was allowed to dry not completely but with little moisture and then dissolved with 300µl of TE buffer (pH 8.0). This was incubated at 65º C for 15 minutes to dissolve and stabilize the DNA. After complete dissolution DNA concentration was measured using Biophotometer (Eppendorf Ltd) and stored at -20º C in appropriate box.

3.3 DETERMINATION OF DNA CONCENTRATION:

3.3.1 Reagents

1M Tris-EDTA (TE) Buffer pH 8.0

1M Tris – 5 ml

0.5M EDTA – 1 ml

Made up the volume to 500 ml with distilled water. Adjust pH to 8.0.

3.3.2 Measurement of DNA concentration: DNA samples were diluted to 1/25 and 1/50 dilution for measurement in the Biophotometer. The average of the concentrations obtained with these two dilutions is taken as the DNA concentration of the test sample.

An OD of 1.0 at 260 nm wavelength is equivalent to a DNA concentration of 50 ng/µl (50µg/ml). Based on this the concentrations of the test samples was calculated and recorded. 260/280 ratio was also recorded to check the purity of DNA. All DNA samples with a 260/280 ratio of 1.8 – 2.0 were considered good for future use. 260/230 ratios were also recorded to understand the contamination with organic salts. All my samples showed 260/230 ratios greater than 1.8.

3.4 MULTIPLEX PCR FOR DMD GENE DELETION DETECTION:

Multiplex PCR analysis was performed for 30 exons at the central and 5'end hot spot regions. (Chamberlain et al., 1988; Beggs et al., 1990; Abs et al., 1991) The primers for the 30 exons were obtained from *www.dmd.nl* website. Multiplex PCR was done in 6 sets each consisting of 4-6 exons. The exons tested were 1, 3, 4, 6, 8, 12, 13, 16, 17, 19, 20, 21, 22, 32, 34, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 55 and 60.

3.4.1 Reagents

1. 10X PCR buffer (Applied Biosystems, USA)
2. 5mM $MgCl_2$ (Applied Biosystems, USA)
3. dNTP mixture – 2.5 mM (Applied Biosystems, USA)
4. Taq Polymerase (Applied Biosystems, USA) – 5U/µL
5. Sterile distilled water (Invitrogen BioServices, USA)
6. Tris EDTA Buffer (TE Buffer) – Invitrogen BioServices, USA
7. Primers 100 µM – Primer sequences as per www.dmd.nl
8. Agarose - SRL
9. TBE Buffer

10X TBE Stock

Tris base – 54g

Boric Acid– 27.2g

EDTA dipotassium salt – 3.72g

Dissolved in distilled water and made up the volume to 500mL.

Adjusted the pH to 8.0.

1X TBE

Stock (10X) was diluted 10 times in distilled water.

10. Ethidium Bromide Solution (Bangalore Genei)

Stock solution - 10 mg/mL

Working Concentration (0.5 μg/mL)

Stock (10mg/ml) was diluted 5 times with distilled water. Mixed 8-10 μL of the working solution with 25 mL of the agarose solution in TBE buffer to obtain the final working concentration (0.5 μg/mL).

11. Bromophenol Blue Solution

4g of sucrose/10 mL of sterile distilled water.

0.01g bromophenol blue/10mL 1X TBE.

Mixed the two solutions and stored at 4^{o}C.

3.4.2 Primers

Primer Sequences (as in www.dmd.nl - Leiden Muscular Dystrophy Pages accessed in July 2006 given in table 3.1)

Table 3.1: Primer sequences for multiplex PCR from www.dmd.nl

S. NO	Exon	Primer Sequence mPCR Primers
1	exon 1 F	5'-AAGATCTAGACAGTGGATACATAACAAATGCATG- 3'
2	exon 1 R	5'-TCTCCGAAGGTAATTGCCTCCCAGATCTGAGTCC- 3'
3	exon 3 F	5'- TCATCCATCATCTTCGGCAGATTAA- 3'
4	exon 3 R	5'- CAGGCGGTAGAGTATGCCAAATGAAAATCA- 3'
5	exon 4 F	5'- TTGTCGGTCTCTCTGCTGGTCAGTG- 3'
6	exon 4 R	5'- CAAAGCCCTCACTCAAACATGAAGC- 3'
7	exon 6 F	5'- CCACATGTAGGTCAAAAATGTAATGAA- 3'
8	exon 6 R	5'- GTCTCAGTAATCTTCTTACCTATGACTATGG- 3'
9	exon 8 F	5'- GTCCTTTACACACTTTACCTGTTGAG- 3'
10	exon 8 R	5'- GGCCTCATTCTCATGTTCTAATTAG- 3'
11	exon 12 F	5'- GATAGTGGGCTTTACTTACATCCTTC- 3'
12	exon 12 R	5'- GAAAGCACGCAACATAAGATACACCT- 3'
13	exon 13 F	5'- AATAGGAGTACCTGAGATGTAGCAGAAAT- 3'
14	exon 13 R	5'- CTGACCTTAAGTTGTTCTTCCAAAGCAG- 3'

15	exon 16 F	5’- TCTATGCAAATGAGCAAATACACGC- 3’
16	exon 16 R	5’- GGTATCACTAACCTGTGCTGTACTC- 3’
17	exon 17 F	5’- GACTTTCGATGTTGAGATTACTTTCCC- 3’
18	exon 17 R	5’- AAGCTTGAGATGCTCTCACCTTTTCC- 3’
19	exon 19 F	5’- TTCTACCACATCCCATTTTCTTCCA- 3’
20	exon 19 R	5’- GATGGCAAAAGTGTTGAGAAAAAGTC- 3’
21	exon 20 F	5'- TGGCTTTCAGATCATTTCTTTC- 3'
22	exon 20 R	5'- AAATACCTATTGATTATGCTCC- 3'
23	exon 21 F	5'- GCAAAATGTAATGTATGCAAAG- 3'
24	exon 21 R	5'- ATGTTAGTACCTTCTGGATTTC- 3'
25	exon 22 F	5'- AGGAAAACATGGCAAAGTGTG- 3'
26	exon 22 R	5'- TGCTCAATGGGCAAACTACC- 3'
27	exon 32 F	5’- GACCAGTTATTGTTTGAAAGGCAAA- 3’
28	exon 32 R	5’- TTGCCACCAGAAATACATACCACACAATG- 3’
29	exon 34 F	5’- GTAACAGAAAGAAAGCAACAGTTGGAGAA- 3’
30	exon 34 R	5’- CTTTCCCCAGGCAACTTCAGAATCCAAA- 3’
31	exon 41 F	5’- GTTAGCTAACTGCCCTGGGCCCTGTATTG- 3’
32	exon 41 R	5’- TAGAGTAGTAGTTGCAAACACATACGTGG- 3’
33	exon 42 F	5’- CACACTGTCCGTGAAGAAACGATGATGG- 3’
34	exon 42 R	5’- CTTCAGAGACTCCTCTTGCTTAAAGAGAT- 3’
35	exon 43 F	5’- GAACATGTCAAAGTCACTGGACTTCATGG- 3’
36	exon 43 R	5’- ATATATGTGTTACCTACCCTTGTCGGTCC- 3’
37	exon 44 F	5’- CTTGATCCATATGCTTTTACCTGCA- 3’
38	exon 44 R	5’- TCCATCACCCTTCAGAACCTGATCT- 3’
39	exon 45 F	5’- AAACATGGAACATCCTTGTGGGGAC- 3’
40	exon 45 R	5’- CATTCCTATTAGATCTGTCGCCCTAC- 3’
41	exon 46 F	5’- GCTAGAAGAACAAAAGAATATCTTGTC- 3’
42	exon 46 R	5’- CTTGACTTGCTCAAGCTTTTCTTTTAG- 3’
43	exon 47 F	5’- CGTTGTTGCATTTGTCTGTTTCAGTTAC- 3’
44	exon 47 R	5’- GTCTAACCTTTATCCACTGGAGATTTG- 3’
45	exon 48 F	5’- TTGAATACATTGGTTAAATCCCAACATG- 3’
46	exon 48 R	5’- CCTGAATAAAGTCTTCCTTACCACAC- 3’
47	exon 49 F	5’- GTGCCCTTATGTACCAGGCAGAAATTG- 3’
48	exon 49 R	5’- GCAATGACTCGTTAATAGCCTTAAGATC- 3’
49	exon 50 F	5’- CACCAAATGGATTAAGATGTTCATGAAT- 3’
50	exon 50 R	5’- TCTCTCTCACCCAGTCATCACTTCATAG- 3’
51	exon 51 F	5’- GAAATTGGCTCTTTAGCTTGTGTTTC- 3’
52	exon 51 R	5’- GGAGAGTAAAGTGATTGGTGGAAAATC- 3’
53	exon 52 F	5’- AATGCAGGATTTGGAACAGAGGCGTCC- 3’
54	exon 52 R	5’- TTCGATCCGTAATGATTGTTCTAGCCTC- 3’
55	exon 53 F	5’- TTGAAAGAATTCAGAATCAGTGGGATG - 3’
56	exon 53 R	5’- CTTGGTTTCTGTGATTTTCTTTTGGATTG- 3’
57	exon 55 F	5'- AATTTAGTTCCTCCATCTTTCTCT- 3'
58	exon 55 R	5'- AAATACATCAGGCTGTATAAAAGC- 3'
59	exon 60 F	5’- AGGAGAAATTGCGCCTCTGAAAGAGAACG- 3’
60	exon 60 R	5’- CTGCAGAAGCTTCCATCTGGTGTTCAGG- 3’

The primers were reconstituted with TE buffer to a concentration of 100 μM. Once reconstituted, the primers were mixed well in a vortex mixer and stored at –20^{o}C. Working Primer solution (10 μM concentration): 1 in 10 dilution of the stock primers were made using TE buffer and stored at –20^{o} C.

3.4.3 PCR Procedure

3.4.3.1. Exon sets for multiplex PCR: 6 sets (Table 3.2) of multiplex PCR reactions was standardized to accommodate the 30 exons studied. Sets 1, 6 to amplify 4 exons each; sets 2 and 3 to amplify 6 exons each; and sets 4 and 5 to amplify 5 exons each.

Table 3.2: List of DMD Exons in each set and their amplicon product base pair size

SET 1		**SET 2**		**SET 3**		**SET 4**		**SET 5**		**SET 6**	
Ex	**bp**	**Ex**	**bp**	**Ex**	**bp**	**Ex**	**bp**	**Ex**	**bp**	**Ex**	**bp**
52	113	**34**	171	**4**	196	**47**	181	**46**	148	**60**	139
42	252	**6**	202	**13**	238	**53**	212	**44**	268	**21**	275
32	405	**50**	271	**16**	290	**41**	274	**8**	360	**17**	372
12	502	**43**	357	**51**	388	**22**	388	**3**	410	**48**	506
		20	393	**55**	445	**1**	535	**19**	459		
		49	439	**45**	547						

3.4.3.2 PCR Reaction Mixture: Table 3.3 gives the reaction mixture for sets 1 and 6, table 3.4 gives the reaction mixture for sets 2 and 3 and table 3.5 gives the reaction mixture for sets 4 and 5.

Table 3.3: PCR reaction protocol for DMD exons - Set 1 and 6

Reagent	**Volume (μL) for 1X Reaction**
10X PCR Buffer	2.5
5mM $MgCl_2$ Solution	2.5
DNTP Mix (10mM)	3.0
Taq Polymerase	0.2
D. Water	7.8
Working Primers (1 μL each of the forward and reverse primers of each exon for a final amount of 10 pmoles/reaction)	8.0
DNA (250 ng/μL)	1.0
Total	**25.0**

Table 3.4: PCR reaction protocol for DMD exons - Set 2 and 3

Reagent	Volume (μL) for 1X Reaction
10X PCR Buffer	2.5
5mM $MgCl_2$ Solution	2.5
DNTP Mix (10mM)	3.0
Taq Polymerase	0.2
D. Water	3.8
Primers (1 μL each of the forward and reverse primers of each exon for a final amount of 10 pmoles/reaction)	12.0
DNA (250 ng/μL)	1.0
Total	**25.0**

Table 3.5: PCR reaction protocol for DMD exons - Set 4 and 5

Reagent	Volume (μL) for 1X Reaction
10X PCR Buffer	2.5
5mM $MgCl_2$ Solution	2.5
DNTP Mix (10mM)	3.0
Taq Polymerase	0.2
D. Water	5.8
Primers (1 μL each of the forward and reverse primers of each exon for a final amount of 10 pmoles / reaction)	8.0
DNA (250 ng/μL)	1.0
Total	**25.0**

3.4.3.3. PCR Reaction Conditions

Table 3.6: mPCR thermal cycler protocol

Steps	Temperature	Time	Cycles
Initial Denaturation	94º C	180 seconds	
Denaturation	93º C	60 seconds	28 Cycles
Annealing	60º C	45 seconds	
Elongation	65º C	60 seconds	
Final Elongation	65º C	600 seconds	
Hold	4º C	Hold	

3.4.4 Submarine-Gel Electrophoresis: The resultant PCR product was run an agarose gel incorporated with ethidium bromide in a submarine gel electrophoresis system for 40 minutes at 180V and was viewed and captured in the gel documentation system.

3.5 MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION (MLPA):

The SALSA MLPA KIT P034, P035 contains 50 reactions each of two different probemixes P034 and P035. This MLPA test has been developed to provide an easy to perform method for the detection of copy number changes of exons of the DMD gene on Xp21.2.

One MLPA probe for each of the 79 DMD exons has been made. In addition a probe is present for the alternative exon 1: DP427c. These 80 probes have been divided in two probe mixes P034 and P035. In each probe mix, 5 control probes for other chromosome X sequences are present in addition to the 40 DMD specific probes. Both probe mixes also contain one chromosome Y-specific probe at 118 bp. The P035 mix contains a probe for a 12q14 specific probe at 109 bp which is absent in P034.

MLPA analysis was carried out using P034 and P035 probes (MRC-Holland, Amsterdam, The Netherlands). The procedures were carried out according to the manufacturer's recommendations (www.mlpa.com). 100 ng DNA was denatured and hybridized overnight at 60º C with the SALSA probe mix 034 (DMD exons 1-10, 21-30, 41-50 and 61-70) and 035 (DMD exons 11-20, 31-40, 51-60 and 71-79). Samples were then treated with Ligase 65 for 15 min at 54ºC. The reactions were stopped by incubation at 98º C for 5 min. Finally, PCR amplification was carried out with the specific SALSA FAM PCR primers. Amplification products were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA) with the following modules: capillaries 36 cm, Polymer POP-4, run temperature 60ºC, capillary fill volume 184 steps, pre-run voltage 15 kV, pre-run time 180 sec, injection voltage 3.0 kV, injection time 10-30 sec, run voltage 15 kV, data delay time 1 sec, run time 1500 sec. The obtained data were analysed by using Genemapper 3.7 Software. Five healthy males and five females without family history of dystrophinopathies were analyzed as controls. They were not age matched controls.

Table 3.7: List of Salsa probes and the chromosomal position for P034 DMD probemix

Length (nt)	SALSA Probe #	Chromosomal position
64-70-76-82*		
94	Synthetic Control probe	2q14
118	Synthetic probe NPK003-L0313	Chromosome Y
130	Control probe 1690-L0423	Xq11.2
138	DMD probe 1353-L1001	DMD exon 1
146	DMD probe 1354-L1002	DMD exon 41
154	DMD probe 1355-L1615	DMD exon 21
162	DMD probe 1356-L1004	DMD exon 61
170	DMD probe 1357-L1005	DMD exon 2
178	DMD probe 1711-L1279	DMD exon 42
186	DMD probe 1359-L1007	DMD exon 22
194	DMD probe 1897-L1008	DMD exon 62
202	Control probe 1691-L0465	Xp22
210	DMD probe 1361-L1009	DMD exon 3
218	DMD probe 1362-L1010	DMD exon 43
226	DMD probe 1363-L1011	DMD exon 23
234	DMD probe 1364-L1012	DMD exon 63
242	DMD probe 1365-L1013	DMD exon 4
250	DMD probe 1366-L1014	DMD exon 44
258	DMD probe 1958-L1518	DMD exon 24
266	DMD probe 1368-L1016	DMD exon 64
274	Control probe 1768-L1617	Xq28
282	DMD probe 1954-L1574	DMD exon 5
290	DMD probe 1370-L1287	DMD exon 45
298	DMD probe 1371-L1019	DMD exon 25
306	DMD probe 1372-L1020	DMD exon 65
314	DMD probe 1373-L1021	DMD exon 6
322	DMD probe 1374-L1288	DMD exon 46
330	DMD probe 1375-L1023	DMD exon 26
338	DMD probe 1376-L1024	DMD exon 66
354	DMD probe 1713-L1281	DMD exon 7
362	DMD probe 1378-L1026	DMD exon 47
370	DMD probe 1379-L1616	DMD exon 27
378	DMD probe 1960-L1520	DMD exon 67
386	DMD probe 1715-L1283	DMD exon 8
394	DMD probe 1382-L1030	DMD exon 48
402	DMD probe 1716-L1284	DMD exon 28
410	DMD probe 2482-L2711	DMD exon 68**
418	Control probe 1770-L1334	Xq28
426	DMD probe 1385-L1033	DMD exon 9
434	DMD probe 1717-L1285	DMD exon 49
442	DMD probe 1387-L1035	DMD exon 29
450	DMD probe 1388-L1036	DMD exon 69
458	DMD probe 1718-L1286	DMD exon 10
466	DMD probe 1390-L1038	DMD exon 50
474	DMD probe 1391-L1039	DMD exon 30
482	DMD probe 1392-L1040	DMD exon 70
490	Control probe 1692-L1531	Xq28

** Not ligation-dependent, this indicates the amount of DNA used.*

Table 3.8: List of Salsa probes and the chromosomal position for P035

Length (nt)	SALSA Probe #	Chromosomal position
64-70-76-82*		
94	Synthetic Control probe.	Chr. 2q14
109	Synthetic probe PPK004-L004	Chromosome 12q14
118	Synthetic probe NPK003-L0313	Chromosome Y !!
130	Control probe 1690-L0423	Xq11.2
138	DMD probe 1393-L1041	DMD exon 11
146	DMD probe 1394-L1042	DMD exon 51
154	DMD probe 1395-L1371	DMD exon 31
162	DMD probe 1396-L1044	DMD exon 71
170	DMD probe 1397-L1608	DMD exon 12
178	DMD probe 2059-L1571	DMD exon 52
186	DMD probe 1399-L1609	DMD exon 32
194	DMD probe 1949-L1610	DMD exon 72
202	Control probe 1691-L0465	Xp22
210	DMD probe 1899-L1049	DMD exon 13
218	DMD probe 1892-L1050	DMD exon 53
226	DMD probe 1900-L1051	DMD exon 33
234	DMD probe 1893-L1052	DMD exon 73
242	DMD probe 1950-L1573	DMD exon 14
250	DMD probe 1894-L1054	DMD exon 54
258	DMD probe 1901-L1055	DMD exon 34
266	DMD probe 1902-L1611	DMD exon 74
274	Control probe 1768-L1617	Xq28
282	DMD probe 1410-L1057	DMD exon 15
290	DMD probe 1411-L1058	DMD exon 55
298	DMD probe 1412-L1059	DMD exon 35
306	DMD probe 1413-L1060	DMD exon 75
314	DMD probe 2060-L1572	DMD exon 16
322	DMD probe 1415-L1062	DMD exon 56
330	DMD probe 1416-L1063	DMD exon 36
338	DMD probe 1417-L1612	DMD exon 76
354	DMD probe 1952-L1065	DMD exon 17
362	DMD probe 1419-L1066	DMD exon 57
370	DMD probe 3038-L2508	DMD exon 37
378	DMD probe 1421-L1068	DMD exon 77
386	DMD probe 1891-L1069	DMD exon 18
394	DMD probe 1423-L1070	DMD exon 58
402	DMD probe 1896-L1071	DMD exon 38
410	DMD probe 1425-L1072	DMD exon 78
418	Control probe 1770-L1334	Xq28
426	DMD probe 1426-L1073	DMD exon 19
434	DMD probe 1427-L1074	DMD exon 59
442	DMD probe 1955-L1613	DMD exon 39
450	DMD probe 1429-L1076	DMD exon 79
458	DMD probe 1430-L1077	DMD exon 20
466	DMD probe 1431-L1614	DMD exon 60
474	DMD probe 1432-L1079	DMD exon 40
482	DMD probe 1433-L1080	DMD exon DP427C
490	Control probe 1692-L1531	Xq28

* Not ligation-dependent, this indicates the amount of DNA used.

3.5.1 Analysis of MLPA data: The Genemapper results were exported to excel sheet and the data were organized based on product sizes and the peak heights of each probe were used to calculate the dosage quotient using the Andrew's software (NGRL, UK). The Andrew's software compares peak heights with internal controls and also with peaks of 5 control samples. This software determines the relative probe signals of each probe by dividing each measured peak area (As) by the sum of all 45 peaks area (ΣAs) of that sample. The relative peak area (As/ΣAs) was then divided by the relative peak area of the corresponding probe obtained from a control DNA sample (Ac/ΣAc). Results are given in terms of normalized ratio and normalized peak heights.

3.6 QUANTITATIVE MULTIPLEX PCR

Quantitatiave multiplex PCR was standardized with the same conditions used for mPCR, except that the starting DNA quantity used was 250 µg/ml for all the samples. Male and female controls were run with the test samples and the gel was run using 12 µL of the PCR product. The resulting band was viewed in the Gel documentation system (Biorad, USA). The band intensities were analysed using the Quantity one software (Biorad, USA) and the adjusted band volumes of the test samples were compared with that of male and female controls. The results were interpreted only if the male control band volumes were half of that of the female controls.

3.7 QUANTITATIVE MULTIPLEX FLUORESCENCE PCR (QMFPCR)

QmfPCR was performed for 51 exons and the procedure carried out as described by Yau et al. Two fluorescently labeled multiplex PCR assays were done to amplify 21 exons from the proximal deletion hotspot of the dystrophin gene (5' assay) and 22 exons from the central deletion hotspot (3' assay). The 5' multiplex assay amplifies 21 dystrophin gene exons, namely the muscle specific promoter and part of exon 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 17, 19, 21, 24, 25, 29, 30, 32 and 37. The 3' multiplex assay amplifies 22 exons: 42, 43, 44, 45, 46, 47, 48, 49, 51, 52, 53, 54, 55, 56, 58, 59, 60, 62, 63, 68, 71 and 75. All forward primers in the assays were labeled with either the fluorescent phosphoramidite 6-FAM (5' assay) or HEX (3' assay) (Applied Biosystems). Primer sequences were obtained from Leiden Muscular Dystrophy,

http://www.dmd.nl, accessed January 2008. Primer sequences and the sizes of the products generated are shown in table 3.9.

Table 3.9: List of Exons and primer sequences in QFMPCR-Multiplex 1

Primer Name	Primer Sequence	Product size (bp)
Ex1F	GAAGGCGGGTCACTTGCTTGTGCGCAG	419
Ex1R	CAATCTACCTAATTAGTGAGCTTG	
Ex2F	ACACTAACACATCATAATGGAAAG	243
Ex2R	GATTTTTAAGATACACAGGTACATAGTCC	
Ex3F	CATCTTCGGCAGATTAATTATGC	330
Ex3R	CAGTACCTAGTCATTCTACTAGATGTC	
Ex4F	TATTAATGCCTCACAGGCTCTGT	277
Ex4R	CCAAAGCCCTCACTCAAACATGAAG	
Ex5F	AAGCTTCAATGCTAAGTCTCTGAA	302
Ex5R	ACACATTTGTTTCACACGTCAAGG	
Ex6F	CCAATGAATCAGAATAGACTCCTAGCC	414
Ex6R	GAGTCTAAATCACCACTTTTACAAG	
Ex7F	AAGGACTATGGGCATTGGTTGTCA	315
Ex7R	TGTGTAGAAATGACAAGTCTCAGA	
Ex8F	GGACATTCATGGACAATTCACTGTTC	482
Ex8R	GCAAAATTGAAAAGGTTTAGTCTGTCTC	
Ex9F	GTAGTCCTTTCGGGTTACTTATGG	321
Ex9R	AACAAACCAGCTCTTCACGAGGAGA	
Ex11F	GCCTGCTTCTGAAGAACTTGACTTAAG	444
Ex11R	AAGCTTCCAAAACTTGTTAGTCTTC	
Ex12F	GATAGTGGGCTTTACTTACATCCTTC	405
Ex12R	TAATTCTCTCCCATCAACCATGTCATCT	
Ex13F	GGAGTACCTGAGATGTAGCAGAAAT	384
Ex13R	CAGCACTTCAGCTGATTATGAGTG	
Ex17F	GCTATTTTGATCTGAAGGTCAATCTACC	354
Ex17R	GAGTTTTCTCCACTTCATTTGCAG	
Ex19F	GATTCACGTGATAAGCTGACAGAGTG	429
Ex19R	TTCTACCACATCCCATTTTCTTCCA	
Ex21F	CAGATATTTGTGAAGGGTATTAAGC	389
Ex21R	TATTGTTTCATGTTAGTACCTTCTGG	
Ex24F	GCCTGTGTTTAGACATAACACAATG	243
Ex24R	CATACAAAATTATTCATATTAAAGG	
Ex25F	AGACTGTTAGGCAGTCATCTATATC	398
Ex25R	AGTAACGGTGAAGGGAGACATTAGG	
Ex29F	GTAAATCAGAAGATACTGAGCATTTGC	301
Ex29R	AGGCCTGTATCTGCTATACATTAATGC	
Ex30F	CATTTATTGTTCAGCAGGATTAC	344
Ex30R	CCCATGGAAAACTAGTTGAATAA	
Ex31F	AGAGGTGGTTGAGGAGAGTTTCTGA	249
Ex31R	GCCCAACGAAAACACGTTCCTTAG	
Ex37F	CTGCATGTGCTTGCTCTCATTTTCTTAC	427
Ex37R	GAAAACCTTGCTGTGGGGTCTACTTG	

Table 3.10: List of Exons and primer sequences in QFMPCR - Multiplex 2

Primer Name	Primer Sequence	Product size (bp)
Ex42F	CAATTGTCAGCTGTAGAATGAGACC	348
Ex42R	TGAATGATCAGTATGATCACCTTG	
Ex43F	GCAACACCATTTGCTACCTTTGGGA	321
Ex43R	AAATCATTTCTGCAAGTATCAAG	
Ex44F	GCAGGAAACTATCAGAGTGATATCTTTGTC AG	360
Ex44R	TCCATCACCCTTCAGAACCTGATCT	
Ex45F	CTTTCTTTGCCAGTACAACTGCATGTG	369
Ex45R	GCTTATAATCTCTCATGAAATATTC	
Ex46F	CAGTTTGCATTAACAAATAGTTTGAG	346
Ex46R	GAAAAACACTTTAGCAAGGAACTATG	
Ex47F	CATTTTGATAGACTAATCAATAGAAGC	289
Ex47R	CTTGCAACATTTAACACATGTGACG	
Ex48F	TTGAATACATTGGTTAAATCCCAACATG	463
Ex48R	GTCTTTAATAATGATACCAAATGAG	
Ex49F	GTGCCCTTATGTACCAGGCAGAAATTG	310
Ex49R	GTCCACGTCAATGGCAAATGTACAACAGG	
Ex51F	GAAATTGGCTCTTTAGCTTGTGTTTC	388
Ex51R	GGAGAGTAAAGTGATTGGTGGAAAAT	
Ex52F	GTGTTTTGGCTGGTCTCACA	370
Ex52R	CATTATGGACTGAAAATCTCAGCAC	
Ex53F	GAATCCTGTTGTTCATCATCCTAGCC	422
Ex53R	GTATAATTTTATCAAATGTAACCAG	
Ex54F	TTCTGACCTGAGGATTCAGAAGCTG	347
Ex54R	GAAAAACAAATCCTCATGGTCCATCCAG	
Ex55F	TATATTACAATTTAGTTCCTCCATC	383
Ex55R	GTTTTGTCCCTGGCTTGTCAG	
Ex56F	CTCCAAATTCACATTCATCGCTTG	336
Ex56R	GAGATACCAGTTACTTGTGCTAAG	
Ex58F	GAGATAGAAATTGACCTGGGAGTTTC	328
Ex58R	GAGAGCTATCCAGACCCTGGCAGCA	
Ex59F	GGTTACCCTCTTGTTCAACTGTACTCT	461
Ex59R	GGGAAGATAACACTGCACTCAAGT	
Ex60F	GCAAACATTACTGGCACTGCACCCTAA	374
Ex60R	CCTATCCTCACAAATATTACCATG	
Ex62F	GCTGAGCAAACAGACCAATATCAGTGT	289
Ex62R	CACAGGTATTGTAGGCCAGGCTAATGTCGC	
Ex63.1F	GCAAAAATCATGTTGTTGTTATTG	301
Ex63.1R	TGGATAGGAAGGTGCCACTGCTTTCA	
Ex68F	CGAACTGATATACACCTCCTTTGCC	326
Ex68R	GATAAAAGATCAAGTCATAAAAAGGTG	
Ex71F	GCTATTGCTTTCCATGGTTCATAC	236
Ex71R	TAAACAGAACAAAAGAGAACCAAG	
Ex75F	AGTCAGATGCATCTATCTACCATGG	492
Ex75R	CACTTTGCAGGCACATACCAAGCAC	

Amplifications were performed in 25 µl volumes containing 125 ng genomic DNA, 0-2 pmol/L of each primer (except in the 5' assay where 0.4 µmol/L of the

primers for Pm and exon 9 were used), 1x Taq polymerase buffer (67 mmol/L Tris HCI, pH 8.8, 16.6 mmol/L $(NH4)_2SO4$, 6.7 mmol/L $MgCl_2$, 170 µg/ml BSA, 10 mmol/L 3-mercaptoethanol), 0.5 mmol/L dNTPs, and 1.5 units of Taq DNA polymerase (Amplitaq). After an initial denaturation of six minutes at 96°C a "hot start" was carried out by the addition of Taq polymerase, followed by 18 cycles of denaturation for 48 seconds at 93°C, annealing for 48 seconds at 62°C (5' assay) or 59°C (3' assay, subsequently reduced to 58°C), and extension for three minutes at 70°C, with a final extension for five minutes at 70°C.

PCR product (3 µL) was mixed with 3.5 µL of formamide loading buffer (95% formamide in 1 x TBE (89 mol/L Tris, 89 mmol/L borate, 2 mmol/L EDTA, pH 853) with 5 mg/ml dextran blue) and 0.5 µl internal lane size standard (GENESCAN-500 ROX) (Haider et al.). The multiplex products, formamide dye, and size standard were denatured for seven minutes at 96°C, then electrophoresed by capillary electrophoresis in DNA sequencer ABI 3130 (Applied Biosystems). Data were analysed automatically using the Genescan and Genotyper Analysis Software (Applied Biosystems) to produce an electrophoretogram from each sample, with peaks showing the size (bp) of each amplification product and areas under the peaks representing the amount of fluorescence signal from labeled primers incorporated into the products.

In order to determine gene dosage for every exon amplified in an assay the peak areas from a sample were compared against one another, and against those from controls, to obtain a series of dosage quotients (DQ). Peak area data from samples and controls was transferred automatically from the Genotyper program into a simple Excel (Microsoft) spreadsheet. Dosage quotients for pairs of exons in a sample were then calculated by dividing the ratio of the two exons' peak areas from the sample by the corresponding ratio from the controls. For example, the dosage quotient for exons 45 and 48: $DQ^{E45/E48}$= (sample exon 45 peak area/sample exon 48 peak area) / (control exon 45 peak area/control exon 48 peak area).

Thus, a pair of double copy or single copy loci will give theoretical DQ values of 1.0, whereas in the case of a deletion carrier the theoretical DQ value for a deleted locus will diverge from 1.0 to values of 0.5 and 2.0, depending on whether the peak area for the single copy locus is a numerator or denominator in

the equation. In the case of a duplication carrier the theoretical dosage quotients will be 1.5 and 0.67, again depending on whether the peak area from a duplicated locus is the numerator or denominator, respectively.

3.8 CA- (STR) SEGREGATION ANALYSIS

CA- (STR) segregation analysis were performed for 11 markers spanning the DMD gene, 19n8, 3'm, 1671, 1623, i50, i49, i45, i44, 7n4, 5n4, and 5n3. The method was performed according to the method described in Carsana et al. using previously reported primer oligonucleotides (Leiden Muscular Dystrophy, *http://www.dmd.nl*, accessed January 2008). The forward primers were labeled with 5-carboxyfluorescein (FAM), PET, NED, or VIC fluorochromes.

Multiplex polymerase chain reaction mixtures (25 µl) contained 200 ng of genomic DNA, 0.25 mmol/L dNTP mixture, 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$ (2 mmol/L $MgCl_2$ in multiplex reaction B), 30 ng/µL dimethyl sulfoxide, and 0.5 U of *Taq* polymerase. Amplification reactions were performed using a touch-down protocol (denaturation at 95°C for 3 minutes; 39 cycles with denaturation at 95°C for 20 seconds, annealing at 62°C for 40 seconds, -0.5°C per cycle for 14 cycles and at 55°C for 40 seconds for 25 cycles, and polymerization at 72°C for 45 seconds; final extension: 72°C for 7 minutes). These PCR conditions were designed to provide a robust amplification of the 15 STRs under the same thermal profile.

PCR products (0.5 µL from each multiplex reaction) were mixed with 0.5 µL of Gene-Scan-500 LIZ size standard (Applied Biosystems) and were separated by capillary gel electrophoresis (15 kV at 60°C for 30 minutes) on the ABI Prism 3130 Genetic Analyzer (Applied Biosystems) using the POP-7 polymer. The Genemapper 3.7 (Applied Biosystems) software was used for data analysis and creating a macro that allowed us to label the peaks and identify the alleles of each marker automatically.

Table 3.11: List of Markers, Repeat, localization in DMD gene, base pair length and primer sequences for STR-(CA) segregation analysis

Marker Name	Repeat	Localization in DMD gene	Reference	Length in bp	Forward/Reverse primer
19n8	$(GT)_{18}$	200 kb 3' of exon 79	(King et al., 1995; King et al., 1994)	148	aacgacttcccccactctgt/ agccccattctgtacatcaaat
3'm (3'DYS MSC)	c.461(CA)8 (TACA) 1_2 (CA)6	exon 79 (11,550)	(Matsumoto and Niikawa, 2004)	133	gaaagattgtaaactaaagtgtgc/ ggatgcaaaacaatgcgctgcctc
DI671	c.9808-4883_9808-4814 (CT)17_36	intron 67	(Matsumoto and Niikawa, 2004)	239-277	tcgccccttcagaagtcact/ gtccagcagatcaatcgtccagc
DI623	9225-23589_9225-23542 (GAA)9_27	intron 62	(Matsumoto and Niikawa, 2004)	141-195	acctgcctagtcaaggta/ cactgccatggtgaatgatc
i50 (STR 50)	complex $(CA)_{16}$	intron 50	(Clemens et al., 1991)	241	aaggttcctccagtaacagatttgg/ tatgctacatagtatgtcctcagac
i49 (STR 49)	$(AC)_{24}$	intron 49	(Clemens et al., 1991)	249	cgtttaccagctcaaaatctcaac/ catatgatacgattcgtgttttgc
i45 (STR 45)	$(CA)_{28}$	intron 45	(Clemens et al., 1991)	172	gaggctataattctttaactttggc/ ctctttccctctttattcatgttac
i44 (STR 44)	$(TG)_6GG$ $(TG)_{15}$ $TA(TG)_2(AG)_{14}$	intron 44	(Clemens et al., 1991)	196	tccaacattggaaatcacatttcaa/ tcatcacaaatagatgtttcacag
5'-7n4	$(CA)_8TA$ $(CA)_{19}$	intron 25	(King et al., 1995)	165	gtgaagctacaaaaatattagag/ caacaatatctcaccatacttg
5'-5n4	$(AC)_{24}$	intron 4	(King et al., 1995)	133	gaagggaaaatgatgaataaaact/ gtcagaactttgtcacctgtc
5'-5n3	$(TG)_{23}$	intron 2	(King et al., 1995; King et al., 1994)	112	ttcagtttctctcggtgttcct/ tacacctgcacatgtgatgaaa

Subscripts denote the number of repeats.

3.9 DYSTROPHIN GENE SEQUENCING

The DMD gene was screened for mutations by PCR amplification and direct DNA sequencing using ABI Prism 3130 genetic analyser for the affected patients who either had a family history of the disorder or immunohistochemistry showing dystrophin deficiency. Seventy eight sets of primers which included intronic and overlapping exonic regions for amplification of 79 exons. The amplified products

were electrophoresed in 2% agarose gel for verification and followed by sequencing.

Table 3.12: PCR reaction protocol for the exon amplification of DMD gene

Initial denaturation	94° C	5 min	1 cycle
Denaturation	94° C	60 sec	35 cycles
Annealing temperature	68° C	45 sec	
Extension	72° C	60 sec	
Final Extension	72° C	7 min	1 cycle

3.9.1 DNA sequencing

Table 3.13: Cycle sequencing protocol

Order	Reagents	Volume (in μL)
1	Amplified product	1.0
2	10 μM Primer	2.0
3	Sequencing buffer	1.5
4	Ready Reaction mix	2.0
5	Autoclaved Milli Q water	3.5

Table 3.14 Reaction conditions of cycle sequencing

Initial denaturation	96°C	60 seconds
Denaturation	96°C	10 seconds
Annealing	50°C	5 seconds
Extension	60°C	4 minutes

Carry the reaction for 25 cycles.

3.9.2 Purification of extension products:

The extension products were purified to remove the unincorporated dye terminators before the samples are analysed by electrophoresis. Excess dye terminators obscure data in early part of the sequence and interfere with base calling.

Reagents required: 50 mM EDTA

3 M Sodium acetate (pH4.8)

Absolute ethanol

70 % ethanol

A one in four dilution of 50 mM EDTA was done with MQ water. To 10 μL of autoclaved MQ water, 2 μL of diluted EDTA solution was added. To this 50 μL of absolute ethanol and 2 μL of 3M sodium acetate was added. To the above mixture 10 μL of cycle sequencing product was added and mixed. This was kept at room temp for 15 minutes and centrifuged at 8000 rpm for 20 minutes. The supernatant was discarded and to the pellet add 250 μL of 70% ethanol. This was vortexed and centrifuged at 8000 rpm for 10 minutes. After this the supernatant was discarded and the vial was covered with parafilm, perforated and dried at 37°C. Before loading this to the ABI PRISM genetic analyser, 10 – 15 μL of Hi-di formamide / template suppressor reagent was added, vortexed and denatured at 95°C for 5 minutes.

Table 3.15: List of DMD exon sequencing primers, their base pair length and annealing temperature.

Exon	Forward / reverse primer	Length (in bp)	Annealing T_m (in ^oC)
Dp427m promoter region (-674 - -149)	F 5'GAAGATCTAGAacagtggatacataacaaatgcatg3' F5'ttctccgaaggtaattgcctcccCAGATCTGAGTCC3'	535	--
1	F 5'GCAGGTCCTGGAATTTGA 3' R 5'caaactaaacgttatgccaca3'	405	55
2	F 5'cactaacacatcataatgg3' R 5'gatacacaggtacatagtc3'	269	52
3	F 5'tcatccatcatcttcggcagattaa3' R 5'caggcggtagagtatgccaaatgaaaatca3'	444	55
4	F 5'ttgtcggtctctctgctggtcagtg3' R 5'ccaaagccctcactcaaac3'	233	55
5	F 5'caactaggcatttggtctc3' R 5'ttgtttcacacgtcaaggg3'	261	55
6	F 5'tggttcttgctcaaggaatg3' R 5'tggggaaaaatatgtcatcag3'	335	55
7	F 5'ctatgggcattggttgtc3' R 5'aaaagcagtggtagtccag3'	296	55
8	F 5'tcgtcttcctttaactttg3' R 5'tcttgaatagtagctgtcc3'	343	55
9	F 5'tctatccactcccccaaacc3' R 5'aacaaaccagctcttcac3'	318	55
10	F 5'ggaacaatctgcaaagac3' R 5'aaaggatgacttgccattataac3'	350	55
11	F 5'caaataaaactcaaaaccacacc3' R 5'cttccaaaacttgttagtcttc3'	337	55
12	F 5'ctttcaaagaggtcataatagg3' R 5'catctgtgttactgtgtatagg3'	305	55
13	F 5'gcaaatcatttcaacacac3'	387	55

	R 5’tctttaaatcacagcacttc3’		
14+15	F 5’tggcaaattattcatgccatt3’ R 5’tgatccaagcaaaaataaacatt3’	548	52
16	F 5’atgcaacccaggcttattc3’ R 5’ctgtagcatgataattggtatcac3’	286	55
17	F 5’ttttcctttgccactccaag3’ R 5’caccaccaacaaaactgctg3’	362	55
18	F 5’tgtcaggcaggagtctcagat3’ R 5’cggagtttacaagcagcaca3’	339	55
19	F 5’gatggcaaaagtgttgagaaaaagtc3’ R 5’ttctaccacatcccattttcttcca3’	495	55
20	F 5’tggctttcagatcatttctttc3’ R 5’aaatacctattgattatgctcc3’	393	55
21	F 5’gcaaaatgtaatgtatgcaaag3’ R 5’atgttagtaccttctggatttc3’	355	55
22	F 5’aggaaaacatggcaaagtgtg3’ R 5’tgctcaatgggcaaactacc3’	370	55
23	F 5’tcatctactttgtttacatgtttgaa3’ R 5’acagtgtatcgttagggaaaaa3’	433	52
24	F 5’ttgggcctgtgtttagacata3’ R 5’aaatccaccccagctgtaaaa3’	327	55
25	F 5’tgtggcagtaatttttttcag3’ R 5’aggaaatcttagttaagtacg3’	296	55
26	F 5’taataatgtttcatcactgtc3’ R 5’tgttgcatttctttcttttttc3’	335	55
27	F 5’tgggatgttgtgagaaagaa3’ R 5’tgaccatgtattgacatataattga3’	365	55
28	F 5’gaagttttaataatgaaatggcaaaa3’ R 5’gtacctcttttaatactgcatat3’	311	55
29	F 5’ccaatgtatttagaaaaaaaaggag3’ R 5’gcaaattagattaaagagattttcac3’	279	52
30	F 5’tacagaaaagctatcaagag3’ R 5’aaaaacaaaagaatggaagc3’	297	55
31	F 5’atggtagaggtggttgagga3’ R 5’tataatgcccaacgaaaaca3’	296	52
32	F 5’cagttattgtttgaaaggcaaa3’ R 5’cttcttaatgaggaaagtcaagg3’	322	55
33	F 5’tggaatagcaattaaggg3’ R 5’gctaagactctaatcatac3’	393	55
34	F 5’cagaaatataaaagttccaaataagtg3’ R 5’catgttaatacttccttacaaaatc3’	374	55
35	F 5’ccgtttcataagcattaaatc3’ R 5’agcttctagccttttctc3’	307	55
36	F 5’tgtctaaccaataatgccatg3’ R 5’ctggtgtacaatttggaca3’	257	55
37	F 5’ctttctcactcttctcgctcac3’ R 5’ttcgcaagagaccatttagcac3’	377	55
38	F 5’ggtttatgtttcttataaaaagtaa3’ R 5’atttatttccactcctagtt3’	267	52
39	F 5’taaaaaccaaaatgaagactg3’ R 5’ttaaataagcatacacattgaac3’	365	55
40	F 5’tacaaaaagatgagggac3’ R 5’aatagaaacaagaacatcaac3’	387	55
41	F 5’gttagctaactgccctgggccctgtattg3’	311	55

	R 5’tagagtagtagttgcaaacacatacgtgg3’		
42	F 5’atggaggaggtttcactgtt3’ R 5’ccatgtgaaagtcaaaatgc3’	408	55
43	F 5’tgcaacaccatttgctacc3’ R 5’atcatttctgcaagtatcaag3’	357	55
44	F 5’gttacttgaaactaaactctgcaaatg3’ R 5’acaacaacagtcaaaagtaatttccatc3’	444	55
45	F 5’ttctttgccagtacaactgc3’ R 5’tctgctaaaatgttttcattcc3’	357	55
46	F 5’ccagtttgcattaacaaatagtttgag3’ R 5’agggttaagaagaaataaagttgtgag3’	409	55
47	F 5’tgatagactaatcaatagaagcaaagac3’ R 5’aacaaaacaaaacaacaatccacatacc3’	399	55
48	F 5’ttgaatacattggttaaatcccaacatg3’ R 5’cctgaataaagtcttccttaccacac3’	543	55
49	F 5’gtgcccttatgtaccaggcagaaattg3’ R 5’gcaatgactcgttaatagccttaagatc3’	475	55
50	F 5’caccaaatggattaagatgttcatgaat3’ R 5’tctctctcacccagtcatcacttcatag3’	307	55
51	F 5’gaaattggctctttagcttgtgtttc3’ R 5’ggagagtaaagtgattggtggaaaatc3’	424	55
52	F 5’gtgttttggctggtctcaca3’ R 5’catgcatcttgctttgtgtgt3’	298	55
53	F 5’tcctccagactagcatttac3’ R 5’ttagcctgggtgacagtg3’	485	55
54	F 5’gtattctgacctgaggattc3’ R 5’catggtccatccagtttc3’	378	55
55	F 5’aatttagttcctccatctttctct3’ R 5’aaatacatcaggctgtataaaagc3’	445	55
56	F 5’attctgcacatattcttcttcctgc3’ R 5’ggatgatttacgtagacatgtgag3’	353	55
57	F 5’caatggaattgttagaatcatca3’ R 5’cactggattactatgtgcttaacat3’	320	55
58	F 5’ttttgagaagaatgccacaagcc3’ R 5’aaaatatgagagctatccagaccc3’	315	55
59	F 5’aaagaatgtggcctaaaacc3’ R 5’ttgtgggaagataacactgc3’	433	55
60	F 5’taaatattctcatcttccaatttgc3’ R 5’ttactgtaacaaaggacaacaatg3’	267	55
61	F 5’cattgttttaattgttcctcatt3’ R 5’ttcaactcttaattcttttgttttt3’	270	55
62	F 5’taatgttgtctttcctgtttgcg3’ R 5’atacaggttagtcacaataaatgc3’	221	55
63	F 5’tactcatggtaaatgctaaagtc3’ R 5’tagcaaagtaactttcacactgc3’	229	55
64	F 5’ttctgatggaataacaaatgct3’ R 5’cattctaggcaaactctaggc3’	322	55
65	F 5’tatgagagagtcctagctagg3’ R 5’taagcctcctgtgacagagc3’	383	55
66	F 5’gtctagtaattgttttctgctttg3’ R 5’ataagaacagtctgtcatttccc3’	246	55
67	F 5’gaagtaaccccactactgtggaa3’ R 5’aaacgaagctctgtgggttt3’	405	55
68	F 5’taatcgaactgatatacacctcc3’	387	55

	R 5’actaacagcaactggcacagg3’		
69	F 5’gaacgtggtagaaggtttattaaa3’ R 5’ctaactctcacgtcaggctg3’	267	55
70	F 5’tggtcattagttttgaaatcatc3’ R 5’catcaaacaagagtgtgttctg3’	273	55
71	F 5’ggctgagaaagcgtgtgtct3’ R 5’gagcgaatgtgttggtggta3’	174	55
72	F 5’gatggtatctgtgactaatcac3’ R 5’atttcaatcaatatttgcctggc3’	181	55
73	F 5’acgtcacataagttttaatgagc3’ R 5’atgctaattcctatatcctgtgc3’	238	55
74	F 5’ataaggggggggaaaaaac3’ R 5’tgcaagtgtatgcactctg3’	290	52
75	F 5’tctttttttacttttttgatgc3’ R 5’agtgctctctgaggtttag3’	380	55
76	F 5’gggtcaaaatttatgagtcctg3’ R 5’ttcatgtccctgtaatacgact3’	330	55
77	F 5’taatcatggccctttaatatctg3’ R 5’gatactgcgtgttggcttcc3’	306	55
78	F 5’ttctgatatctctgcctcttcc3’ R 5’catgagctgcaagtggagagg3’	267	55
79	F 5’agagtgatgctatctatctgcac3’ R 5’TGCATAGACGTGTAAAACCTGCC3’	385	55

(-674 - - 149) denotes the region upstream 674 to upstream 149 of DMD gene.

Sequence of the primers with exonic sequences given in upper case, intronic sequences in lower case

3.10 GENOMEWIDE SNP ASSAY USING AFFYMETRIX SNP ARRAY 6.0

The Affymetrix® Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0 [P/N 901152, 901015] was used in conjunction with the Genome-Wide Human SNP Array 6.0. Briefly, total genomic DNA (500 ng) was digested with Nsp I and Sty I restriction enzymes and ligated to adaptors that recognize the cohesive 4 bp overhangs. All fragments resulting from restriction enzyme digestion, regardless of size, are substrates for adaptor ligation. A generic primer that recognizes the adaptor sequence was used to amplify adaptor-ligated DNA fragments. PCR conditions have been optimized to preferentially amplify fragments in the 200 to 1,100 bp size range. PCR amplification products for each restriction enzyme digest are combined and purified using polystyrene beads. The amplified DNA was then fragmented, labeled and hybridized to a Genome-Wide Human SNP Array 6.0. The arrays that passed the QC call rate threshold were analyzed using the Birdseed algorithm at the default setting of 0.1.

The Genome-Wide Human SNP Array 6.0 was used in conjunction with Affymetrix® Genotyping Console, which implements a novel genotype calling algorithm called Birdseed. Birdseed is an evolution of the RLMM genotype calling algorithm1. It performs a multiple-chip analysis to estimate a signal intensity for each allele of each SNP, fitting probe-specific effects to increase precision (like the BRLMM-P algorithm developed for the Genome-Wide Human SNP Array 5.0). It then makes genotype calls by fitting a Gaussian mixture model in the two-dimensional A-signal vs. B-signal space, using SNP-specific models to improve accuracy.

FLUIDICS PROTOCOL USED - GenomeWideSNP6_450

LIBRARY FILES USED - GenomeWideSNP_6

Library files contain information about probe array design layout and other characteristics, probe use and content and scanning and analysis parameters. These files are unique for each probe array type. Library files were available from the Affymetrix website at www.affymetrix.com/support/technical/libraryfilesmain.affx

CHAPTER 4: GENETIC DIAGNOSIS OF DUCHENNE MUSCULAR DYSTROPHY – DELETION AND DUPLICATION ANALYSIS OF THE DMD GENE

4.1 INTRODUCTION:

The first step in the diagnosis of Duchenne muscular dystrophy initially was based on demonstrating the loss of the dystrophin protein in the skeletal muscle tissues of the affected individual by immunohistochemistry. This would be followed by Western blot analysis where the necessary expertise was available. Molecular diagnosis would then follow where the causative DMD gene mutation was tested for by different methods. Identification of mutations in probands aids not only in confirming a clinical diagnosis but also allows carrier testing and contributes towards prevention strategies (Aartsma-Rus et al. 2003). Moreover, the potential therapies being tested for DMD, such as exon skipping and PTC 124 (Ataluren), are absolutely dependent on precise knowledge of the mutation (Abbs and Bobrow, 1992; Schmitz and Famulok, 2007) Further, on a long term perspective the mutation analysis paves a base for phenotypic-genotypic correlation that throws light in understanding the disorder.

Several methods have been devised for DMD gene mutation analysis by different groups with each carrying their own advantages and disadvantages. Multiplex PCR (mPCR) was one such method which was used for picking up whole exon, single and multiple deletions. The basis for the development of this method was on the observation that single or multiple exon deletions were the most common mutations in the DMD gene. (Abbs and Bobrow, 1992) It was also known that these mutations occurred in two hot spot regions, the central and the 5' hot spots. (Abbs and Bobrow, 1992) Based on these observations, the screening of just 19 exons of the 79 was enough to pick mutations in approximately 65% of all DMD cases or 95-98% of all DMD gene deletions.

Although ~95% of deletions can be detected in males using multiplex PCR, other methods must be used to determine duplications, small insertion and deletions and point mutations. The most commonly applied methods are quantitative multiplex PCR and quantitative Southern blotting. The drawback of quantitative

multiplex PCR is that often not all exons are examined, meaning that small and rare mutations are missed. Southern blotting is usually applied to determine the boundaries of the mutation, which is essential for distinguishing DMD from BMD, i.e. frame disrupting from open reading frame changes. Using high-quality Southern blots it is possible to perform a quantitative analysis and detect duplications. However, this technique is time consuming and cumbersome and it is difficult to exactly determine the duplication boundaries and triplications will be missed. Methods that have been used in the diagnosis of DMD have been listed in Table 4.1.

Table 4.1: List of test methods commonly used for the diagnosis of various types of DMD /BMD gene mutations.

Test Method	Mutations detected	% of Males DMD	% of Males BMD
Multiplex PCR, Southern	Deletions	60-65 %	85%
Southern / QPCR	Duplications, Carrier analysis	6%	NR
Mutation scanning/ Seq analysis	Small insertions, deletions, Point mutations	30%	NR

MLPA and MAPH were DNA based methods which were very successful in picking deletions and duplications of whole exons throughout the entire 79 exons of the DMD gene. MAPH is based on a quantitative PCR of short DNA probes recovered after hybridisation to immobilized genomic DNA. Each probe represents a single exon, which is amplified and cloned into a specific vector. By reamplification using primers from the multiple cloning sites it is possible to co-amplify all probes in one reaction using a single pair of primers (Refer figures 1.11 and 1.12).

MLPA based on Multiplex Ligation-dependent Probe Amplification, has recently gained more importance due to its ease, simplicity and accuracy (Schouten et al., 2002). It requires a lower amount of input DNA and is a one-tube assay. Up to 96 samples can be handled simultaneously, with results being available within 24 hours.

4.1.1 Best Practice Guidelines for the Molecular Diagnosis of DMD

Best Practice Guidelines for molecular diagnosis of Duchenne and Becker muscular dystrophy was published recently (Abbs et al, 2010). New therapeutic trials for DMD demand accurate diagnosis of the disorder, especially where the therapy is targeted towards specific mutations. These guidelines aim to help diagnostic laboratories attain that accuracy by describing the minimum standards for acceptable molecular diagnostic testing of DMD. For the different types of clinical referral received by a molecular diagnostic laboratory, the guidelines recommend the appropriate tests to be carried out, interpretation of the results and how those results should be reported. Figure 4.1 shows the accepted algorithm that has to be followed in the diagnosis of DMD.

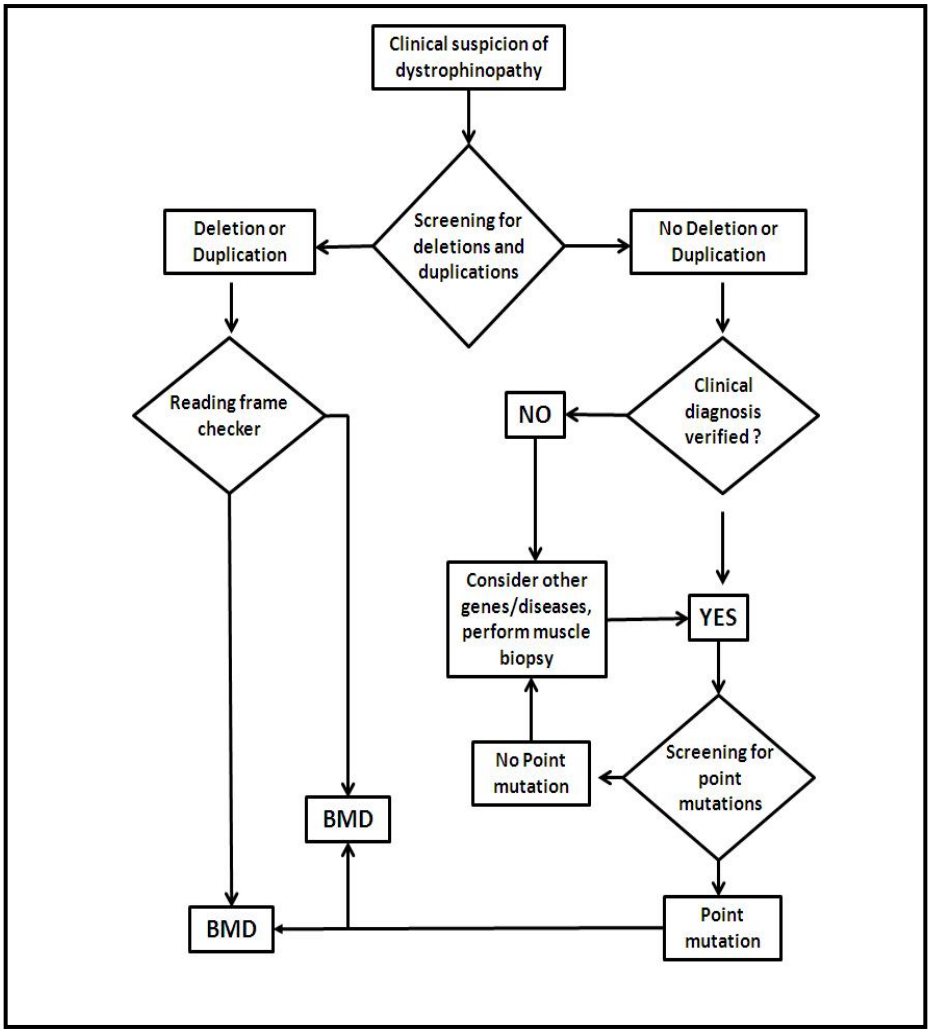

Figure 4.1: Flow chart for the diagnostic work-up of a dystrophinopathy recommended by the Best practice guidelines. *(Abbs et al., 2010)*

4.1.2 DMD diagnosis – Indian Scenario

The method of choice in India for DMD/BMD diagnosis is multiplex PCR which targets about 18 to 32 exons of the DMD gene to look for whole exon deletions (Banerjee and Verma, 1997; Mallikarjuna Rao et al., 2003; Basak et al., 2006; Dastur et al., 2008). Several groups from eastern India, North and Southern India have reported DMD gene deletion analysis using multiplex PCR. Kumari et al have seen deletions in 62.5% of DMD and 60% of BMD cases (Kumari et al., 2003). Southern blotting in these cases yielded more cases missed by multiplex PCR. All the other groups from eastern and southern India yield similar pick up rate by mPCR. None of the studies had used MLPA as a diagnostic tool for DMD.

The Objective of this chapter was to standardize mPCR and MLPA and use a systematic, less-invasive approach for the molecular diagnosis of DMD. Our main aim in this study is to use genomic DNA as a source of diagnosis, delaying the necessity of a muscle biopsy and increase the mutation pick up rate to 75%.

4.2 MATERIALS AND METHODS

4.2.1 Samples: Samples from patients with a clinical diagnosis of DMD/BMD received at the Molecular Diagnostic Facility (MDF) for genetic diagnosis between July 2006 and August 2009 were included in the study. The study was approved by the Institutional Review Board. Blood samples were collected from the patients after obtaining informed consent from them (>18 years) or their parents, as applicable. 3 ml EDTA anticoagulated blood samples were collected and DNA was extracted from them as described in the chapter on Materials and Methods.

The samples were also received at the facility from different centres and clinicians in Chennai, other parts of Tamilnadu and Karnataka. All the samples were from males, clinically suspected for DMD (Query DMD) or BMD (Query BMD). Clinical diagnosis was generally based on age of onset of symptoms, calf muscle hypertrophy, proximal muscle weakness and elevated CPK values. Either the samples with filled in genetic request forms or the patients with request forms completed from the clinicians were sent to the facility. At the facility a complete family history was taken, pedigree drawn and blood samples were collected for DNA analysis. A sample of the genetic request form has been attached in the appendix.

During the study period a total of 571 apparently unrelated cases/samples were received at the facility for diagnosis of DMD/BMD. Clinical diagnosis of 514 of the 571 cases was DMD and the rest 57 were BMD/LGMD. The number of cases with family history details of the 571 cases is tabulated below in Table 4.2.

Table 4.2: Details of the number of cases, clinical diagnosis and family history of patients recruited in the study.

Clinical diagnosis	No of cases	Family history		
		Yes	No	NA
DMD	514	76	393	45
BMD	57	15	42	--

NA – Not available

4.2.2 Multiplex PCR (mPCR): Multiplex PCR was done as the first step in genetic diagnosis of DMD, for 30 exons of the hot spot regions in the DMD gene.

As mentioned earlier, this is the first recommended technique and the most cost effective method to pick up most of the deletion mutations. The standardization of mPCR has been described in the results and the final protocol used for samples is given in the Material and Methods chapter. All the 571 samples were tested for DMD gene deletions by mPCR.

4.2.3 MLPA: MLPA has been used as the second step in diagnosis in this study. MLPA was performed as described and analysis was done using the NGRL software, as directed by the manufacturer.

Cases which showed deletion with clear exonic borders i.e. a definitive molecular diagnosis was not tested by MLPA. Only those cases which did not show a deletion, and which showed deletions with unclear exonic borders, non contiguous deletions and single exon deletions were tested by MLPA. Some of the cases showing single exon deletion by mPCR were also reconfirmed by MLPA. (Table 4.3)

With the above criteria, MLPA was done for a total of 315 cases including 196 no deletion (162 DMD and 34 BMD), 103 borders nor clear (91 DMD and 8 BMD) and 16 single exon deletion cases (all DMD). MLPA was done using the protocol recommended by the manufacturer as given in the Materials and Methods chapter.

Table 4.3: Details of the number of samples for which MLPA was done

Clinical diagnosis	No of cases	No deletion for MLPA			Borders not clear for MLPA/non contiguous		Single exon deletions	
		Del	No del	Not done	Borders identified	Not done	Confirmed	Not done
DMD	514	41	121	6	88/7	4/1	16	46
BMD	57	22	35	1	8/0	--	--	--

4.2.4 Validation of mPCR and MLPA results

The sizes of the mPCR products were validated by running the products along with 100bp DNA ladder. The neucleotide sizes of the products were calculated using the Quantity one software and verified. (Figure 4.2)

Validation of the mPCR results was done by testing samples from other institutes like SGPGIMS, Chandigarh in India and Centre for Human and Clinical Genetics, Leiden University Medical Centre. DNA samples of known DMD cases tested by this laboratory were sent to us for inter-lab comparison of method and the results correlated with ours. mPCR and MLPA was also validated using samples from Centre for Human and Clinical Genetics, Leiden University Medical Centre. All our results correlated with their results. (Table 4.4)

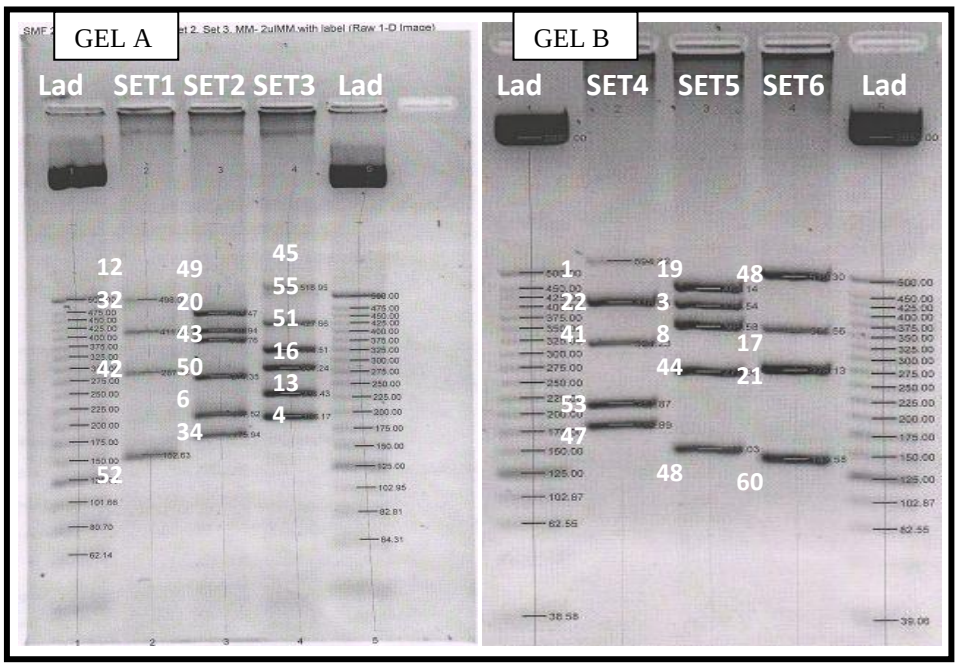

Figure 4.2: Validation of mPCR using DNA ladders. *The multiplex PCR products were run in 2% agarose gel along with the 100bp ladders on both the sides. The size of the products were calculated using the Quantity One (Biorad, USA) software to confirm the product sizes. Here the 6 set of mPCR products were run and validated.*

GEL A:

Lane 1 & 5: 100bp ladder

Lane 2: Exons 12, 32, 42 and 52

Lane 3: Exons 49, 29, 43, 50, 6 and34

Lane 4: Exons 4, 13, 16, 51, 55 and 45

GEL B:

Lane 1 & 5: 100bp ladder

Lane 2: Exons 47, 53, 41, 22 and 1

Lane 3: Exons 48, 44, 8, 3 and 19

Lane 4: Exons 60, 21, 17 and 48

Table 4.4: Results of the quality assurance samples from Leiden University Medical Centre.

Sample	Sex	mPCR results	MLPA results
1	M	Exons 22 - 44 Deleted	Not done
2	M	Exons 45 - 49 Deleted	Not done
3	M	Exons 45 - 48 Deleted	Not done
4	M	Exon 45 Deleted	Exon 45 Deleted
5	M	Exons 3 - 22 Deleted	Exons 3-29 Deleted
6	M	Exons 8 - 32 Deleted	Exons 8-32 Deleted
7	M	No deletion	Exons 13-17 Duplicated
8	M	No deletion	Exon 2 Duplicated
9	M	No deletion	Exons 2-30 Duplicated
10	M	No deletion	Exons 35-44 Duplicated
11	F	No deletion	Exons 48-52 Deleted
12	F	No deletion	Exons 30-44 Deleted
13	F	No deletion	Exons 8-12 Deleted
14	F	No deletion	Exons 10-44 Deleted
15	F	No deletion	No deletion/No duplication
16	F	No deletion	No deletion/No duplication
17	F	No deletion	Exons 31-41 Duplicated
18	F	No deletion	No deletion/No duplication
19	F	No deletion	No deletion/No duplication

MLPA was not done for samples which had a definitive diagnosis with mPCR.

4.2.5 Mutation screening by gene sequencing

DMD gene sequencing from genomic DNA was attempted for 4 cases which were confirmed to DMD with a muscle biopsy. Muscle biopsy was done by the clinicians before the patients could arrive at our facility for diagnosis. Immunohistochemistry results showing an absence of dystrophin protein were confirmed as DMD. DMD gene sequencing for all the 79 exons was attempted at Cochin Hospital, Paris in Dr. France Leturcq's lab. The method outlined in the materials and methods chapter was used.

Reading frame check

Framedness of the deletions and duplications obtained in our study were checked using the Reading frame checker in www.dmd.nl website. Entering the exonic borders of deletions/duplications in this checker gives the reading frame at the DNA level.

4.4 RESULTS:

The exons were first selected for mPCR were based on the Chamberlain's and Begg's panel, for 18-22 exons in the hot spot regions, which identifies approximately 95-98% of all DMD gene deletions. In most of the cases the assignment of framedness of the deletion becomes impossible due to the unclear borders of the deletion. This assigning of framedness becomes necessary to differentiate DMD from BMD. To enable us to have a better understanding of the framedness we decided to include a few more exons in the hot spot regions in our diagnostic protocol. This also helped us to limit the number of samples taken over for MLPA, which is a more expensive method. Based on this approach, we decided to perform multiplex PCR for 30 exons from the 5' and the central hot spot regions in as many as 6-7 sets. The exons selected for mPCR were exon 1, 3, 4, 6, 8, 12, 13, 16, 17, 19, 20, 21, 22, 32, 34, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 55, and 60. Different combinations of these exons were tried with different Taq Polymerase enzymes and different annealing temperatures. To make the diagnostic procedure easier it was attempted to have the annealing temperature for all the exons same so that a single PCR protocol would suffice, thus bringing down the turn around time. The method was standardized such that the PCR primer concentrations and the thermal cycler program were the same for all the exons tested. The separation of the products with 2% agarose gel was enough to separate the products in the multiplex PCR and did not require Poly acrylamide gel electrophoresis.

Of the 571 apparently unrelated males tested for DMD gene mutations, 514 were clinically diagnosed as DMD and 51 as BMD. Pedigree details are available for 469 cases, of which 76 (16.2%) had family history of DMD and 393 (83.8%) did not show a family history. For the rest 45 cases details on their family history was not available.

Age at onset was available for 450 cases of 514 received with a clinical diagnosis of DMD and the average was 5 years (range 1 to 20 years). Among these 332 cases (64.5%) showed an age of onset of 5 or below while for the rest 35.5% it was more than 6 years. For cases received with a clinical diagnosis of BMD, 50 of 57 cases had age of onset details and the average was 13.5 years (range 3 to 40 years). Of these 46 cases (80.7%) showed an age of onset of more than 6 years.

Creatine phosphokinase (CPK) is an enzyme present in the muscle tissue that catalyses the phosphorylation of creatine. The serum level of creatine, which reflects tissue catabolism due to cell trauma, is usually raised in D/BMD patients. We had CPK data available for 287 of 514 DMD cases and the mean CPK was 14885U/L (range 14 to 21,400 U/L). All cases except 6 showed values above the normal range. Among the BMD cases 31 of the 57 had CPK values, all of which were above normal range with a mean of 7300 U/L (range 309 to 34,000 U/L).

Among the DMD cases, status of ambulant/non-ambulant was not available for 63 of 514 cases at the time of diagnosis. Of these 322 were ambulant and 129 were non-ambulant at the time of diagnosis. Age at loss of ambulation data was available for 110 cases, where the average age at loss of ambulation was 9.35 years and 84 cases were non-ambulant before 10 years of age.

As a policy, we ask the clinicians not to recommend muscle biopsy for D/BMD patients before molecular diagnosis by mPCR/MLPA to avoid invasive procedures. But, some of the cases still come to us only after muscle biopsy done. Among the 70 DMD cases for which muscle biopsy was done, only for 10 cases immunohistochemistry for dystrophin was done. For the rest 60 cases the common diagnosis was suggestive of muscular dystrophy/myopathy or suggestive of DMD. For those which IHC was done, the results were varied and some were not consistent with the molecular diagnosis. For one case, B71, IHC results were suggestive of other MD and not DMD, but MLPA results showed exon 2 duplication. For another case, B488, IHC results were consistent with BMD, C terminal negative few fibers present, but the molecular analysis showed an out of frame deletion of exons 45 to 54 consistent with DMD. These results either point to the ineffectiveness of IHC methods in Indian centres to accurately diagnose DMD or an exception to the reading frame rule or post translational modifications.

Consanguinity is not a risk factor for X-linked recessive disorders. However, as part of our protocol we get details of consanguinity for cases received at our facility. Consanguinity was generally not very common in the DMD families in this study. Among the 465 cases for which details were available, only 75 cases (16%) were a product of consanguineous marriage. Among the BMD cases 13 of 57 cases (22.8%) were born of consanguineous marriages.

4.4.1 DMD gene deletion using mPCR

In this study DMD gene exon deletion was tested in all the cases using multiplex PCR for the proximal and central hot spot regions. Multiplex PCR was able to pick up deletions in 368 of the total 571 cases, which accounted for 65.7% of all cases.

Of the 514 DMD cases 346 (68.3%) showed a deletion by mPCR. There were 62 single exon deletions (16.5% of all mutation positive cases). Deletion of exons 44 and 45 accounted for more than 50 per cent of single exon deletions. Most (81.8%) of the deletions were confined to the central hot spot region between exons 44 and 55 (284 of 347 deletion cases). The most common of the deletions was that of exon 45 in 27 samples, followed by exons 45-52 deletion in 26 samples and 45-50 deletion in 25 samples. Almost 28% (96 of 347 cases) of the cases had exon 45 (or intron 44) as the deletion breakpoint. However, deletion of specific exons to assess the framedness was not clear for 96 cases (88 DMD and 8 BMD) and hence the reading frame rule could not be applied, using the mPCR results. Among the DMD cases for which the deletion of exons to apply the reading frame rule was clear, 240 showed out-of-frame deletions and 8 showed in-frame deletions and 7 were non-contiguous deletions whose framedness could not be assessed. MLPA was done for 84 of the 88 borders not clear cases, resulting in 74 out-of-frame and 10 in-frame cases. Among the 7 non-contiguous deletions MLPA was done for 6 cases, from which 5 turned out to be contiguous out-of-frame deletions and 2 were non-contiguous deletions.

Of the 57 cases that were diagnosed as BMD clinically, there were 22 samples which showed DMD gene deletion with mPCR and 35 cases showed no deletion. Of the 22 cases 14 were in-frame deletions and the rest 8 were without unclear borders of deletion. These may be exceptions to the reading frame rule, and need

to be studied further at the mRNA level and for the protein characteristics. The commonest breaking point at the 5' region was intron 44 (96 cases) and that in the 3' region was intron 51 (72 cases). The most frequently deleted exons were exon 49 and exon 50. The fact that our samples were primarily referred by pediatricians could be the reason for low numbers of BMD cases. Figure 4.3 summarizes the mPCR results of all the samples and table 4.5 shows the number of cases with a diagnosis with mPCR testing.

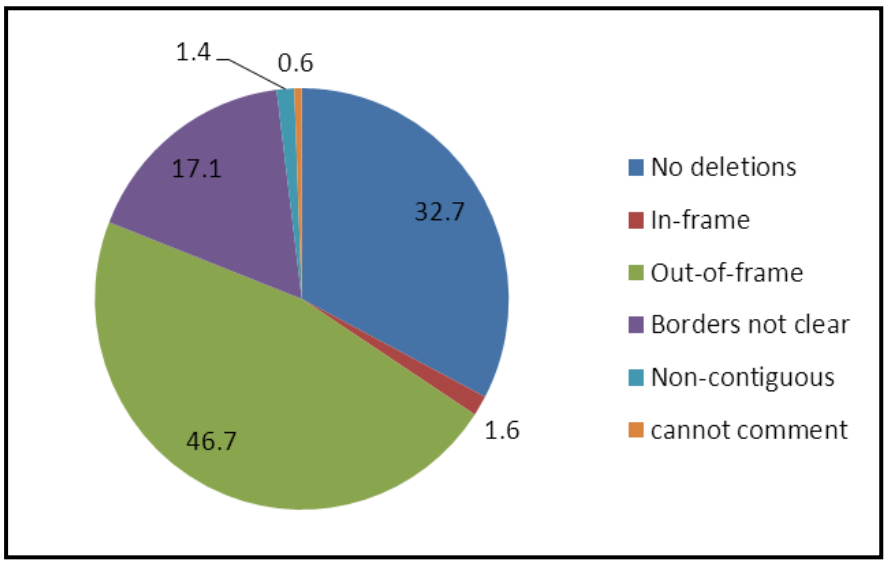

Figure 4.3: mPCR results showing distribution of deletions based on reading frame. *Only 46% of the total cases are fully benefitted by mPCR, whereas the rest of the cases require further analysis by downstream methods to obtain complete molecular diagnosis.*

Samples that showed no deletion, single exon deletion and deletion with borders not clear by mPCR were taken forward to do MLPA.

Table 4.5: Results of mPCR with respect to the framedness of the mutation in DMD (514 cases)/BMD (57 cases).

	DMD		BMD	
mPCR result	**No. of Cases**	**Percentage**	**No. of Cases**	**Percentage**
No deletions	168	32.7	35	61.4
In-frame	8	1.6	14	24.6
Out-of-frame	240	46.7	0	0.0
Borders not clear	88	17.1	8	14.0

Non-contiguous	7	1.4	0	0.0
cannot comment	3	0.6	0	0.0
Total	**514**		**57**	

4.4.2 DMD gene deletion/duplication analysis by MLPA

Multiplex ligation-dependent probe amplification (MLPA) is a method described a few years back, (Schouten, J. P. et al. 2002) which is now gaining importance due to its simplicity and efficiency to pick deletions and duplications in every exon of the DMD gene. It is now being used for several genes where copy number changes are known to be the commonest mutations. Internal QC standard deviation, a check of the data quality, was within range (<0.1) for all the samples. The deletion, normal and duplication DQs were within the given range and were interpreted accordingly.

A total of 306 cases qualified for MLPA from mPCR. Of the 306 cases, MLPA was done only for 294 cases. We were unable to conduct MLPA for 4 borders not clear, 1 non-contiguous and 7 no deletion cases by mPCR. Table 4.6 summarizes the number of samples taken for MLPA from mPCR based on mPCR results.

Table 4.6: Details of the number of samples analysed by MLPA based on mPCR results.

S.No	mPCR results	DMD	BMD	TOTAL
1	No deletion	162	34	**196**
2	Borders not clear	84	8	**92**
3	Non-contiguous	6	0	**6**
	TOTAL	**252**	**42**	**294**

4.4.2.1 MLPA Analysis of deletion-negative samples

A total of 168 DMD samples which showed no deletion by mPCR were available to be taken to the next step of molecular diagnosis, *viz* MLPA. MLPA was done for 162 of the 168 cases and mutations were picked in 41 samples (25% of no

deletion samples). Of these 41 samples, 32 (82%) showed duplications of single or multiple exons. The commonest duplication was that of exon 2 with 6 cases, followed by exon 3-7 (with 3 cases) and exons 8-11 (with 2 cases). Twenty three of the 41 samples (56%) having duplications had their origin in the proximal end of the DMD gene. Five of the 8 duplications have their origin at the 3' region of the gene. Duplications account for 5.3 per cent of all cases suspected to have DMD/BMD. Two of the duplications identified were complex rearrangements involving two separate regions of the DMD gene (Dup Ex 20 & 57 and Dup Ex 45-48 & 53-55). There was also a long duplication spreading from exon 11-40.

Nine samples showed deletion of exons, eight of which were single exon deletions and one multiple exon deletion. Six of the eight deletions missed by mPCR and picked by MLPA were at the distal end of the gene. Single exon deletions by MLPA should be confirmed by native PCR. The single exons picked by MLPA in our study were further tested for the exon deletion by exon specific PCR the results of which are tabulated in table 4.7.

Table 4.7: List of samples for which mPCR showed no deletion and MLPA showed single exon deletion.

S. No	Sample ID	mPCR results	MLPA results	PCR results	Sequencing required (Yes/No)
1	B185	No deletion	Exon 62 deleted	Exon 62 deleted	No
2	B211	No deletion	Exon 64 deleted	Exon 64 not deleted	**Yes**
3	B223	No deletion	Exon 32 deleted	Exon 32 not deleted	**Yes**
4	B287	No deletion	Exon 59 deleted	Exon 59 deleted	No
5	B522	No deletion	Exon 30 deleted	Exon 30 not deleted	**Yes**
6	B524	No deletion	Exon 52 deleted	Exon 52 deleted	No
7	B566	No deletion	Exon 5 deleted	NOT DONE	NOT DONE
8	B614	No deletion	Exon 54 deleted	Exon 54 deleted	No

Results of PCR for the single exons are also given along with which samples require exon screening for mutations.

4.4.2.2 DMD gene sequencing

DMD gene sequencing was attempted for one of the above 3 samples, for the particular exon which was deleted in MLPA. Other than this, we also attempted gene sequencing for 3 more samples where we screened the complete 79 exons of the DMD gene. We selected these samples based on the muscle immuno-

histochemistry results, which had already been done before coming to our lab for diagnosis. We took samples which were confirmed as DMD by muscle IHC results. Results of these samples are tabulated below in table 4.8.

Table 4.8: Results of DNA sequencing of samples showing single exon deletion by MLPA.

S.No	Sample ID	Exon sequenced	Sequence results	Figure number
1	B211	Exon 64	Sequencing result quality from forward and reverse primers were not enough to read the total exon sequence. Repeat sequencing with alternate set of primers required.	--
2	B223	Exon 32	Deletion of AA in exon 32 at position g955062-955063 (genomic DNA)/ c4471-4472 (cDNA)/ p1491 (amino acid), resulting in frameshift, which in turn results in stop codon TAA, 54 neucleotides and 19 codons downstream at exon 33. (c.4471_4472del)Mutation already reported.	Figure 4.4a
3	B522	Exon 3	Sequencing result quality from forward and reverse primers were not enough to read the total exon sequence. Repeat sequencing with alternate set of primers required.	--
4	B49	Exons 14 & 15	Substitution of T to C in exon 14 at position g770776 (genomic DNA)/ c1615 (cDNA)/ p539 (amino acid), resulting in stop codon TAG. Mutation already reported. (c.1615C>T)	Figure 4.4b
5	B97	Exon 23	Substitution of G to A in exon 23 at position g876037 (genomic DNA)/ c3087 (cDNA)/ p1029 (amino acid), resulting in stop codon TAG. Mutation already reported. (c.3087G>A)	Figure 4.4c
6	B108	Exon 62	Deletion of CAAA in exon 62 at position g2020992-2020995 (genomic DNA)/ c9204-9207 (cDNA)/ p3068-3069 (amino acid), resulting in frameshift, which in turn results in stop codon TGA, 56 neucleotides and 19 codons downstream at exon 63. (c.9204_9207del) Novel mutation.	Figure 4.4d

Of the 4 point mutations identified one (CAAA deletion in exon 62) was found to be a novel mutation, whereas the other three were already reported (www.dmd.nl database as accessed on September 2012).

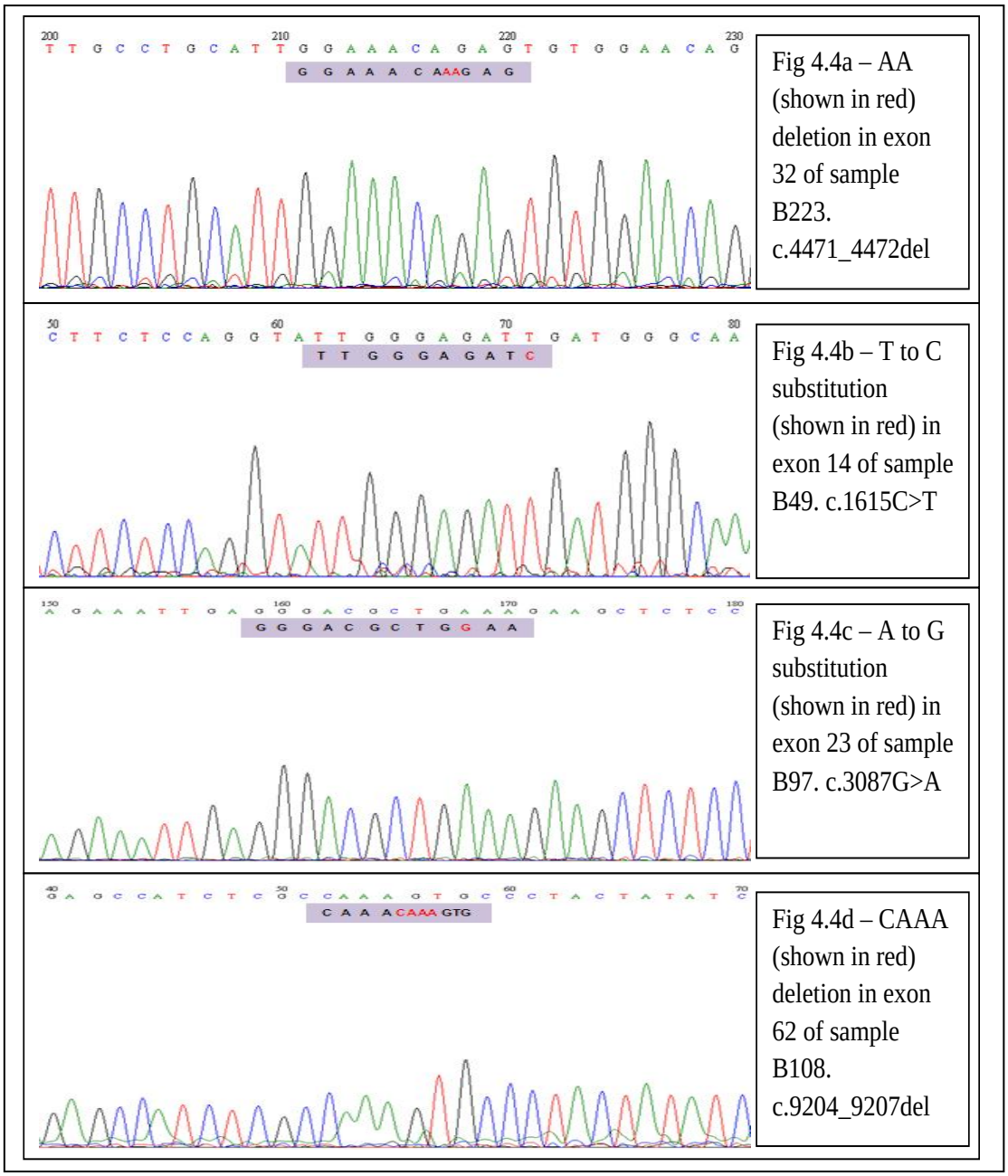

Figure 4.4: DNA sequencing results of single exon deletion cases by MLPA. *Shaded sequence denotes the reference sequence. The once shown in red are the deleted neucleotides as compared to the sample sequence.*

It can be seen that, single exon deletions in MLPA not picked by PCR mostly turns out to be due to a point mutation in the probe binding region of the exon. Among the cases clinically diagnosed as BMD, 34 cases showing no deletion by mPCR were taken for MLPA, of which only one case showed a duplication mutation (Exon 13 duplication). The rest of the cases showed no deletion.

4.4.2.3 Detection of deletion borders by MLPA: Among the deletions detected by mPCR, exon borders of the deletion were not clear for 88 DMD and 8 BMD cases. Of these, MLPA was done for 84 DMD and 8 BMD cases. Borders of all the 92 cases tested were confirmed by MLPA (Table 4.9). Of the 84 DMD cases, 74 turned out to be out-of-frame deletions and 10 were in-frame deletions. Among the BMD cases, 4 turned out to be in-frame and 4 were out-of-frame deletions. These results become important to check the framedness of the deletion and hence to attempt to understand the genotype-phenotype correlation. One of the deletions covering exons 10 to 62 (53 of the 79 exons deleted) is a novel deletion where 2/3rd of the exons are deleted spanning almost 1.38 Mb of the gene.

Six out of 7 non-contiguous deletions was also tested by MLPA. Four out of the seven cases turned out to be contiguous mutations, all of which were out-of-frame deletions (Table 4.9). This could be due to single neucleotide changes at primer binding sites of the introns in these particular samples, and could be confirmed with alternative primers for those exons.

Table 4.9: List of samples with unclear exon deletion borders by mPCR, which were analysed by MLPA.

S. No	Patient ID	Results of mPCR	Framedness (mPCR)	Results of MLPA	Framedness (MLPA)	Molecular diagnosis (based on framedness)
DUCHENNE MUSCULAR DYSTROPHY						
1	**B18**	**Exons 12-60 Deleted**	Borders not clear	**Exons 10-62 Deleted**	OUT-OF-FRAME	**DMD**
2	**B30**	**Exons 3-13 Deleted**	Borders not clear	**Exons 3-13 Deleted**	IN-FRAME	**BMD**
3	**B31**	**Exons 3-13 Deleted**	Borders not clear	**Exons 3-13 Deleted**	IN-FRAME	**BMD**
4	**B44/ O12**	**Exon 60 Deleted**	Borders not clear	**Exons 56-61 Deleted**	OUT-OF-FRAME	**DMD**
5	**B56**	**Exons 46-53 Deleted**	Borders not clear	**Exons 46-55 Deleted**	OUT-OF-FRAME	**DMD**
6	**B58**	**Exons 8-17 Deleted**	Borders not clear	**Exons 8-17 Deleted**	OUT-OF-FRAME	**DMD**
7	**B63**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-54 Deleted**	OUT-OF-FRAME	**DMD**
8	**B69**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-53 Deleted**	IN-FRAME	**BMD**
9	**B95**	**Exons 49-53**	Borders not	**Exons 49-53**	IN-FRAME	**BMD**

		Deleted	clear	**Deleted**		
10	**B107**	**Exon 55 Deleted**	Borders not clear	**Exon 55 Deleted**	OUT-OF-FRAME	**DMD**
11	**B123**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-54 Deleted**	OUT-OF-FRAME	**DMD**
12	**B125**	**Exons 46-55 Deleted**	Borders not clear	**Exons 46-55 Deleted**	OUT-OF-FRAME	**DMD**
13	**B126**	**Exons 41-45 Deleted**	Borders not clear	**Exons 35-45 Deleted**	OUT-OF-FRAME	**DMD**
14	**B149**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-54 Deleted**	OUT-OF-FRAME	**DMD**
15	**B156**	**Exons 51-53 Deleted**	Borders not clear	**Exons 51-54 Deleted**	OUT-OF-FRAME	**DMD**
16	**B167**	**Exons 3-41 Deleted**	Borders not clear	**Exons 3-41 Deleted**	IN-FRAME	**BMD**
17	**B169**	**Exons 41-43 Deleted**	Borders not clear	**Exons 38-43 Deleted**	OUT-OF-FRAME	**DMD**
18	**B173**	**Exons 53-55 Deleted**	Borders not clear	**Exons 53-55 Deleted**	OUT-OF-FRAME	**DMD**
19	**B187**	**Exons 46-55 Deleted**	Borders not clear	**Exons 46-55 Deleted**	OUT-OF-FRAME	**DMD**
20	**B191**	**Exons 19-22 Deleted**	Borders not clear	**Exons 18-26 Deleted**	OUT-OF-FRAME	**DMD**
21	**B195**	**Exons 34-45 Deleted**	Borders not clear	**Exons 33-45 Deleted**	OUT-OF-FRAME	**DMD**
22	**B197**	**Exons 3-6 Deleted**	Borders not clear	**Exons 3-7 Deleted**	OUT-OF-FRAME	**DMD**
23	**B202**	**Exons 19-22 Deleted**	Borders not clear	**Exons 18-29 Deleted**	OUT-OF-FRAME	**DMD**
24	**B209**	**Exons 3-4 Deleted**	Borders not clear	**Exons 3-4 Deleted**	IN-FRAME	**BMD**
25	**B212**	**Exons 52-53 Deleted**	Borders not clear	**Exons 52-54 Deleted**	OUT-OF-FRAME	**DMD**
26	**B225**	**Exons 51-53 Deleted**	Borders not clear	**Exons 51-53 Deleted**	OUT-OF-FRAME	**DMD**
27	**B232**	**Exons 6-22 Deleted**	Borders not clear	**Exons 5-27 Deleted**	IN-FRAME	**BMD**
28	**B261**	**Exons 3-6 Deleted**	Borders not clear	**Exons 3-7 Deleted**	OUT-OF-FRAME	**DMD**
29	**B265**	**Exon 8 Deleted**	Borders not clear	**Exons 8-9 Deleted**	OUT-OF-FRAME	**DMD**
30	**B266**	**Exon 8 Deleted**	Borders not clear	**Exons 8-9 Deleted**	OUT-OF-FRAME	**DMD**
31	**B268**	**Exons 3-8 Deleted**	Borders not clear	**Exons 3-11 Deleted**	OUT-OF-FRAME	**DMD**
32	**B305**	**Exons 3-8 Deleted**	Borders not clear	**Exons 2-11 Deleted**	OUT-OF-FRAME	**DMD**
33	**B307**	**Exons 51-53 Deleted**	Borders not clear	**Exons 51-53 Deleted**	OUT-OF-FRAME	**DMD**
34	**B333**	**Exons 53-55 Deleted**	Borders not clear	**Exons 53-55 Deleted**	OUT-OF-FRAME	**DMD**
35	**B344**	**Exons 48-53 Deleted**	Borders not clear	**Exons 48-54 Deleted**	OUT-OF-FRAME	**DMD**

36	**B350**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-54 Deleted**	OUT-OF-FRAME	**DMD**
37	**B356**	**Exons 3-8 Deleted**	Borders not clear	**Exons 3-11 Deleted**	OUT-OF-FRAME	**DMD**
38	**B358**	**Exons 8-34 Deleted**	Borders not clear	**Exons 8-36 Deleted**	OUT-OF-FRAME	**DMD**
39	**B363**	**Exons 3-6 Deleted**	Borders not clear	**Exons 3-7 deleted**	OUT-OF-FRAME	**DMD**
40	**B404**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-54 Deleted**	OUT-OF-FRAME	**DMD**
41	**B407**	**Exon 53 Deleted**	Borders not clear	**Exon 53 Deleted**	OUT-OF-FRAME	**DMD**
42	**B411**	**Exons 48-55 Deleted**	Borders not clear	**Exons 48-58 Dleted**	OUT-OF-FRAME	**DMD**
43	**B416**	**Exons 8-44 Deleted**	Borders not clear	**Exons 8-44 Deleted**	OUT-OF-FRAME	**DMD**
44	**B419**	**Exons 49-53 Deleted**	Borders not clear	**Exons 49-54 Deleted**	OUT-OF-FRAME	**DMD**
45	**B420**	**Exons 8-16 Deleted**	Borders not clear	**Exons 8-16 Deleted**	OUT-OF-FRAME	**DMD**
46	**B421**	**Exons 41-43 Deleted**	Borders not clear	**Exons 38-43 Deleted**	OUT-OF-FRAME	**DMD**
47	**B423**	**Exon 6 Deleted**	Borders not clear	**Exons 6-7 deleted**	OUT-OF-FRAME	**DMD**
48	**B432**	**Exons 48-55 Deleted**	Borders not clear	**Exons 48-54 Deleted**	OUT-OF-FRAME	**DMD**
49	**B433**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-54 Deleted**	OUT-OF-FRAME	**DMD**
50	**B441**	**Exons 3-13 Deleted**	Borders not clear	**Exons 2-13 Deleted**	OUT-OF-FRAME	**DMD**
51	**B443**	**Exons 41-43 Deleted**	Borders not clear	**Exons 39-43 Deleted**	OUT-OF-FRAME	**DMD**
52	**B447**	**Exons 51-53 Deleted**	Borders not clear	**Exons 51-54 Deleted**	OUT-OF-FRAME	**DMD**
53	**B455**	**Exon 53 Deleted**	Borders not clear	**Exons 53-54 Deleted**	OUT-OF-FRAME	**DMD**
54	**B462**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-54 Deleted**	OUT-OF-FRAME	**DMD**
55	**B475**	**Exon 53 Deleted**	Borders not clear	**Exons 53-54 Deleted**	OUT-OF-FRAME	**DMD**
56	**B488**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-54 Deleted**	OUT-OF-FRAME	**DMD**
57	**B489**	**Exons 51-53 Deleted**	Borders not clear	**Exons 51-53 Deleted**	OUT-OF-FRAME	**DMD**
58	**B494**	**Exons 32-44 Deleted**	Borders not clear	**Exons 28-44 Deleted**	IN-FRAME	**BMD**
59	**B500**	**Exons 12-47 Deleted**	Borders not clear	**Exons 10-47 Deleted**	IN-FRAME	**BMD**
60	**B515**	**Exons 51-53 Deleted**	Borders not clear	**Exons 51-54 Deleted**	OUT-OF-FRAME	**DMD**
61	**B519**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-52 Deleted**	OUT-OF-FRAME	**DMD**
62	**B526**	**Exons 48-53 Deleted**	Borders not clear	**Exons 48-54 Deleted**	OUT-OF-FRAME	**DMD**

63	**B542**	**Exons 19-20 Deleted**	Borders not clear	**Exons 18-20 Deleted**	OUT-OF-FRAME	**DMD**
64	**B588**	**Exon 8 Deleted**	Borders not clear	**Exons 8-9 Deleted**	OUT-OF-FRAME	**DMD**
65	**B611**	**Exons 51-53 Deleted**	Borders not clear	**Exons 51-53 Deleted**	OUT-OF-FRAME	**DMD**
66	**B620**	**Exons 41-52 Deleted**	Borders not clear	**Exons 35-52 Deleted**	OUT-OF-FRAME	**DMD**
67	**B622**	**Exons 8-22 Deleted**	Borders not clear	**Exons 8-22 Deleted**	OUT-OF-FRAME	**DMD**
68	**B636**	**Exons 19-21 Deleted**	Borders not clear	**Exons 18-21 Deleted**	OUT-OF-FRAME	**DMD**
69	**B641**	**Exon 19 Deleted**	Borders not clear	**Exons 18-19 Deleted**	OUT-OF-FRAME	**DMD**
70	**B652**	**Exons 12-34 Deleted**	Borders not clear	**Exons 12-34 Deleted**	OUT-OF-FRAME	**DMD**
71	**B666**	**Exons 46-55 Deleted**	Borders not clear	**Exons 46-55 Deleted**	OUT-OF-FRAME	**DMD**
72	**B675**	**Exons 48-53 Deleted**	Borders not clear	**Exons 48-54 Deleted**	OUT-OF-FRAME	**DMD**
73	**B682**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-54 Deleted**	OUT-OF-FRAME	**DMD**
74	**B684**	**Exons 8-44 Deleted**	Borders not clear	**Exons 8-44 Deleted**	OUT-OF-FRAME	**DMD**
75	**B693**	**Exons 12-17 Deleted**	Borders not clear	**Exons 10-17 Deleted**	OUT-OF-FRAME	**DMD**
76	**B726**	**Exons 19-22 Deleted**	Borders not clear	**Exons 18-26 Deleted**	OUT-OF-FRAME	**DMD**
77	**B742**	**Exon 19 Deleted**	Borders not clear	**Exon 19 Deleted**	OUT-OF-FRAME	**DMD**
78	**B743**	**Exons 51-53 deleted**	Borders not clear	**Exons 51-53 deleted**	OUT-OF-FRAME	**DMD**
79	**B760**	**Exons 51-53 deleted**	Borders not clear	**Exons 51-53 deleted**	OUT-OF-FRAME	**DMD**
80	**B766**	**Exons 8-12 Deleted**	Borders not clear	**Exons 8-12 Deleted**	OUT-OF-FRAME	**DMD**
81	**B772**	**Exons 3-22 Deleted**	Borders not clear	**Exons 3-28 Deleted**	IN-FRAME	**BMD**
82	**B777**	**Exons 48-53 Deleted**	Borders not clear	**Exons 48-54 Deleted**	OUT-OF-FRAME	**DMD**
83	**B803**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-54 Deleted**	OUT-OF-FRAME	**DMD**
84	**B811**	**Exons 8-34 Deleted**	Borders not clear	**Exons 8-37 Deleted**	OUT-OF-FRAME	**DMD**
85	**B286**	**Exons 45-46 & 48-50 Deleted**	**Non-contiguous**	**Exons 45-50 Deleted**	OUT-OF-FRAME	**DMD**
86	**B301**	**Exon 46 & Exons 48-51 Deleted**	**Non-contiguous**	**Exons 46-51 Deleted**	OUT-OF-FRAME	**DMD**
87	**B303**	**Exons 46, 48-52 & 55 Deleted**	**Non-contiguous**	**Exons 46-55 Deleted**	OUT-OF-FRAME	**DMD**
88	**B257**	**Exons 52, 55 & 60 Deleted**	**Non-contiguous**	**Exons 52-62 Deleted**	OUT-OF-FRAME	**DMD**

89	**B110**	**Exons 45-50 & 53 Deleted**	**Non-contiguous**	**Exons 45-50 & 53-54 Deleted**	**Non-contiguous**	**Cannot comment**
90	**B332**	**Exons 45-50 & 53 Deleted**	**Non-contiguous**	**Exons 45-50 & 53-54 Deleted**	**Non-contiguous**	**Cannot comment**
BECKER MUSCULAR DYSTROPHY						
91	**B29**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-53 Deleted**	IN-FRAME	**BMD**
92	**B414**	**Exons 3-6 Deleted**	Borders not clear	**Exons 3-7 deleted**	OUT-OF-FRAME	**DMD**
93	**B437**	**Exon 3 Deleted**	Borders not clear	**Exon 3 deleted**	IN-FRAME	**BMD**
94	**B546**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-53 Deleted**	IN-FRAME	**BMD**
95	**B570**	**Exons 3-4 Deleted**	Borders not clear	**Exons 3-7 Deleted**	OUT-OF-FRAME	**DMD**
96	**B663**	**Exons 3-8 Deleted**	Borders not clear	**Exons 3-11 Deleted**	OUT-OF-FRAME	**DMD**
97	**B733**	**Exons 45-53 Deleted**	Borders not clear	**Exons 45-53 Deleted**	IN-FRAME	**BMD**
98	**B734**	**Exons 48-53 Deleted**	Borders not clear	**Exons 48-54 Deleted**	OUT-OF-FRAME	**DMD**

Single exon deletions by mPCR were confirmed by MLPA on 21 samples. Twenty of these 21 samples showed the same deletion by MLPA. One sample which showed exon 60 deletions by mPCR showed exon 56-61 deletion by MLPA.

4.4.3 DMD/BMD Molecular diagnosis algorithm

The algorithm where MLPA followed mPCR resulting in whole exon deletion and duplication detection is the algorithm recommended by the Best Practice Guidelines. This will be followed by IHC and point mutation detection, to complete DMD diagnosis where almost 99% of the cases are diagnosed completely. Our approach to diagnosis stops at this point owing to inability to perform the downstream assays.

However, we were able to perform diagnostics for most of our cases till this point and below we have summarized the results. For convenience and to understand the cases which are exceptions to the reading frame rule, results of samples which were received with clinical diagnosis of DMD and BMD are given separately.

4.4.3.1 Query DMD samples

Table 4.10 shows the spectrum of mutations picked up in our study in patients with a clinical diagnosis of DMD, using mPCR and MLPA.

Table 4.10: Mutation spectrum of query DMD cases organized based on the number of exons deleted, the framedness, and the techniques used to detect with novel findings

Number of Exon deleted	Exons deleted/ Duplicated (n=382)	No of cases	Framedness	Technique used	Novel (Yes/ No)
1 exon deleted					
n=72	**Exon 17 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exon 19 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exon 3 Deleted	1	IN-FRAME	mPCR & MLPA	No
	Exon 43 Deleted	1	OUT-OF-FRAME	mPCR	No
	Exon 44 Deleted	11	OUT-OF-FRAME	mPCR	No
	Exon 45 deleted	27	OUT-OF-FRAME	mPCR	No
	Exon 5 Deleted	1	IN-FRAME	MLPA	No
	Exon 50 Deleted	9	OUT-OF-FRAME	mPCR	No
	Exon 51 Deleted	7	OUT-OF-FRAME	mPCR	No
	Exon 52 Deleted	8	OUT-OF-FRAME	mPCR	No
	Exon 53 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exon 54 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exon 55 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exon 59 Deleted	1	OUT-OF-FRAME	MLPA	Yes
	Exon 62 Deleted	1	OUT-OF-FRAME	MLPA	No
2 exons deleted					
n=50	**Exons 1-2 Deleted**	3	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 3-4 Deleted	1	IN-FRAME	mPCR & MLPA	No
	Exons 18-19 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 42-43 Deleted	4	OUT-OF-FRAME	mPCR	No
	Exons 45-46 Deleted	1	IN-FRAME	mPCR	No
	Exons 46-47 Deleted	18	OUT-OF-FRAME	mPCR	No
	Exons 49-50 Deleted	15	OUT-OF-FRAME	mPCR	No
	Exons 51-52 Deleted	1	IN-FRAME	mPCR	No
	Exons 53-54 Deleted	2	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 6-7 deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 8-9 Deleted	3	OUT-OF-FRAME	mPCR & MLPA	No
3 exons deleted					
n=43	**Exons 18-20 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 44-46 Deleted	1	OUT-OF-FRAME	mPCR	No
	Exons 45-47	2	IN-FRAME	mPCR	No

	Deleted				
	Exons 46-48 Deleted	7	OUT-OF-FRAME	mPCR	No
	Exons 48-50 Deleted	19	OUT-OF-FRAME	mPCR	No
	Exons 50-52 Deleted	4	OUT-OF-FRAME	mPCR	No
	Exons 51-53 Deleted	6	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 52-54 Deleted	1	OUT-OF-FRAME	mPCR	No
	Exons 53-55 Deleted	2	OUT-OF-FRAME	mPCR & MLPA	No
4 exons deleted					
n=20	**Exons 18-21 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 39-43 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 42-45 Deleted	1	OUT-OF-FRAME	mPCR	No
	Exons 46-49 Deleted	2	OUT-OF-FRAME	mPCR	No
	Exons 47-50 Deleted	4	OUT-OF-FRAME	mPCR	No
	Exons 48-51 Deleted	2	IN-FRAME	mPCR	No
	Exons 49-52 Deleted	5	OUT-OF-FRAME	mPCR	No
	Exons 50-53 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 51-54 Deleted	3	OUT-OF-FRAME	mPCR & MLPA	No
5 exons deleted					
n=34	**Exons 3-7 deleted**	3	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 8-12 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 46-50 Deleted	15	OUT-OF-FRAME	mPCR	No
	Exons 48-52 Deleted	13	OUT-OF-FRAME	mPCR	No
	Exons 51-55 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 49-53 Deleted	1	IN-FRAME	mPCR & MLPA	No
6 exons deleted					
n=39	**Exons 38-43 Deleted**	2	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 45-50 Deleted	26	OUT-OF-FRAME	mPCR	No
	Exons 46-51 Deleted	9	OUT-OF-FRAME	mPCR	No
	Exons 49-54 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No

	Exons 56-61 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	Yes
7 exons deleted					
n=12	**Exons 46-52 Deleted**	7	OUT-OF-FRAME	mPCR	No
	Exons 48-54 Deleted	5	OUT-OF-FRAME	mPCR & MLPA	No
8 exons deleted					
n=28	**Exons 10-17 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 45-52 Deleted	27	OUT-OF-FRAME	mPCR	No
9 exons deleted					
n=6	**Exons 18-26 Deleted**	2	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 3-11 Deleted	2	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 45-53 Deleted	1	IN-FRAME	mPCR & MLPA	No
	Exons 8-16 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
10 exons deleted					
n=18	**Exons 2-11 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 3-12 Deleted	1	IN-FRAME	mPCR & MLPA	No
	Exons 8-17 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 45-54 Deleted	10	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 46-55 Deleted	5	OUT-OF-FRAME	mPCR & MLPA	No
11 exons deleted					
n=5	**Exons 3-13 Deleted**	2	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 35-45 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 48-58 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 52-62 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
12 exons deleted					
n=2	**Exons 2-13 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 18-29 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
13 exons deleted					
n=2	**Exons 33-45 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	Yes
	Exons 62-74 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	Yes
15 exons deleted					
n=2	**Exons 3-17 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	No
	Exons 8-22 Deleted	1	OUT-OF-FRAME	mPCR & MLPA	No
17 exons deleted					
n=1	**Exons 28-44 Deleted**	1	IN-FRAME	mPCR & MLPA	No

18 exons deleted					
n=1	**Exons 35-52 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	Yes
23 exons deleted					
n=3	**Exons 12-34 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	Yes
	Exons 22-44 Deleted	1	OUT-OF-FRAME	mPCR	Yes
	Exons 5-27 Deleted	1	IN-FRAME	mPCR & MLPA	Yes
24 exons deleted					
n=1	**Exons 20-43 Deleted**	1	OUT-OF-FRAME	mPCR	No
25 exons deleted					
n=1	**Exons 20-44 Deleted**	1	OUT-OF-FRAME	mPCR	No
26 exons deleted					
n=1	**Exons 3-28 Deleted**	1	IN-FRAME	mPCR & MLPA	Yes
27 exons deleted					
n=1	**Exons 17-43 Deleted**	1	OUT-OF-FRAME	mPCR	No
29 exons deleted					
n=1	**Exons 8-36 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	yes
30 exons deleted					
n=1	**Exons 8-37 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	No
37 exons deleted					
n=2	**Exons 8-44 Deleted**	2	OUT-OF-FRAME	mPCR & MLPA	No
38 exons deleted					
n=1	**Exons 10-47 Deleted**	1	IN-FRAME	mPCR & MLPA	Yes
39 exons deleted					
n=1	**Exons 3-41 Deleted**	1	IN-FRAME	mPCR & MLPA	No
53 exons deleted					
n=1	**Exons 10-62 Deleted**	1	OUT-OF-FRAME	mPCR & MLPA	Yes
non-contiguous deletions					
n=3	**Exon 20 & Exons 45-52 Deleted**	1	Cannot comment	mPCR & MLPA	Yes
	Exons 45-50 & 53-54 Deleted	2	Cannot comment	mPCR & MLPA	Yes
DUPLICATIONS					
1 exon duplicated					
n=9	**Exon 2 Duplicated**	6	OUT-OF-FRAME	MLPA	No
	Exon 12 Duplicated	1	OUT-OF-FRAME	MLPA	No
	Exon 19 Duplicated	1	OUT-OF-FRAME	MLPA	No
	Exon 44	1	OUT-OF-FRAME	MLPA	No

	Duplicated				
2 exons duplicated					
n=2	**Exons 8-9 Duplicated**	1	OUT-OF-FRAME	MLPA	No
	Exons 61-62 Duplicated	1	OUT-OF-FRAME	MLPA	No
3 exons duplicated					
n=2	**Exons 60-62 Duplicated**	1	OUT-OF-FRAME	MLPA	No
	Exons 2-4 Duplicated	1	OUT-OF-FRAME	MLPA	No
4 exons duplicated					
n=4	**Exons 8-11 Duplicated**	2	OUT-OF-FRAME	MLPA	No
	Exons 61-64 Duplicated	1	OUT-OF-FRAME	MLPA	No
	Exons 64-67 Duplicated	1	OUT-OF-FRAME	MLPA	No
5 exons duplicated					
n=3	**Exons 3-7 Duplicated**	3	OUT-OF-FRAME	MLPA	No
7 exons duplicated					
n=1	**Exons 3-9 Duplicated**	1	IN-FRAME	MLPA	No
9 exons duplicated					
n=2	**Exons 8-16 Duplicated**	1	OUT-OF-FRAME	MLPA	No
	Exons 64-72 Duplicated	1	OUT-OF-FRAME	MLPA	Yes
10 exons duplicated					
n=2	**Exons 8-17 Duplicated**	1	OUT-OF-FRAME	MLPA	No
	Exons 3-12 duplicated	1	OUT-OF-FRAME	MLPA	No
13 exons duplicated					
n=1	**Exons 50-62 Duplicated**	1	OUT-OF-FRAME	MLPA	No
14 exons duplicated					
n=1	**Exons 3-16 Duplicated**	1	IN-FRAME	MLPA	No
30 exons duplicated					
n=1	**Exon 11-40 duplicated**	1	IN-FRAME	MLPA	Yes

Non-contiguous duplication					
n=4	**Exons 52-62 & 66-79 Duplicated**	1		MLPA	Yes
	Exons 45-48 & 53-55 Duplicated	1		MLPA	Yes
	Exons 3-9 & 18-44 Duplicated	1		MLPA	Yes
	Exons 20&57 duplicated	1		MLPA	Yes

Overall, MLPA was required to be done for 266 cases (51% of DMD), and was done for 252 cases (49% of all DMD). At the end of this study 127 cases (121 tested by mPCR & MLPA and 6 tested only by mPCR), which is 24.7% of all DMD cases, did not show any deletion or duplication and qualify for downstream analysis of DMD gene mutation detection like sequencing (Table 4.11).

Table 4.11: Grouping the number of cases based on molecular diagnosis and methods used.

S.No	mPCR/MLPA results	Number of cases	Methods used
1	Single/Multi exon deletions	341	mPCR &/or MLPA
2	Single or multi exon duplications	32	MLPA
3	Non-contiguous deletions	6	mPCR &/or MLPA
4	Borders not clear by mPCR (not confirmed by MLPA)	4	mPCR
5	Non-contiguous deletions (not confirmed by MLPA)	1	mPCR
6	No Deletions	118	mPCR & MLPA
7	No Deletions (not confirmed by MLPA)	6	mPCR
8	Small insertions/deletions/substitutions	4	Direct sequencing &/or MLPA

In this study, out of the 514 cases tested 352 cases (68.4%) showed whole exon deletions (of which 1 has been shown to have point mutation and two have to be tested and confirmed), 32 cases (6.2%) showed whole exon duplications (contiguous and con-contiguous). Two hundred and forty eight cases (48.4%) got a confirmatory diagnosis with mPCR, and MLPA was necessary to confirm diagnosis in 131 (25.5%) cases. MLPA was not done for 8 cases for which mPCR gave a diagnosis but with borders not confirmed.

Of the deletions 329 were out-of-frame, 17 were in-frame, 3 were cases where the framedness could not be identified due to the involvement of exon 1 in the deletion (Exons 1-2 deleted), three were non-contiguous and four were ones whose borders of deletion were not confirmed **(see table 4.10).** Figures 4.5 show some of the MLPA results obtained for the DMD cases.

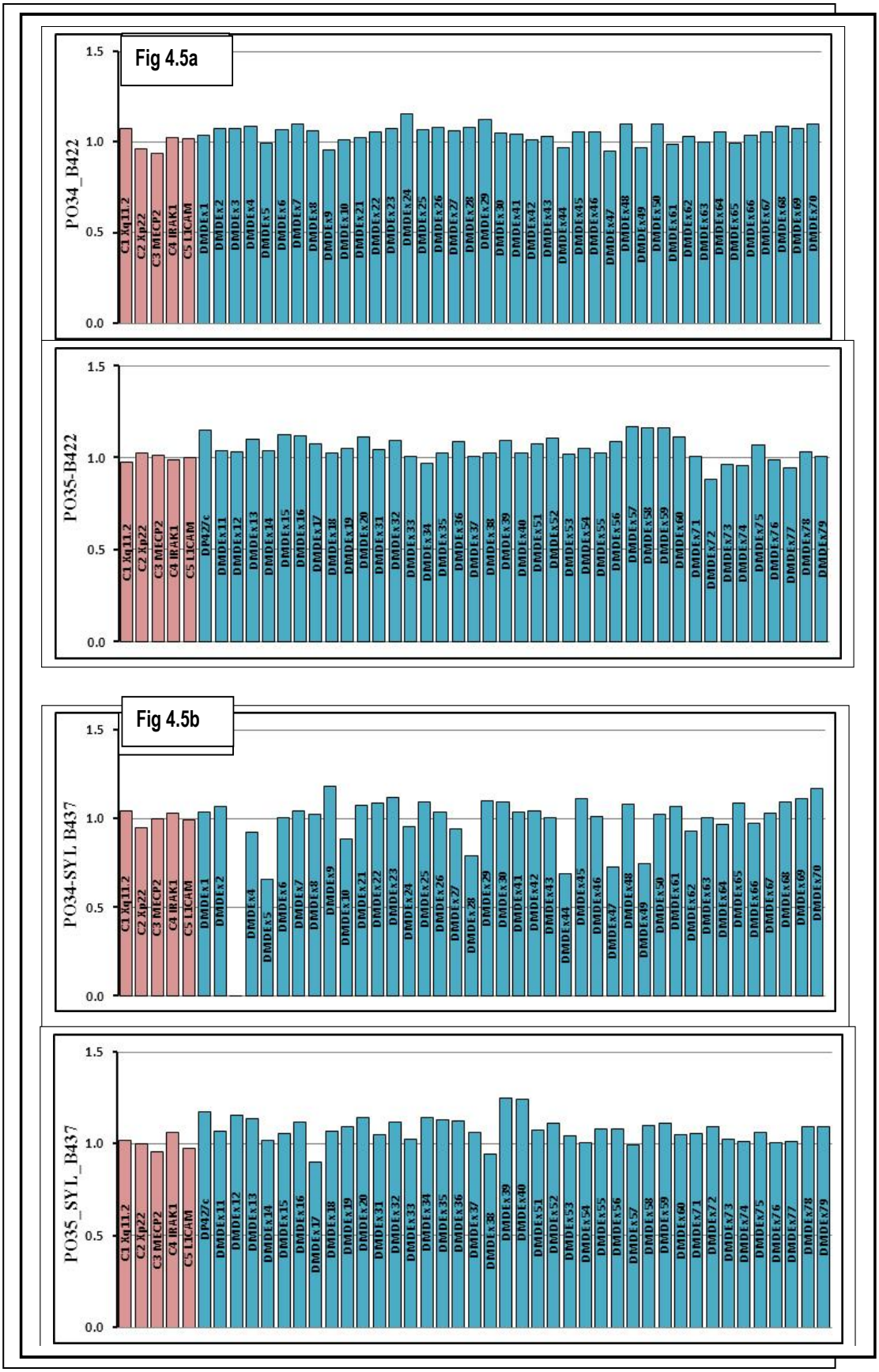
Fig 4.5a
PO34_B422
PO35-B422
Fig 4.5b
PO34-SYL_B437
PO35_SYL_B437
1.5
1.0
0.5
0.0
C1 Xq11.2
C2 Xp22
C3 MECP2
C4 IRAK1
C5 L1CAM
DP427c
DMDEx1
DMDEx2
DMDEx3
DMDEx4
DMDEx5
DMDEx6
DMDEx7
DMDEx8
DMDEx9
DMDEx10
DMDEx11
DMDEx12
DMDEx13
DMDEx14
DMDEx15
DMDEx16
DMDEx17
DMDEx18
DMDEx19
DMDEx20
DMDEx21
DMDEx22
DMDEx23
DMDEx24
DMDEx25
DMDEx26
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DMDEx28
DMDEx29
DMDEx30
DMDEx31
DMDEx32
DMDEx33
DMDEx34
DMDEx35
DMDEx36
DMDEx37
DMDEx38
DMDEx39
DMDEx40
DMDEx41
DMDEx42
DMDEx43
DMDEx44
DMDEx45
DMDEx46
DMDEx47
DMDEx48
DMDEx49
DMDEx50
DMDEx51
DMDEx52
DMDEx53
DMDEx54
DMDEx55
DMDEx56
DMDEx57
DMDEx58
DMDEx59
DMDEx60
DMDEx61
DMDEx62
DMDEx63
DMDEx64
DMDEx65
DMDEx66
DMDEx67
DMDEx68
DMDEx69
DMDEx70
DMDEx71
DMDEx72
DMDEx73
DMDEx74
DMDEx75
DMDEx76
DMDEx77
DMDEx78
DMDEx79

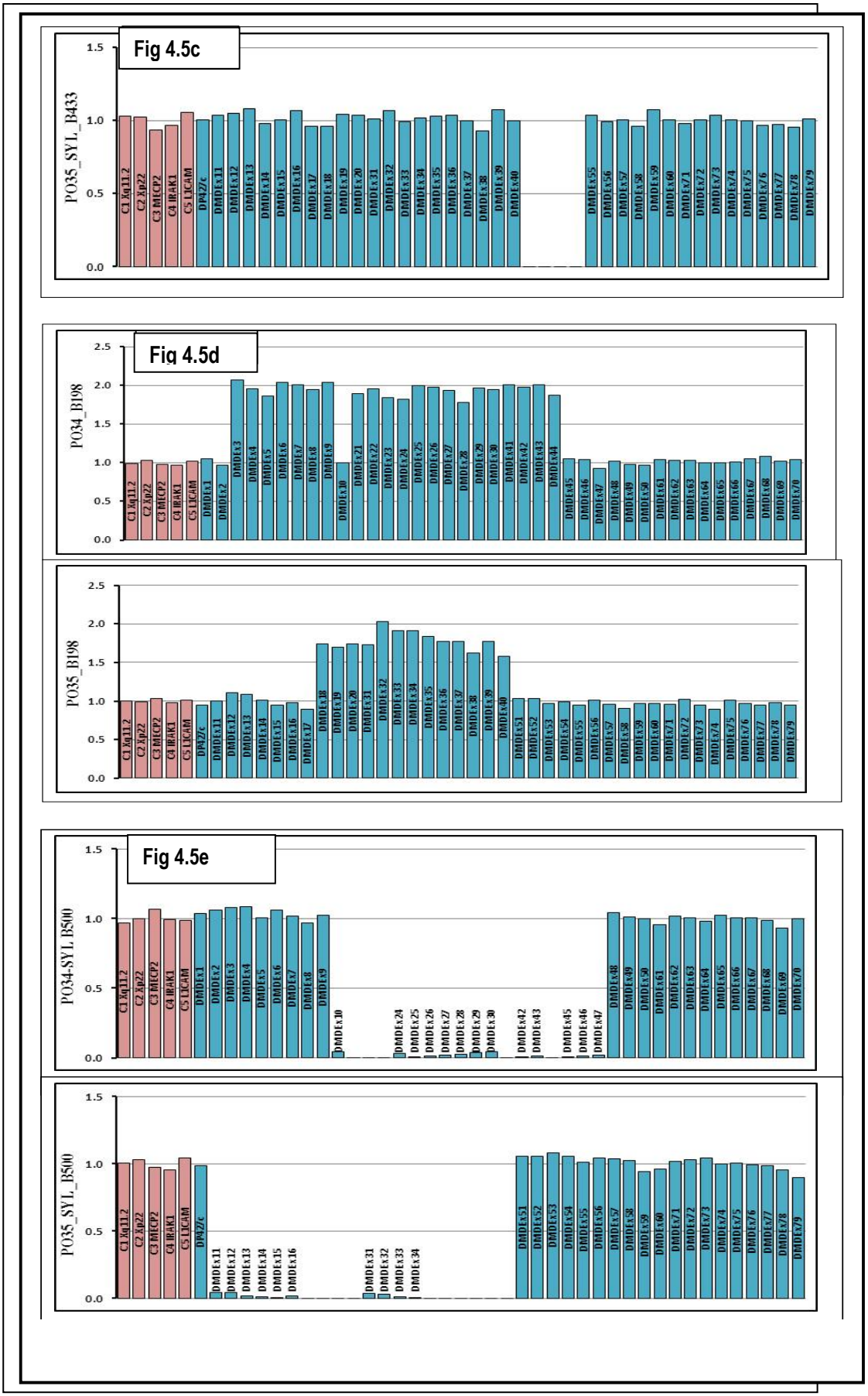


Fig 4.5c
PO35_SYL_B433
Fig 4.5d
PO34_B198
PO35_B198
Fig 4.5e
PO34-SYL B500
PO35_SYL_B500

Figure 4.5: Results of MLPA analysis on D/BMD samples

Figure 4.5a*: Sample showing no deletion and no duplication in the DMD gene exons. PO34 and PO35 probes are shown along with the control probes. The DQs of all the exons are the same and almost equal to 1.0.*

Figure 4.5b*: Figure showing a single exon deletion of exon 3, PO 34 probe showing the deletion and Probe PO35 showing no deletion.*

Figure 4.5c*: Representative figure showing a sample with exons 45-54 deleted, PO34 probe shows exons 45-50 deletion and PO35 probe shows exons 51-54 deletion.*

Figure 4.5d*: Representative figure showing noncontiguous duplication of exons 3-9 and 18- 44. Probe PO34 showing duplication of exons 3-9, 21-30 and 41-44 in probe PO34, and exons 18-20 and 31-40 in probe PO35*

Figure 4.5e*: Representative figure showing long contiguous deletion of exons 10-47. Probe PO34 showing deletion of exons 10, 21-30 and 41-47 and probe PO35 showing exons 11-20 and 31-40.*

4.4.3.2 Single exon deletions in DMD

Single exon deletions in the DMD gene accounted for 72 cases (14.0 %) (Figure 4.6).

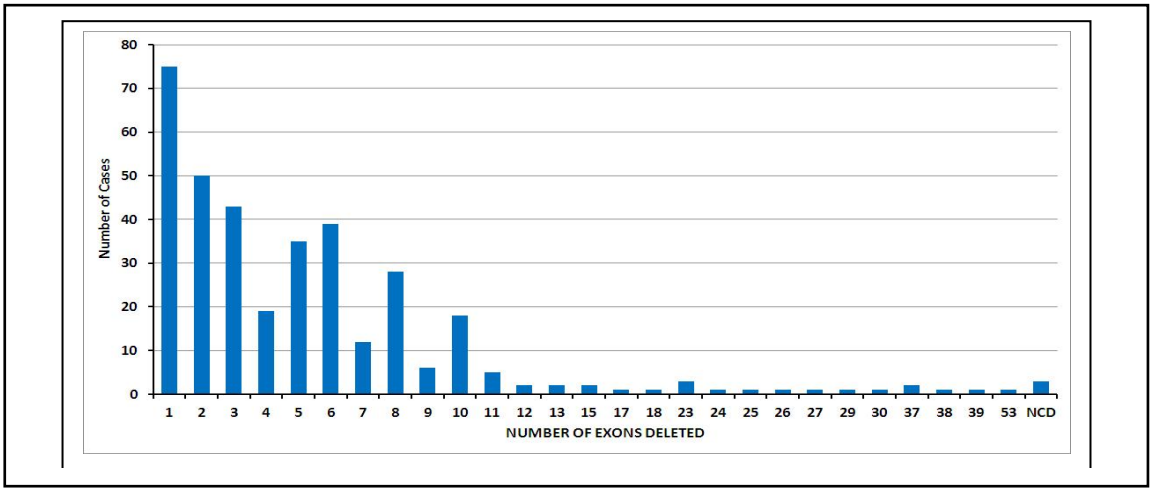


Figure 4.6: Dystrophin gene deletions in query DMD cases, arranged in terms of number of exons deleted. *The commonest deletions are single exon deletions. It can also be observed that deletions involving smaller number of*

exons are more in the study, at the same time there are 27 cases with more than 10 exons deleted. NCD refers to non-contiguous deletions.

Among the single exon deletion, the commonest deletion was exon 45 deletion, which was seen in 27 cases (36% of all single exon deletions). The other common single exon deletions are that of exons 44, 50, 51 and 52 (Figure 4.7). Most of these single exon deletions were picked by mPCR. However, exon 5, 30, 32, 59, 62, 64 and one sample each with exon 52 and 54 deletions were picked only by MLPA. To rule out point mutations at the probe binding site for these samples, PCR was done for those individual exons as described in materials and methods, the results are given earlier. The interpretation was based on the two results;

- If exon deletion is seen in both MLPA and PCR, this was considered to be a whole exon deletion (missed by mPCR since these exons were not in the mPCR panel).

- If the exon is deletion is MLPA and not deletion in PCR, this could be due to point mutations in the probe binding site, and sequencing is necessary to confirm the same.

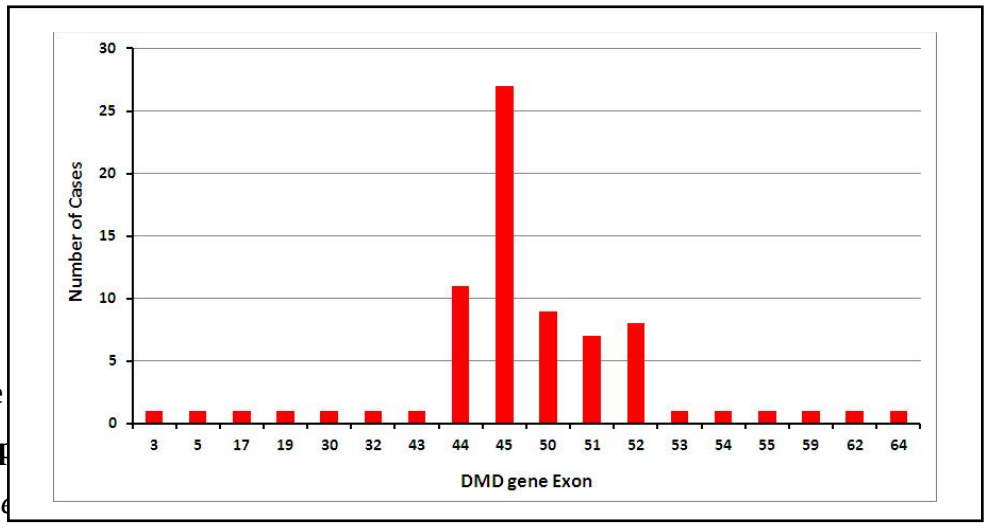


Figure
DMD p
the othe

4.4.3.3 Exon wise distribution of DMD deletions observed and duplications

It was that DMD deletions were spread throughout the gene covering most of the 79 exons except exons 76 to 79 (Figure 4.8). The exon wise distribution was comparable with the data published earlier, with the central hotspot region being the most affected. There were not many mutations to separate the proximal hot spot region with the other regions preceding the central hot spot region. Exon 50

was the most deletion exon (125 cases) followed by exons 49 down to 45. Deletions in the distal end of the DMD gene were very few.

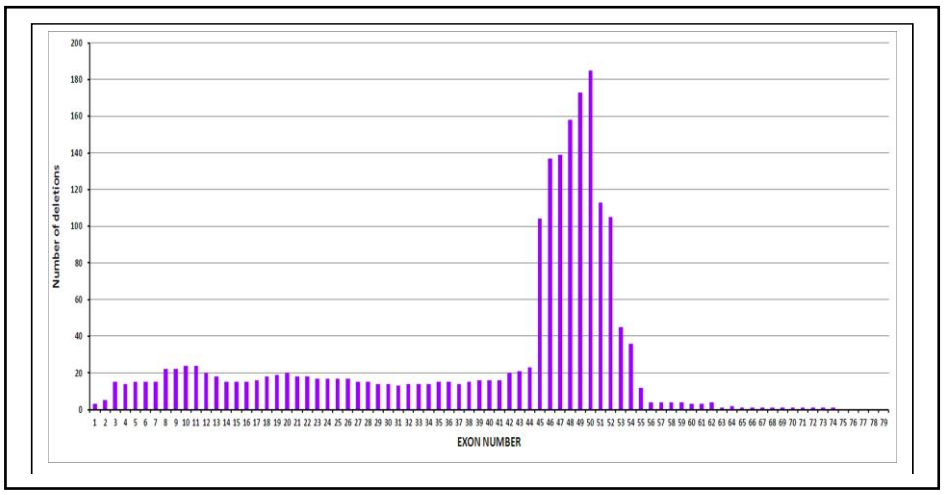


Figure 4.8: Exon wise distribution of deletions in dystrophin gene among query DMD patients. *The central hot spot region is the most deleted region in the DMD gene.*

Exon wise distribution of duplications was not similar to the deletions, and was spread throughout the gene with all except exon1 duplicated (Figure 4.9)

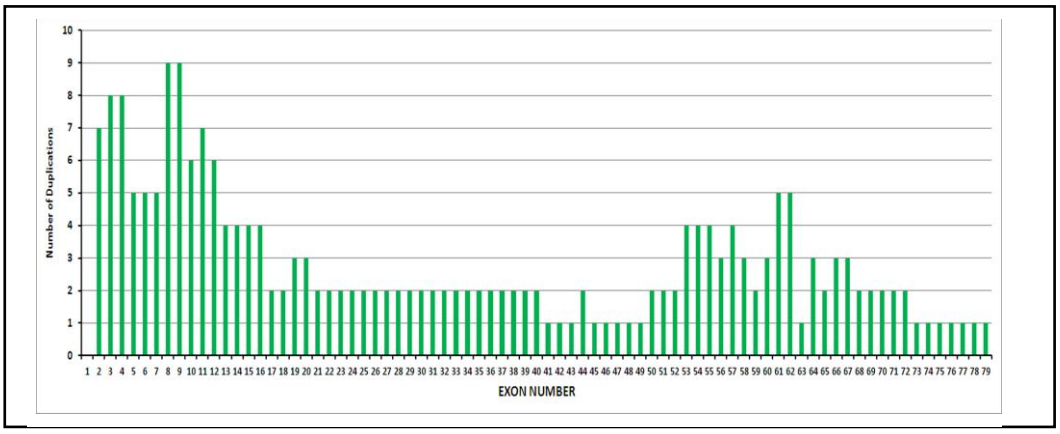


Figure 4.9: Exon wise distribution of duplications in dystrophin gene among query DMD patients. *The whole gene except exon 1 is involved and there are no hot spots for duplication.*

Single exon duplications contributed to 9 cases, of which exon 2 deletion was seen in 6 cases.

4.4.3.4 Non-contiguous mutations: In our study 4 non-contiguous mutations were picked up, which included 1 deletion and 2 duplications (Table 4.10). From

the clinical data available, the severity of the cases was checked based on the age at onset. It was seen that 2 cases, showing exons 45-50 and 53-54 deletion and exons 20 and 57 duplication had an age at onset of 8 yr. The other case having a double duplication of exons 45-48 and 53-55 had an age at onset of 3 ½ yr, suggesting a severe phenotype. Figure 4.5d shows a representative picture of MLPA analysis results of the samples showing non-contiguous mutations.

Overall mutation distribution in DMD patients in our study resembles other studies with a lot of mutations in the central hot spot region and proximal end of the DMD gene.

4.4.3.5 Novel mutations in the study: Twenty one novel mutations (not listed in the *www.dmd.nl* database as accessed in September 2012) identified in the study are listed in Table 4.12. Fourteen of them were deletions, 6 were duplications and one was a point mutation. MLPA was necessary for picking up all these mutations.

Table 4.12: **List of novel mutations identified in the study based on exon deletion and duplication.**

S. No	Exons deleted/ Duplicated (n=382)	No of cases	Framedness	Fragment deleted/ duplicated	Technique used
DELETIONS					
1	**Exons 3-28 Deleted**	1	IN-FRAME	c.94-?_3921+?del	mPCR & MLPA
2	**Exons 5-27 Deleted**	1	IN-FRAME	c.265-?_3786+?del	mPCR & MLPA
3	**Exons 8-36 Deleted**	1	OUT-OF-FRAME	c.650-?_5154+?del	mPCR & MLPA
4	**Exons 10-47 Deleted**	1	IN-FRAME	c.961-?_6912+?del	mPCR & MLPA
5	**Exons 10-62 Deleted**	1	OUT-OF-FRAME	c.961-?_9224+?del	mPCR & MLPA
6	**Exons 12-34 Deleted**	1	OUT-OF-FRAME	c.1332-?_4845+?del	mPCR & MLPA
7	**Exons 22-44 Deleted**	1	OUT-OF-FRAME	c.2804-?_6438+?del	mPCR
8	**Exons 33-45 Deleted**	1	OUT-OF-FRAME	c.4519-?_6614+?del	mPCR & MLPA
9	**Exons 35-52 Deleted**	1	OUT-OF-FRAME	c.4846-?_7660+?del	mPCR & MLPA
10	**Exons 56-61 Deleted**	1	OUT-OF-FRAME	c.8218-?_9163+?del	mPCR & MLPA
11	**Exon 59 Deleted**	1	OUT-OF-FRAME	c.8668-?_8937+?del	MLPA
12	**Exons 62-74 Deleted**	1	OUT-OF-FRAME	c.9164-?_10553+?del	mPCR & MLPA
13	**Exon 20 & Exons 45-52 Deleted**	1	Cannot Comment	c.2381-?_2622+?del; c.6439-?_7660+?del	mPCR & MLPA
14	**Exons 45-50 & 53-54 Deleted**	2	Cannot Comment	c.6439-?_7309+?del; c.7661-?_8027+?del	mPCR & MLPA
DUPLICATIONS					
15	**Exons 64-72 Duplicated**	1	OUT-OF-FRAME	c.9287-?_10329+?dup	MLPA
16	**Exon 11-40 duplicated**	1	IN-FRAME	c.1150-?_5739+?dup	MLPA
17	**Exons 52-62 & 66-79 Duplicated**	1	Cannot Comment	c.7543-?_9224+?dup; 9564-?_(*2691_?)dup	MLPA
18	**Exons 45-48 & 53-55 Duplicated**	1	Cannot Comment	c.6439-?_7098+?dup; c.7661-?_8217+?dup	MLPA
19	**Exons 3-9 & 18-44 Duplicated**	1	Cannot Comment	c.94-?_960+?dup; 2169-?_6438+?dup	MLPA
20	**Exons 20&57 duplicated**	1	Cannot Comment	c.2381-?_2622+?dup; c.8391-?_8547+?dup	MLPA

4.4.4 Samples with clinical diagnosis of Becker Muscular Dystrophy

A total of 57 cases were received at our facility with a clinical diagnosis of BMD. The number of cases was less since most of the clinicians sending samples to our center were pediatricians and pediatric neurologists. The spectrum of mutations among the BMD cases is tabulated in table 4.13.

Table 4.13: Spectrum of mutations detected in the DMD gene in query BMD patients.

Number of exons deleted/duplicated	Exons deleted/duplicated	No of cases	Framedness	Method used for diagnosis
1 exon deleted	Exon 3 deleted	**1**	IN-FRAME	mPCR & MLPA
3 exons deleted	Exons 45-47 Deleted	**8**	IN-FRAME	mPCR
4 exons deleted	Exons 45-48 Deleted	**4**	IN-FRAME	mPCR
5 exons deleted	Exons 45-49 Deleted	**2**	IN-FRAME	mPCR
	Exons 3-7 deleted	**2**	OUT-OF-FRAME	mPCR & MLPA
9 exons deleted	Exons 45-53 Deleted	**2**	IN-FRAME	mPCR & MLPA
	Exons 45-53 Deleted	**1**	BORDERS NOT CLEAR	mPCR
	Exons 3-11 Deleted	**1**	OUT-OF-FRAME	mPCR & MLPA
7 exons deleted	Exons 48-54 Deleted	**1**	OUT-OF-FRAME	mPCR & MLPA
DUPLICATIONS	Exon 13 duplicated	**1**	IN-FRAME	MLPA

Twenty three cases of the 57 (40.3%) had DMD gene mutations, 22 of them being deletions and 1 duplication. The commonest deletion was exon 45-47 deletion, which was seen in 8 cases. Seventeen cases showed in-frame deletions (77.2%) and 4 were out-of-frame deletions (18%). For one case whose deletion borders were not clear, MLPA was not done to confirm the borders.

Exon wise distribution showed only the proximal and central hot spot regions deleted, with exons 45 to 47 being deleted in most cases.

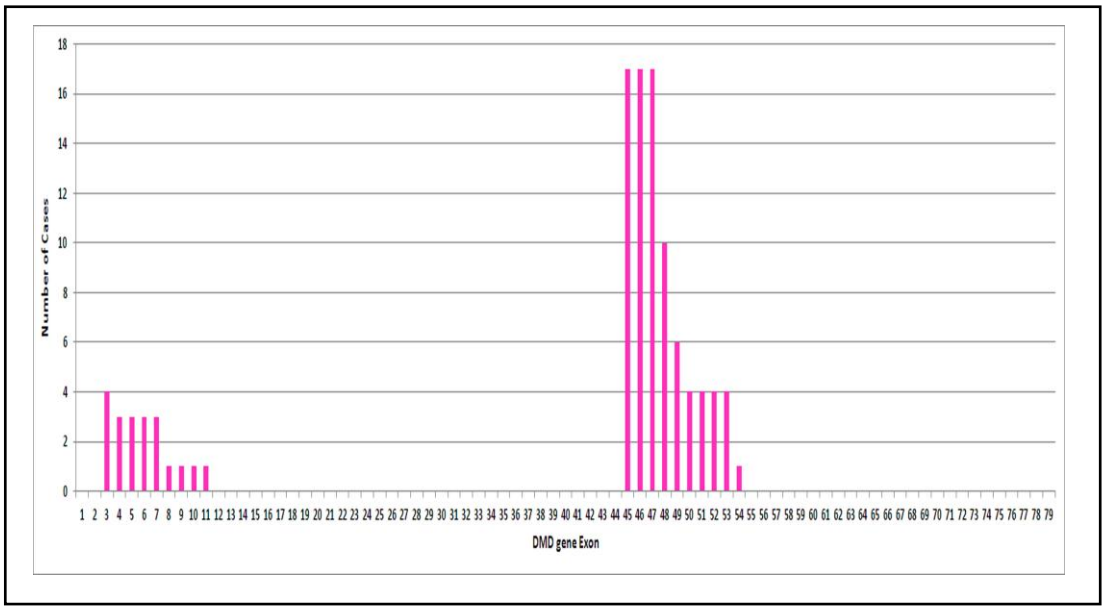


Figure 4.10: Exon wise distribution of dystrophin gene deletions among query BMD cases. *It can be observed that the proximal and central hot spot regions are the only ones affected.*

4.4.5 Exceptions to reading frame rule

Exceptions to the reading frame rule are reported to be seen in 10% of the cases. (Monaco, Bertelson et al. 1988) In our study in frame DMD gene deletions were seen in 18 cases the list of which can be seen in the table 4.14.

Table 4.14: **List of In Frame dystrophin gene deletions in query DMD cases and Out of Frame dystrophin gene deletions in query BMD cases**

S.No.	Sample ID	DMD exons deleted	Framedness	Age of onset of symptoms
1	**B2**	**Exons 48-51 Deleted**	IN-FRAME	Not available
2	**B7**	**Exons 3-12 Deleted**	IN-FRAME	2
3	**B45**	**Exons 48-51 Deleted**	IN-FRAME	Not available
4	**B46**	**Exons 51-52 Deleted**	IN-FRAME	Not available
5	**B347**	**Exons 45-47 Deleted**	IN-FRAME	15
6	**B707**	**Exons 45-46 Deleted**	IN-FRAME	8
7	**B804**	**Exon 3 Deleted**	IN-FRAME	5
8	**B813**	**Exons 45-47 Deleted**	IN-FRAME	6
9	**B30**	**Exons 3-13 Deleted**	IN-FRAME	Not available
10	**B31**	**Exons 3-13 Deleted**	IN-FRAME	Not available
11	**B69**	**Exons 45-53 Deleted**	IN-FRAME	6
12	**B95**	**Exons 49-53 Deleted**	IN-FRAME	6
13	**B167**	**Exons 3-41 Deleted**	IN-FRAME	Not available
14	**B209**	**Exons 3-4 Deleted**	IN-FRAME	5
15	**B232**	**Exons 5-27 Deleted**	IN-FRAME	4
16	**B494**	**Exons 28-44 Deleted**	IN-FRAME	2
17	**B500**	**Exons 10-47 Deleted**	IN-FRAME	4
18	**B772**	**Exons 3-28 Deleted**	IN-FRAME	3
Out of Frame dystrophin gene deletions in query BMD cases.				
19	**B414**	**Exons 3-7 deleted**	OUT-OF-FRAME	4
20	**B570**	**Exons 3-7 Deleted**	OUT-OF-FRAME	11
21	**B663**	**Exons 3-11 Deleted**	OUT-OF-FRAME	7
22	**B734**	**Exons 48-54 Deleted**	OUT-OF-FRAME	5

Among the in frame mutations, only exon 3-13 deletion and 45-47 deletion are seen in two samples each, whereas the rest of the mutations are varied. But comparing the data with the age of onset of symptoms, which was available for 12 cases, it was seen that in 7 cases (58%) the age of onset was below 5 years which is usual for DMD, and for 5 cases it was above 5 years (42%). Only in one of the cases the age of onset was above 10 years (B347- exons 45-47), which could be just a BMD case and misdiagnosed as DMD. Ten cases have an age of

onset below or equal to 6 years, and can be considered DMD. The seven cases with in frame deletion and showing early onset of disease are exceptions to the reading frame rule, and the mechanism for this needs to be examined in detail.

Among the 4 BMD cases with out of frame deletions, 2 showed exons 3-7 deletion and one 3-11 and 48-54 deletion (see table 4.14). The age of onset of two of those are less than 5 and don't obey the reading frame rule.

4.5 DISCUSSION

Duchenne and Becker, muscular dystrophies are X-linked recessive allelic disorders predominantly affecting the males. It is caused due to mutations in the DMD gene which codes for the dystrophin protein. Dystrophin is a cytoskeletal protein of muscle fibres loss of which in humans leads to severe muscle wasting disorder that is inevitably fatal. Dystrophin binds to cytoskeletal F-actin by its NH_2-terminal and to dystroglycan by its COOH-terminal (Grady et al., 1997). Lack of dystrophin in muscle fibres creates a mechanically weakened sarcolemma that becomes susceptible to focal tears on contractile activity (Petrof et al., 1993). Deletions, duplications and point mutations have been reported in the DMD gene, with deletions contributing to 67% of all mutations, duplications 5-7% and the rest by small insertions, deletions and substitutions. Deletions are heterogeneous with respect to size and location. Two deletion rich regions have been identified in the dystrophin gene encompassing 79 exons. The proximal hot spot region covers exons 1-20 and the more active central hot spot region covering exons 40-55. These two regions also represent major meiotic recombination hot spots. (Koenig et al., 1989; Koenig and Kunkel, 1990)

Molecular testing is becoming the primary diagnostic method for DMD and BMD (Yan et al., 2004; Prior and Bridgeman, 2005). Molecular diagnosis has relied on testing for deletions and duplications in the *DMD* gene with a reported sensitivity of detecting 65–70% of all mutations in the gene. With advances in DNA sequencing technology, it is now also possible to offer point mutation analysis of exons and intron/exon boundaries in the *DMD* gene as a clinical diagnostic laboratory service. (Flanigan, 2003) Due to the burden arising from the effort required to perform comprehensive mutation analysis of the large *DMD* gene and the increased demand for this testing, there is a need to assess how to best utilize comprehensive *DMD* gene testing to maximize clinically useful results.

The Best Practice Guidelines meet was held in Naarden, The Netherlands on November 14–16, 2008, to establish consensus Best Practice Guidelines for molecular diagnosis of Duchenne and Becker muscular dystrophy. The meet was

hosted by European Neuro-Muscular Centre (ENMC, www.enmc,org) which is is an international platform organization supporting research for neuromuscular disorders and strives to facilitate communication amongst scientists and clinicians working in the area of neuromuscular disease. The main route whereby ENMC facilitates communication and collaboration is via the funding and organisation of workshops with regard to a range of neuromuscular diseases including Spinal Muscular Atrophies, Duchenne Muscular Dystrophy, Congenital Muscular Dystrophies, CIDP and many others.

Dr.Lakshmi, my guide and Director of Molecular diagnostic facility also attended the meet representing India. During the discussion meet it was first put forward that MLPA will be the first line of testing for DMD gene mutations. But Dr.Lakshmi observed that MLPA testing is expensive when compared to mPCR. And for a developing country like ours where many could not afford molecular testing mPCR testing, which picks up more than 60% of DMD gene mutations, is the right choice as the first test. This was accepted by the panel and the guideline now has mPCR as the first test for DMD gene mutation analysis followed by MLPA (Abbs et al., 2010).

Based on this, we set out to develop a strategy for molecular testing for diagnosis of DMD and BMD using data from analysis of deletions, and duplications in the *DMD* gene on a group of 571 males referred for molecular testing over a 3-year period. In this study presented here, 514 DMD and 57 BMD patients from various regions of South India were selected for DMD gene mutation analysis.

The clinical diagnosis was done by the clinician who referred us the patients, and the signs and symptoms were typical of DMD/BMD. Once the patients are at our facility we take details on the clinical history like;

- Age at onset of symptoms
- H/O difficulty in walking
- H/O difficulty in climbing stairs
- H/O difficulty getting up from supine position
- H/O frequent falls
- Presence of calf pseudohypertrophy

- Presence of Gower's sign
- Ambulant status
- If non-ambulant, age at loss of ambulant status

After this we take details of family history followed by a detailed pedigree.

Majority of the children with a clinical diagnosis of DMD had the first symptoms of disease before the age of 5 years and the average age at loss of ambulation was 9.5 years. Among those with a clinical diagnosis of BMD, majority had an age at onset of symptoms after 6 years of age. Elevated CPK levels were found in most of the cases with the average being 14,885 IU/L. We have tested our systematic approach of using mPCR followed by MLPA as a diagnostic tool to precisely detect deletions and duplications and in some cases point mutations, in the coding region of the DMD gene and also suggested the overall algorithm towards DMD gene analysis. The above attempt is mainly to ensure that an invasive method like a muscle biopsy need not be the first step to confirm the clinical diagnosis.

Of the 571 males tested in this study, 514 had been referred for molecular testing as query DMD and 57 as query BMD. (Table 4.2) As indicated in results, we were able to identify a mutation in 388 of the 514 patients referred as query DMD (75.2%), (Table 4.11) but identified a mutation in only 23 out of the 57 (40.3%) patients referred as query BMD. (Table 4.13) Using the referral and mutation information from this group of patients, a strategy that includes clinical molecular diagnostic testing for DMB/BMD is outlined in figure 4.11. Initial serum creatine phosphokinase kinase analysis would first be useful to differentiate query DMD and BMD patients. For all referrals, regardless of DMD or BMD clinical presentation, deletion analysis should be performed as the initial molecular test using mPCR to test the hot spot exons due to the relatively common occurrence of single-exon deletions. The test sensitivity in our cohort for deletion analysis by mPCR was approximately 67.3% (346/514) for query DMD patients and 38.6% (22/57) for query BMD patients.

Multiplex PCR was able to offer diagnosis for 65.7% of all cases (DMD and BMD), and 67.3% of cases with a diagnosis of DMD, which goes well with earlier published results (Table 4.15). In our study among the DMD cases 18% of the deletions were seen in the proximal region and 16% between exons 21 to 40,

65% in the central hot spot region and 2% in exons 56-79. Deletions in the dystrophin gene contribute more that 60% of the DMD gene. Not only distribution of intronic deletion breakpoints defers significantly among the regional groups, their incidence also varies in different ethnic population (Mioni et al., 1994; Shomrat et al., 1994) (Table 4.16). Many studies in the American and European populations have detected two thirds of the deletions in the central hot spot region of the Dystrophin gene encompassing exons 44-52. In studies from USA mutant alleles with gene deletions are reported in 55-70% of all DMD/BMD cases (Darras et al., 1988). Table 4.15 represents the data on dystrophin gene deletions from various world populations and table 4.16 gives the distribution in terms of proximal and central hot spot regions. It was observed that our data was consistent with most studies in India and other world populations. (Singh et al., 1997; Mallikarjuna Rao et al., 2003)

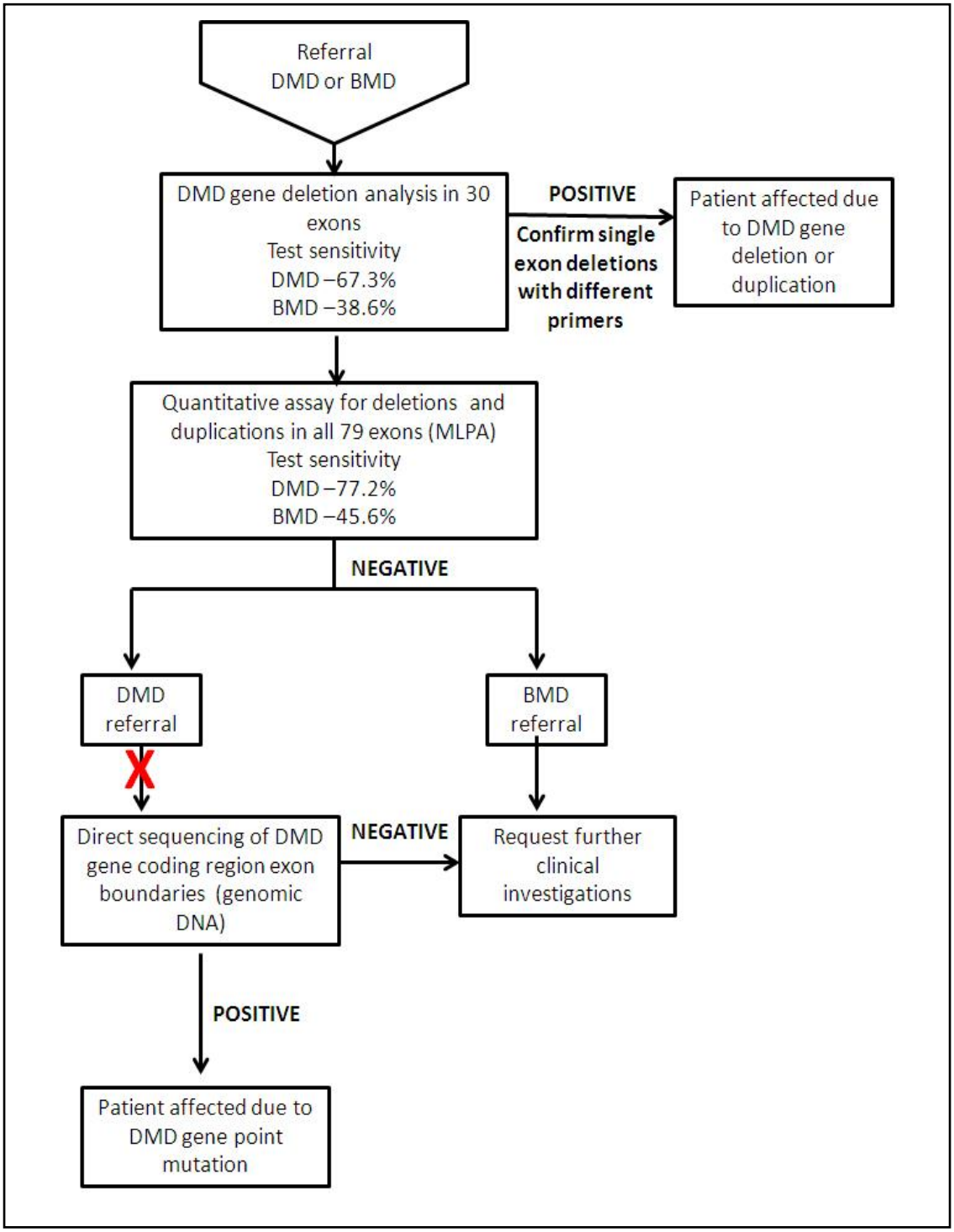


Figure 4.11: Diagnostic strategy used for molecular diagnostic testing of D/BMD used in our study. *Our study stopped with deletion and duplication detection, not extending into point mutation detection strategies (Marked with a red cross).*

Table 4.15: Comparison of data on dystrophin gene deletion in DMD patients from various world populations

Populations		Deletion %	References
North America		55-70 %	(Koenig et al., 1987; Liechti-Gallati et al., 1989)
South America	Venezuela	37 %	(Delgado Luengo et al., 1994)
	Mexico	52 %	(Coral-Vazquez et al., 1993)
Europe	Spain	45.5 %	(Patino et al., 1995)
	Estonia	48 %	(Talkop et al., 1999)
	Czeck Republic	50 %	(Speer et al., 1990)
	German Democratic Republic	39 %	(Speer et al., 1990)
	Hungary	39 %	(Speer et al., 1990)
	Croatia	50 %	(Sertic et al., 1997)
	United Kingdom	43 %	(Roddie and Bundey, 1992)
	Greece	63.3 %	(Florentin et al., 1995)
	Italy	82 %	(Mioni et al., 1994)
Asia / Middle East	Arabs (Kuwaiti and Egyptians)	86 %	(Haider et al., 1998)
	Saudi Arabia	63 %	(Al-Jumah et al., 2002)
	Egypt	55 %	(Effat et al., 2000)
	Israel	37 %	(Shomrat et al., 1994)
	Turkey	52- 58 %	(Dincer et al., 1996)
	Japan	33 – 60 %	(Katayama, Takeshita et al. 1993)
	Russia	23.2 – 41 %	(Baranov, Gorbunova et al. 1993)
	China	37 – 52 %	(Soong et al., 1991; Yuge et al., 1999)
	Singapore	58 – 61 %	(Lai et al., 1992; Low et al., 1996)
	Thailand	55 %	(Mutirangura et al., 1995)
	Philippines	33 %	(Cutiongco et al., 1995)
	South Africa	42 %	(Ballo et al., 1994)
	India	**61 – 73 %**	**(Singh et al., 1997; Mallikarjuna Rao et al., 2003)**
	Srilanka	62.5 %	(Welihinda et al., 1993)
	Pakistan	65 %	(Hassan et al., 2008)

Table 4.16: Comparison of data on deletions in proximal and central hotspot regions of dystrophin gene in DMD patients from various world populations

Populations		Total % of deletions	Deletion % in proximal region	Deletion % in Central Region	References
North America		55 - 70	37	63	(Koenig et al., 1987; Liechti-Gallati et al., 1989)
South America	Venezuela	37	23	77	(Delgado Luengo et al., 1994)
	Mexico	52	13	87	(Coral-Vazquez et al., 1993)
Europe	Estonia	48	18	82	(Talkop et al., 1999)
	Bulgaria	67.8	33	67	(Danieli et al., 1993)
	Czeck Republic	50	00	100	(Speer et al., 1990)
	United Kingdom	50	25	76	(Roddie and Bundey, 1992)
	Italy	82	20	80	(Mioni et al., 1994)
Asia / Middle East	Arabs (Kuwaiti and Egyptians)	86	08	50	(Haider et al., 1998; Effat et al., 2000)
	Israel	37	22	78	(Shomrat et al., 1994)
	Turkey	47 - 58	10.8	89.2	(Erdem et al., 1993; Dincer et al., 1996; Onengut et al., 2000)
	Japan	33 – 60.5	30	70	(Sakuraba, Ishii et al. 1991; Katayama, Takeshita et al. 1993)
	Russia	23.2 - 41	6.3	93.7	(Baranov et al., 1993)
	China (218)	37 - 52	33.6	66.4	(Soong et al., 1991)
	Singapore	40 - 61	38.1	61.9	(Lai et al., 1992; Low et al., 1996)
	Thailand	55	20	80	(Mutirangura et al., 1995)
	Vietname	32.4	27.3	72.7	(Lai et al., 2002)
	India	61 - 73	30.3	69.7	(Singh et al., 1997; Mallikarjuna Rao et al., 2003)
	Pakistan	42.16	41.02	58.97	(Hassan et al., 2008)

Multiplex PCR methods allow the detection of approximately 98 per cent of deletions, which accounts for 65 per cent of all mutations (Chamberlain et al., 1988; Beggs et al., 1990). In our study, though mPCR was able to pick up deletions in 65.7 per cent of all the cases, confirmed molecular diagnosis was achieved only in 265 cases (46.4%, 251 DMD [48.8%] and 14 BMD [24.5%]), due to the fact that precise molecular diagnosis is arrived only if deletions where exon borders are clear and single exon deletions are confirmed. Results from our mPCR method indicated that the commonly used Beggs–Chamberlain exon primer sets would detect 98% of the deletions as reported earlier (Beggs et al., 1990). However, the exon boundaries of the deletion, and hence the framedness of the deletion will not be known for of the deletions. Whereas, our mPCR could detect exon border of deletions for 265 (46.4%) in query DMD and BMD cases, with the Beggs–Chamberlain exon primer sets, only 79 cases (13.9%) would have had clear exonic borders. It is clear that assays commonly done for the hot spot regions do not analyze enough exons of the gene and hence have a lower sensitivity than the mPCR assay for 30 exons developed and followed at our facility.

Mutations affecting the open reading frame, due to frameshift, generate truncated non-functional dystrophin protein giving rise to severe DMD phenotype. However, mutations not affecting the open reading frame may produce a semi-functional dystrophin protein and usually correlate with mild phenotypes (Muntoni et al., 2003). In order to assess the reading frame, it is usually necessary to screen the entire gene for determination of the exons involved in deletion or duplication. Quantitative analysis of the entire *DMD* gene is also required to detect the relatively common single-exon deletions, duplications and exonic boundaries of deletions. Though Southern blot analysis was for long the method of choice to establish the exact breakpoints (Ashton et al., 2008), and since this method was time-consuming and cumbersome, the two recent methods, *i.e.*, MAPH (Trimarco et al., 2008) and MLPA (Schwartz and Duno, 2004; Gatta et al., 2005; Janssen et al., 2005), has greatly simplified this analysis, where MLPA has become the method of choice. The exact exon boundaries of the 88 samples that showed unclear borders by mPCR were confirmed by MLPA, the results of which are tabulated in Table 4.9.

MLPA on our cohort has revealed 42 new mutations (7.35%) not picked by mPCR, 41 in query DMD (7.97%) and 1 in query BMD cases (1.75%). Further, MLPA was able to identify exon boundaries for all the cases where it was necessary. Overall, MLPA aided to accurately diagnose 140 of the 571 cases (24.5%). Most of them were (32 cases) were whole exon duplications. While White *et al* had described close to 87 per cent of duplications in deletion negative / point mutation negative cases, (White et al., 2002) we found duplications in 21.2 per cent of all deletion negative cases and 26.6% of query DMD deletion negative cases. There is no specific region, which shows more duplication, and the mutations identified are generally spread out throughout the gene. It was also able to pick up three single exon deletions, which were not whole exon deletions as tested by exonic PCR, which are likely to be point mutations in the probe binding region of the exon, as confirmed for one case. Thus, combination of mPCR and MLPA is recommended for the diagnosis of 75% of all DMD/BMD cases.

Within our patient group identified with deletions and duplications, a significant number of patients (14%, 72/571 total patients) had single-exon deletions. This goes with most studies across the world. In India there is another study reporting 65.1% of multiple exon deletions, which is lower than the observations in this study (Mallikarjuna Rao et al., 2003). However a few studies from Pakistan and Turkey report a higher percentage of single exon deletions, like those in Pakistan (Hassan et al., 2008) and Turkey (Dincer et al., 1996). Deletion of only a single exon should be confirmed with an alternate primer set or method. Twenty five cases with mPCR results showing single exon deletions were confirmed by MLPA and all but one single exon deletions by MLPA were tested by PCR for specific exons. (Table 4.7)

If a query DMD patient is negative on the deletion/duplication analysis by mPCR and MLPA, direct sequencing of the coding region and intron/exon boundaries should then follow. In our cohort of patients, testing just three cases with known dystrophin deficiency by direct sequencing yielded three point mutations or small deletions which resulted in a stop codon. However, these strategies of checking for dystrophin deficiency before gene sequencing or cDNA sequencing are both invasive procedures and are not recommended by us. Alternatively, latest next generation sequencing technologies where large genomic regions can be

sequenced in one assay for a lower cost per base can be used to sequence the DMD gene.

Though 90 per cent of the cases follow the reading frame rule, 10 per cent show exception to the reading frame rule and have been well documented. (Monaco et al., 1988) In our cohort, 18 query DMD cases which contribute to 5.1% of single or multi exon deletions showed in-frame deletions. And 4 out of 32 deletion positive query BMD cases (12.5%) showed out-of-frame deletions. Overall 5.7% of the cases were exceptions to the reading frame rule. However, clinical details were not enough for understanding the real clinical status of these cases and it was beyond the scope of our study.

Seven non-contiguous deletions were picked by mPCR. But on confirmation by MLPA, 4 of them turned to be contiguous deletions and 3 were non-contiguous mutations. Three non-contiguous duplications were picked by MLPA. This also shows the importance of screening the whole gene, whilst some non-contiguous mutations could be missed. Reports on non-contiguous deletions exhibiting milder phenotypes have been published (Lai et al., 2006; Wang et al., 2008). All of the non-contiguous deletions reported in our study are novel mutations. Though there are reports on non-contiguous deletions and duplications in literature, there are no reports providing the phenotype of non-contiguous duplications. Further detailed clinical study and protein studies looking for residual dystrophin might throw light on the pathophysiology of these non-contiguous mutations.

In our study we have also picked up 21 novel mutations not reported in the Leiden database (www.dmd.nl). The Leiden database has the complete list of all published DMD gene mutations upto date. Most of these (14 cases) are deletions and 6 are duplications and one substitution. The novel mutations are also spread throughout the gene, and the novelty could be attributed only to the extent of the deletion.

Exon skipping of dystrophin gene exons containing a mutation is a promising potential therapy for DMD and other recessive muscular dystrophies (Aartsma-Rus et al., 2002). Skipping specific exons would be expected to restore the reading frame and result in the production of internally deleted, but essentially functional dystrophin as observed in the milder Becker muscular dystrophy, thus

providing significant functional improvement of DMD. Because some deletions and duplications of the dystrophin gene are more common than others, it has been estimated that skipping 12 exons would treat 73.3% of deletions. Among these possibilities, skipping exon 51 was the first choice because it could theoretically be therapeutic for ~20% of dystrophin deletions. Exon skipping can be achieved by antisense oligonucleotides (AONs) or adeno-associated vectors expressing small nuclear ribonucleoproteins (snRNPs). Both target one or more of the donor splice site, acceptor splice site or exonic sequences essential for exon definition during pre-mRNA splicing of specific exons. Upon binding of the AON or snRNP to a target exon, the exon will be spliced out with its flanking introns and the disrupted open reading frame will be restored. Following preclinical proof-of concept, the Department of Human Genetics in Leiden has set up a first study in humans in collaboration with Prosensa B.V. (http://prosensa.eu/). The aim is to obtain clinical proof-of-concept and assess safety and tolerability of a single, local intramuscular injection of PRO051, a 20-Omethyl RNA with a phosphorothioate backbone that targets exon 51 in the tibialis anterior muscle of DMD patients; more details are posted on the website of the Nederlands Trial Register (http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=712). Aartsma-Rus *et al,* have used the following deletions (exons 45-50, 45-54, 48-50, 51-55, and 52) to understand and evaluate the feasibility of exon skipping (Aartsma-Rus et al., 2002). From our study we have 64 children who can be eligible participants towards this therapeutic strategy.

Mutations were not picked up in more cases (34 of 57 cases, 59.6%) of query BMD cases as compared to query DMD. Several of these patients were also under query clinical differential diagnosis of limb girdle disorders. The higher rate of mutation negative query BMD patients may also reflect the use of molecular testing of the *DMD* gene as a method to rule out BMD in patients with muscle disease when results of other investigations are not clear. However, precisely identifying patients truly affected with BMD is essential to offer accurate genetic counselling, as the consequences of counseling an autosomal recessive disease as X-linked disorder could be catastrophic. Although our results indicate that the deletion/duplication analysis of the *DMD* gene in query BMD patients is useful, the clinical utility of direct *DMD* gene coding region

sequencing is of limited value for these patients. Confirmation of lack or deficiency of dystrophin protein is necessary before Dystrophin gene sequencing is considered. It would be reasonable to request further investigations of query BMD patients who are negative for deletion/duplication mutations, including muscle biopsy for analysis of dystrophin protein prior to embarking on direct *DMD* gene sequencing.

Our study has shown that following the systematic approach/algorithm shown in Figure 4.11 we were able to detect single and multiple exon deletions and duplication in almost 75 per cent of the cases suspected to have DMD/BMD. A study on MLPA analysis of DMD cases from China has reported a pick up rate of 73 per cent. It is interesting to note that reports by Trimarco et al showed 85 per cent pick up rate of mutations (only deletions and duplications) using the log-PCR method (Trimarco et al., 2008).

The unidentified mutations in the study cohort could be point mutations, small insertions or deletions. As per the algorithm suggested, it is only 25 per cent of the cases that require confirmation of dystrophin absence by muscle biopsy followed by cDNA sequencing. Our approach is unique as it is; (*i*) Non-invasive diagnosis to close to 75% of patients, (*ii*) replaces muscle biopsy as the first step in diagnosis, and (*iii*) economical and systematic molecular diagnosis in DMD where close to 75 per cent of the clinically suspected DMD/BMD cases are offered precise mutation detection. This systematic approach/algorithm may be used as a precise and cost-effective tool for DMD diagnosis in a developing country like India.

Overall, our results suggest that quantitative testing for deletions and duplications in the *DMD* gene should include all 79 exons to detect relatively common single-exon deletions and duplications, and that this analysis combined with direct *DMD* gene sequencing of the coding region provides nearly complete diagnosis of DMD. The data suggest that many of the patients in which a diagnosis of BMD is being considered may have an autosomal recessive muscular dystrophy rather than a dystrophinopathy, and that extensive sequence analysis of the *DMD* gene from genomic DNA in query BMD patients is of limited value. In this situation, alternative testing, such as protein analysis, would still be required to arrive at an accurate diagnosis and provide appropriate clinical management.

4.6 CONCLUSION

- We were the first group from India to publish data on DMD diagnosis using MLPA.
- We used a systematic algorithm whereby all the cases received at our facility for the diagnosis of DMD were subject to mPCR.
- All cases showing no deletion, or unclear borders of deletion were further subjected to MLPA, thereby reducing the cost of analysis and picking up mutations in almost 75% of the cases.
- Further analysis for the 25% of cases without a diagnosis is required using point mutation detection methods.
- Though no therapy is available for these cases, mutation detection will lead to identification of probable carriers within the family, carrier diagnosis and prevention through genetic counseling and prenatal diagnosis.

Publication:

1. Murugan S, Chandramohan A, Lakshmi BR. Use of multiplex ligation-dependent probe amplification (MLPA) for Duchenne muscular dystrophy (DMD) gene mutation analysis. *Indian J Med Res*. 2010 Sep; 132:303-11.

CHAPTER 5 – CARRIER DIAGNOSIS IN DUCHENNE MUSCULAR DYSTROPHY USING MLPA

5.1 INTRODUCTION

Majority of genetic disorders place a considerable burden on the families perpetuating the condition for the lack of effective treatment. D/BMD are such lethal disorders caused by mutations in the dystrophin gene. DMD is a common disease affecting 1/3500 male births, while BMD is milder and less frequent (Emery, 1991). Due to X-linked nature of the disorder, males carrying the mutated gene are affected, while females become carriers of the disease. Diagnosis of patients with D/BMD is usually definitive based on clinical, pathological and biochemical findings, although it is increasingly being confirmed by molecular analysis.

Though the incidence has been estimated at one in 3500 male births, not all the mothers of affected infants are carriers; a substantial proportion of cases will represent new mutations. According to Haldane, the mutation rate for a potentially lethal X-linked condition in which the biological fitness (fertility) of affected males is practically zero would be one-third. Estimates based on population studies have produced a similarly high figure. If two-third of the mothers of affected infants are carriers, then the investigation of the carrier state and genetic counseling are vitally important for the families concerned (Dubowitz, 1982).

Due to the lack of efficient rehabilitation and treatment of progressive muscular dystrophy, counseling and prenatal diagnosis are options that medical geneticists can offer today, and their decision depends on information of the carrier status. The first essential step in genetic counseling must always be to verify the diagnosis in the index case. Next, a detailed family tree should be constructed before investigation of the possible carrier is begun. Practically, if the mother of an affected boy (*proband*) has another affected relative, she is an obligate carrier. If there is an affected brother or one affected son, she is a possible carrier (Dubowitz, 1982). But in most families there is only one affected patient.

Therefore, female relatives of affected males are candidates for carrier assessment.

However, the ascertainment of carrier status is one of the basic dilemmas in genetic counseling in X-linked recessive disorders because female carriers are usually asymptomatic. Several biochemical and molecular methods have been suggested to solve this dilemma (Panigrahi and Mittal, 2001). Quantitative multiplex PCR, real time PCR, linkage analysis using dinucleotide repeat (CA) markers, southern blotting have all been used. (Den Dunnen et al., 1989; Clemens et al., 1991; Abbs and Bobrow, 1992; Prior et al., 1995; Boulay et al., 1999) Among these, microsatellite based linkage has been used for many disorders and has been found useful. Microsatellites are short tandemly repeated sequences, which have been identified as by products of the Human Genome Project. There are approximately 50,000-100,000 (CA)n loci (a subclass of STRs) in the human genome. These frequently polymorphic loci have been exploited in carrier detection of many genetic diseases by linkage analysis (Tautz and Renz, 1984).

DNA based linkage analysis, using intragenic (CA) repeat markers of dystrophin gene has been found to be a powerful approach for carrier detection of non-deletional as well as deletional D/BMD families. Due to large size of the gene, intragenic meiotic recombinations are also possible. Therefore, several intragenic and flanking STRs of dystrophin locus are being studied to provide accurate carrier status and prenatal diagnosis (Chaturvedi et al., 2000).

The method requires the sample of proband in addition to other family members. The informativeness of the markers depends on the level of heterozygosity in the population. Hence, the markers selected have to be specific for the target population. Usually 4-6 STR markers for the deletion prone regions of dystrophin gene are used and the haplotype of the specific X-chromosome is deduced based on capillary electorphoresis (Alcantara et al., 1999).

Essentially in families where the proband deletion or duplication are known, dosage testing for the deleted/duplicated exons is the recommended method. For all the other cases where the causative mutations are small changes like small deletions, insertions and point mutations, gene sequencing can be attempted.

In this chapter we have studied the carrier status of probable carriers in families where the DMD gene deletion/duplication has been identified for the affected index case. We have studied the usefulness of MLPA in carrier analysis, while also validating and comparing MLPA results with results obtained from other commonly used methods.

5.2 MATERIALS AND METHODS:

5.2.1 Patients and samples

We have prospectively analysed carrier status of female relatives including mother, maternal grandmother, maternal aunt, and sisters of affected males whose DNA analysis results were available and confirmed to have D/BMD. A total of 150 probable carriers were analysed from 110 apparently unrelated families. DMD gene mutations in the patients were detected using mPCR and MLPA as dicussed in chapter 4. Probable carriers from families in which only proband mutation was detected were taken up for the study. For one of the cases (B28), DMD gene mutation was confirmed by direct sequencing in another lab and was found to have a frameshift point mutation (c.7348dupG) in exon 51.

Since most of the mutations, except for B28, were single exon deletions and duplications, MLPA was the method of choice. Direct sequencing was used for the family in which point mutation was identified. Linkage by STR-(CA) segregation analysis and quantitative multiplex fluorescence PCR (qmfPCR) were used to validate MLPA results on 36 samples.

EDTA samples (3 ml quantities) were collected from patients for genetic analysis after obtaining informed consent. DNA was extracted by salting out method, quantified and stored at –20°C until tested.

5.2.2 Multiplex PCR:

Multiplex PCR analysis was performed for 30 exons at the central and 5’end hot spot regions as already detailed (Murugan, Chandramohan et al. 2010).

5.2.3 Quantitative mPCR:

Quantitatiave multiplex PCR was standardized with the same conditions used for mPCR, except that the starting DNA quantity used was 250µg/ml for all the samples. Quantification of band intensity was done as described.

5.2.4 STR-(CA) segregation analysis:

CA analyses were performed, as described, for 36 samples which were also tested by MLPA and qfmPCR..

5.2.5 Quantitative multiplex fluorescence PCR (qmfPCR):

QmfPCR for 51 exons were tested for validation of MLPA results.

5.2.6 Multiplex Ligation-dependent Probe Amplification (MLPA):

MLPA analysis was carried out using PO34 and PO35 probes purchased commercially from MRC, Holland (Amsterdam, Netherlands). The procedures and analysis of MLPA data were carried out according to the manufacturer's recommendations and as described in materials and methods.

5.3 RESULTS:

Carrier diagnosis was initially tried with quantitative mPCR (QmPCR), which followed the same protocol as mPCR used for diagnosis, except that the starting DNA material was quantified and known standardized concentrations were used. DNA quantity was standardized at 50ng. The resulting amplified product was run on 2% agarose gel and was viewed and captured in the gel documentation system (Biorad, USA). Quantity one software (Biorad, USA) was used for calculating the band intensities and the results were compared with male and female control values. Figure 5.1 shows one family tested for carrier status with QmPCR. But we faced difficulty in reproducing the results on repeated analysis. Hence, MLPA, a more robust method for quantitative exon assay was tried for carrier diagnosis in DMD.

MLPA was performed as per manufacturer's recommendation. A total of 149 cases from 109 families were tested for carrier status in this study. Of the proband mutations, 99 were single or multiple exon deletions, 9 were duplications and one point mutation (Table 5.1). A total of 56 different mutations were seen in the 109 families studied. Among the deletions, single exon deletions were in 21 families, and the rest 78 families had multiple exon deletions ranging from 2 exons to 37 exons with small deletions ranging from 2 exons to 6 exons contributing to most of the cases [59 cases (75.6% of the rest of the cases)]. Eighty five of the deletion cases (85.9%) have their deletions in the central hot spot region of the DMD gene ranging from exons 40 to 55, ten cases in the proximal hot spot region and the rest five in the region between exons 20-40. The duplications were very few with no definite pattern and the only point mutation case in our study harbored the mutation in exon 51.

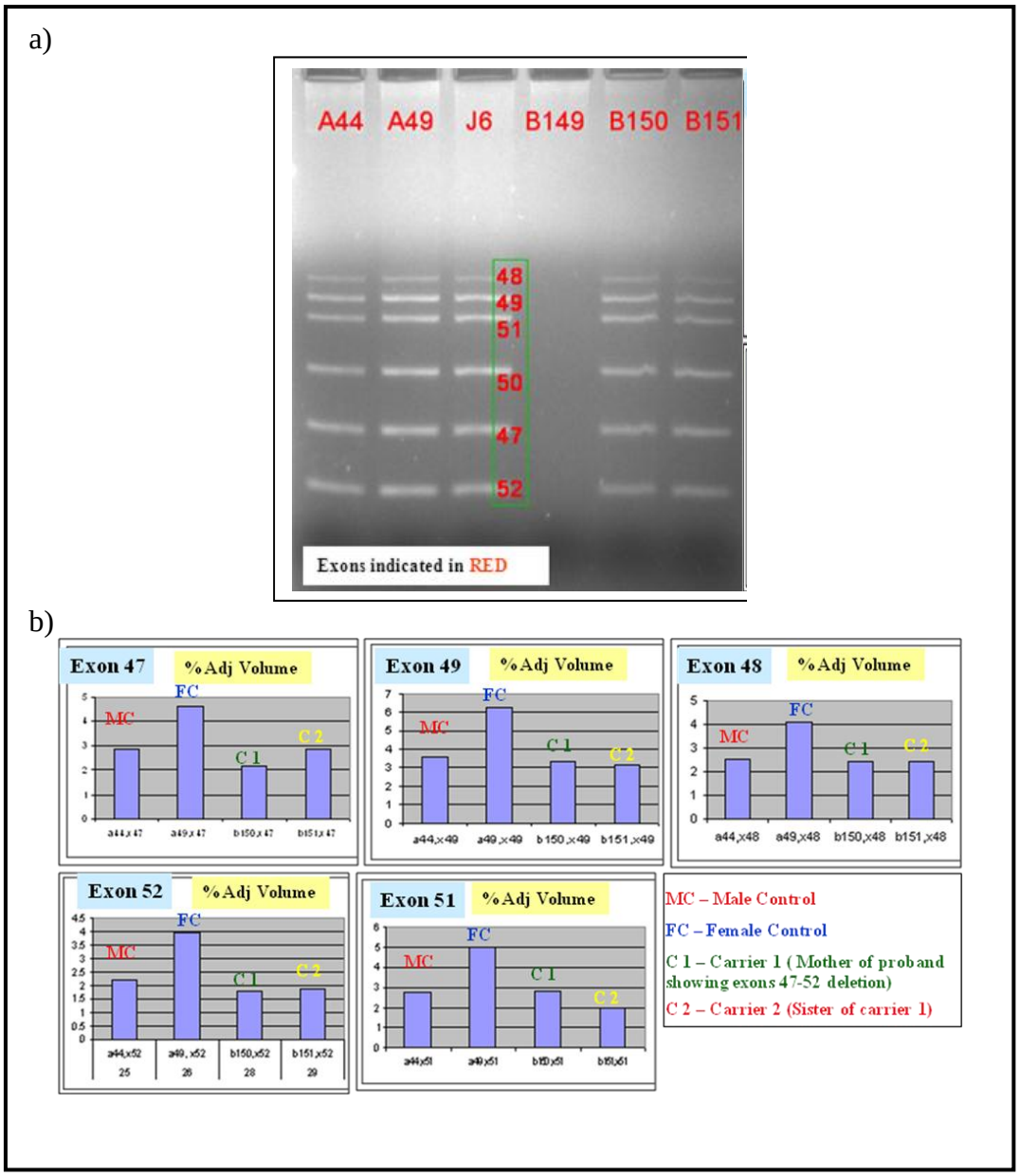


Figure 5.1: QmfPCR for DMD gene exons 47-52, for a family and band intensity analysis. *a & b) Exons tested are indicated in red and are deleted in the proband (B149 – Lane 4 in a). Intensity differences between male (A44 – Lane 1 in a) and female controls (A49 – Lane3 in a) are obviously seen from the gel picture, and also from the intensity values in the bar chart for each of the exons (figure b). J6 (Lane 3) is a control sample for duplications in exons 47 to 52. It is clear that the intensities of each of the exons in the mother's (B150 – Lane 5) and Aunt's (B151 – Lane 6) samples are same as that of the male control which is also seen in the intensity values in the bar charts, confirming both to be carries for deletion of exons 47 to 52.*

Table 5.1: List of deletion, duplication and point mutation in index cases

Number of exons deleted	Index case mutation (n=109)	No of cases
1 exon deletion		
n=21	Exon 44 Deleted	2
	Exon 45 deleted	9
	Exon 50 deleted	3
	Exons 51 deleted	5
	Exon 52 Deleted	1
	Exon 53 deleted	1
2 exons deleted		
n=12	Exon 8-9 deleted	1
	Exons 49-50 deleted	4
	Exons 46-47 Deleted	4
	Exon 46-47 Deleted	2
	Exons 42-43 Deleted	1
3 exons deleted		
n=13	Exon 44-46 deleted	1
	Exon 48-50 deleted	1
	Exons 46-48 Deleted	4
	Exons 48-50 deleted	4
	Exons 51-53 Deleted	2
	Exons 45-47 Deleted	1
4 exons deleted		
n=5	Exon 9-12 deleted	1
	Exons 49-52 deleted	2
	Exons 45-48 Deleted	1
	Exons 47-50 Deleted	1
5 exons deleted		
n=17	Exons 45-53 deleted	3
	Exon 46-50 deleted	2
	Exon 48-52 deleted	2
	Exon 49-53 deleted	1
	Exon 3-7 deleted	1
	Exons 48-52 deleted	5
	Exons 46-50 Deleted	2
	Exon 8-12 Deleted	1
6 exons deleted		
n=12	Exon 45-50 deleted	1
	Exon 46-51 deleted	1
	Exons 3-8 deleted	2

	Exons 45-50 deleted	7
	Exons 48-53 deleted	1
7 exons deleted		
n=2	Exons 46-52 deleted	2
8 exons deleted		
n=6	Exon 45-52 deleted	1
	Exons 10-17 Deleted	1
	Exons 45-52 Deleted	4
9 exons deleted		
n=3	Exon 3-11 deleted	1
	Exon 20, Exons 45-52 Deleted	1
	Exons 18-26 Deleted	1
10 exons deleted		
n=3	Exons 45-54 Deleted	2
	Exons 46-55 Deleted	1
17 exons deleted		
n=1	Exons 28-44 Deleted	1
23 exons deleted		
n=1	Exons 22-44 Deleted	1
27 exons deleted		
n=1	Exons 17-43 Deleted	1
28 exons deleted		
n=1	Exons 8-34 deleted	1
37 exons deleted		
n=1	Exons 8-44 Deleted	1
DUPLICATIONS		
1 exon duplication		
n=2	Exon 2 duplicated	2
3 exon duplication		
n=1	Exons 2-4 duplicated	1
4 exon duplication		
n=3	Exons 8-11 Duplicated	2
	Exon 18-21 duplication	1
5 exon duplication		
n=1	Exon 3-7 duplicated	1
7 exon duplication		
n=1	Exons 45-48 & 53-55 duplicated	1
25 exon duplication		
n=1	Exon 52-75 duplicated	1
POINT MUTATION		
n=1	Exon 51-point mutation	1

Sixteen families had family history. Among the families with history of the disorder, 15 were deletion mutations and 1 was duplication (Table 5.3). There was no definite mutation pattern in families with a history of the disorder. Of the 15 families, proband mutations in 12 families (14.1% of all families with proband mutation in distal hot spot region) were in the central hot spot region and 2 were in the proximal region (20% of all families with proband mutation in proximal hot spot region) of the DMD gene. In six families (40%) the proband mutation was single exon deletion. One family had a duplication of DMD gene exons.

Carrier analysis was tested by MLPA for all the cases except one for which the proband showed a point mutation. MLPA results were validated with CA repeat analysis and QmfPCR.

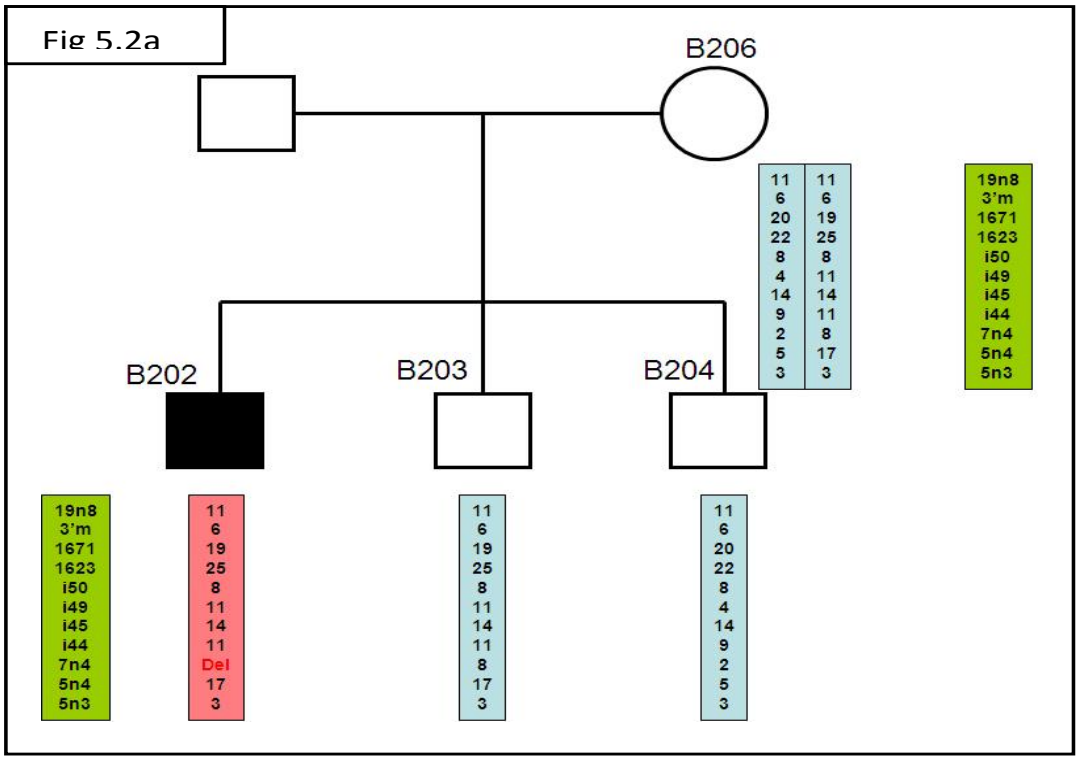
5.3.1 Validation of MLPA results by STR-(CA) segregation analysis

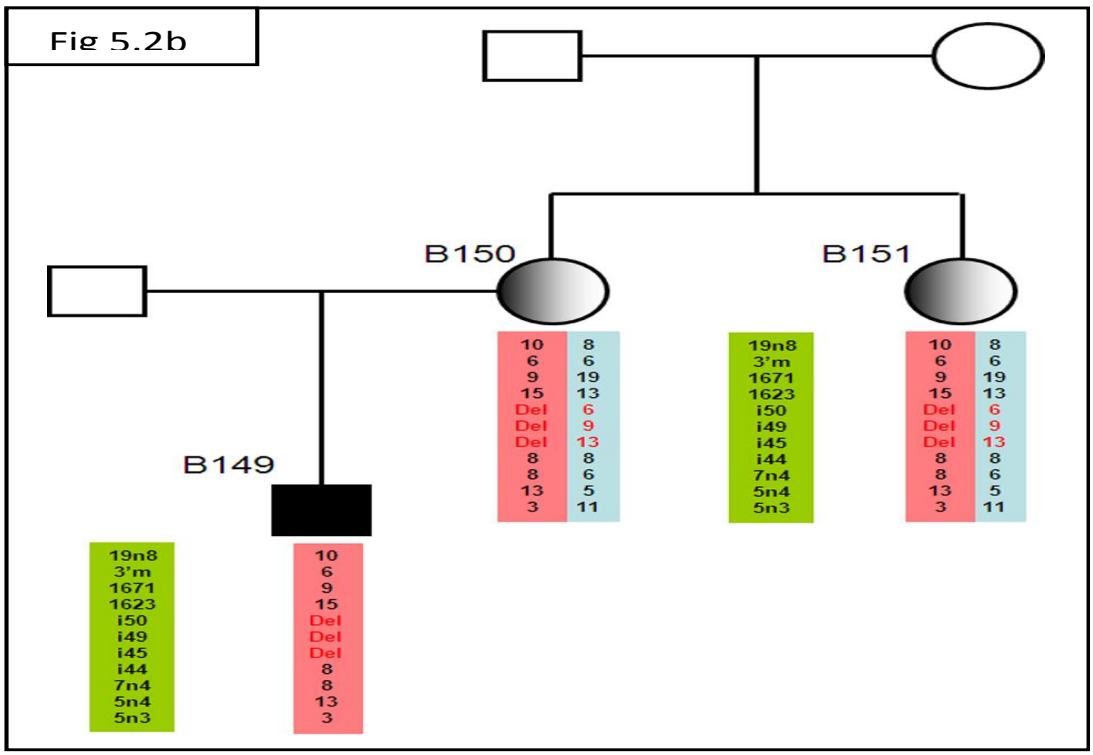
MLPA results of 33 probable carriers from 23 families whose index cases showed DMD gene deletions or duplications were validated by STR-(CA) segregation analysis and qmfPCR, the results of which are shown in table 5.2.

Table 5.2: Comparison of MLPA, CA repeat analysis and QfmPCR results

S.No.	Family No.	Carrier ID	CA repeat analysis result	qmfPCR result	MLPA result
1	1	B118	Carrier	Carrier	Carrier
2		B120	Normal	Normal	Normal
3		B121	Carrier	Carrier	Carrier
4		B234	Normal	Normal	Normal
5	2	B190	Normal	Normal	Normal
6	3	B192	Carrier	Carrier	Carrier
7		B240	Normal	Normal	Normal
8	4	B194	Carrier	Carrier	Carrier
9	5	B206	Normal	Normal	Normal
10	6	B215	Normal	Normal	Normal
11	7	B230	Normal	Normal	Normal
12	8	B235	Non-informative	Carrier	Carrier
13	9	B242	Normal	Normal	Normal
14		B243	Normal	Normal	Normal
15	10	B246	Non-informative	Carrier	Carrier
16	11	B247	Normal	Normal	Normal
17		B248	Normal	Normal	Normal
18	12	B249	Normal	Normal	Normal
19	13	B262	Non-informative	Normal	Normal
20	14	B264	Non-informative	Normal	Normal
21	15	B267	Non-informative	Carrier	Carrier
22	16	B269	Carrier	Carrier	Carrier
23		B270	Normal	Normal	Normal
24	17	B275	Normal	Normal	Normal
25		B276	Normal	Normal	Normal
26	18	B278	Normal	Normal	Normal
27		B279	Carrier	Carrier	Carrier
28	19	B282	Carrier	Carrier	Carrier
29		B283	Carrier	Carrier	Carrier
30	20	B284	Normal	Normal	Normal
31	21	B86	Non-informative	Normal	Normal
32	22	B207	Not Done	Normal	Normal
33	23	B312	Carrier	Carrier	Carrier

STR-(CA) segregation analysis was done for 32 of the 33 cases in the study. The assay was conducted for 11 markers (19n8, 3'm, 1671, 1623, i50, i49, i45, i44, 7n4, 5n4 and 5n3) flanking the whole DMD gene. It was seen that it was helpful in assessing the carrier status in most of the cases when it was combined with one of the direct methods.





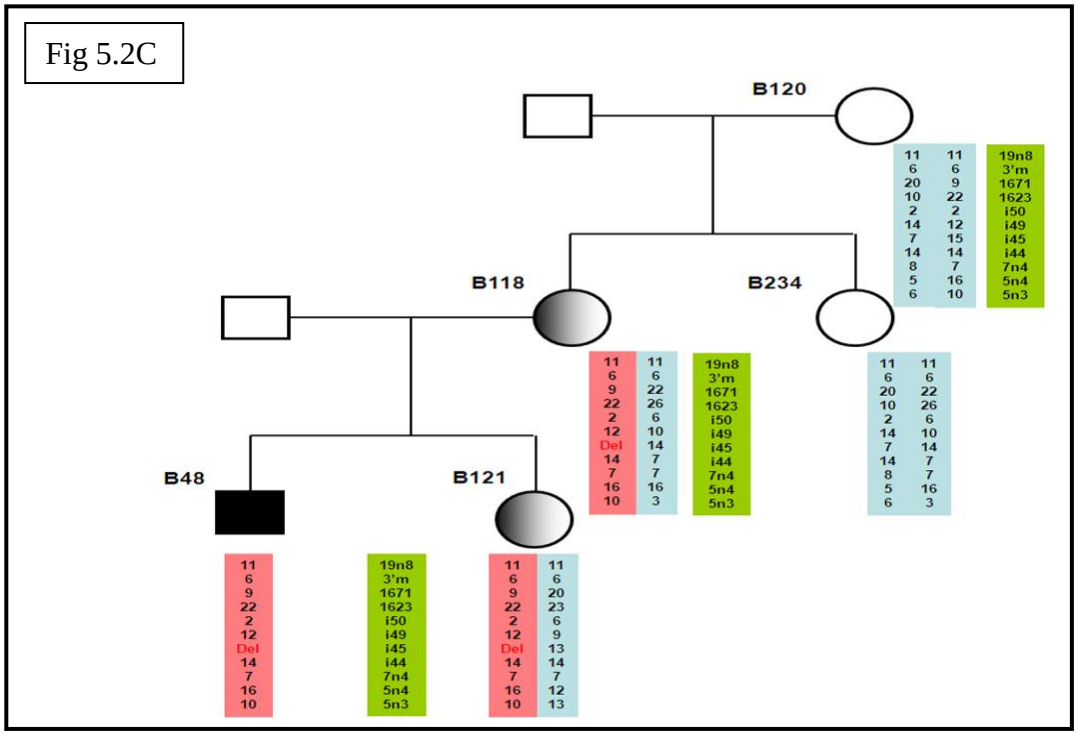


Figure 5.2 a, b, & c: STR-(CA) segregation analysis. *5.2a showing STR-(CA) segregation analysis results in a family where the haplotype in the proband is not seen in the mother and neither in the unaffected sibs. 5.2b showing a family where the proband haplotype is seen in both the mother and the aunt confirming both to be carriers. 5.2c shows a family where the sister and the mother carry the affected haplotype, whereas the aunt and the grandmother are normal. It can also be observed in all these cases that the deletion in the DMD gene is evident.*

STR-(CA) segregation analysis was non-informative in 6 cases (17.6%). Many of the markers showed homozygosity for most of the cases suggesting the need to use alternative markers designed specifically for the Indian population. A representation of STR-(CA) segregation analysis results in one of the families is shown in Figure 5.2c.

5.3.2 Validation of MLPA results by QmfPCR

QmfPCR was done for 59 exons covering most of the hot spot regions and is based on fluorescently tagged primers and quantitative PCR, resulting in peak heights corresponding to the dosage on capillary electrophoresis. QmfPCR analysis was done for 33 cases and its results were same as that of MLPA. Some of the QmfPCR results are shown in the figure 5.3.

Fig 5.3a

B151	Peak Area		Exons																						
Exons	Control	Patient	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	58	60	62	63	68	71	75	GSG-6
42	26384	17826	1.0	0.8	1.0	1.0	1.1	1.0	0.9	1.0	1.1	0.8	1.1	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.2	1.0	1.0	0.9	1.0
43	12529	10259	1.2	1.0	1.2	1.2	1.3	1.2	1.1	1.2	1.3	1.0	1.3	1.3	1.3	1.3	1.2	1.2	1.2	1.3	1.4	1.3	1.2	1.1	1.3
44	23916	16382	1.0	0.8	1.0	1.0	1.1	1.0	0.9	1.0	1.1	0.8	1.1	1.1	1.1	1.1	1.0	1.0	1.0	1.1	1.2	1.1	1.0	0.9	1.1
45	20128	13529	1.0	0.8	1.0	1.0	1.1	1.0	0.9	1.0	1.1	0.8	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.2	1.0	1.0	0.9	1.0
46	15717	9630	0.9	0.7	0.9	0.9	1.0	0.9	0.8	0.9	1.0	0.7	1.0	1.0	0.9	0.9	0.9	0.9	0.9	1.0	1.1	1.0	0.9	0.8	0.9
47	8999	6217	1.0	0.8	1.0	1.0	1.1	1.0	1.0	1.0	1.1	0.8	1.1	1.1	1.1	1.1	1.0	1.0	1.0	1.1	1.2	1.1	1.0	0.9	1.1
48	19994	14537	1.1	0.9	1.1	1.1	1.2	1.1	1.0	1.1	1.1	0.9	1.2	1.1	1.1	1.1	1.1	1.1	1.2	1.3	1.1	1.1	1.1	1.0	1.1
49	19925	13571	1.0	0.8	1.0	1.0	1.1	1.0	0.9	1.0	1.1	0.8	1.1	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.2	1.1	1.0	0.9	1.1
50	19062	12165	0.9	0.8	0.9	0.9	1.0	0.9	0.9	0.9	1.0	0.8	1.0	1.0	1.0	1.0	1.0	0.9	0.9	1.0	1.1	1.0	1.0	0.9	1.0
51	1446	1217	1.2	1.0	1.2	1.3	1.4	1.2	1.2	1.2	1.3	1.0	1.3	1.3	1.3	1.3	1.3	1.2	1.2	1.4	1.5	1.3	1.3	1.2	1.3
52	13184	8316	0.9	0.8	0.9	0.9	1.0	0.9	0.9	0.9	1.0	0.7	1.0	1.0	1.0	1.0	1.0	0.9	0.9	1.0	1.1	1.0	0.9	0.9	1.0
53	12529	8046	1.0	0.8	0.9	1.0	1.0	0.9	0.9	0.9	1.0	0.8	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.1	1.0	1.0	0.9	1.0
54	23618	15383	1.0	0.8	1.0	1.0	1.1	0.9	0.9	1.0	1.0	0.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	0.9	1.0
55	23939	15610	1.0	0.8	1.0	1.0	1.1	0.9	0.9	1.0	1.0	0.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	0.9	1.0
56	15217	10035	1.0	0.8	1.0	1.0	1.1	1.0	0.9	1.0	1.0	0.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.0	1.0	0.9	1.0
58	17507	11947	1.0	0.8	1.0	1.0	1.1	1.0	0.9	1.0	1.1	0.8	1.1	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.2	1.1	1.0	0.9	1.1
60	9403	6354	1.0	0.8	1.0	1.0	1.1	1.0	0.9	1.0	1.1	0.8	1.1	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.2	1.0	1.0	0.9	1.0
62	9164	5704	0.9	0.8	0.9	0.9	1.0	0.9	0.9	0.9	1.0	0.7	1.0	1.0	1.0	1.0	0.9	0.9	0.9	1.0	1.1	1.0	0.9	0.9	1.0
63	15220	8767	0.9	0.7	0.8	0.9	0.9	0.8	0.8	0.8	0.9	0.7	0.9	0.9	0.9	0.9	0.9	0.8	0.9	0.9	1.0	0.9	0.9	0.8	0.9
68	24783	15980	1.0	0.8	0.9	1.0	1.1	0.9	0.9	0.9	1.0	0.8	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.1	1.0	1.0	0.9	1.0
71	19278	12909	1.0	0.8	1.0	1.0	1.1	1.0	0.9	1.0	1.0	0.8	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.2	1.0	1.0	0.9	1.0
75	9815	7168	1.1	0.9	1.1	1.1	1.2	1.1	1.0	1.1	1.1	0.9	1.2	1.1	1.1	1.1	1.1	1.2	1.2	1.3	1.1	1.1	1.1	1.0	1.1
GSG-6	17505	11313	1.0	0.8	0.9	1.0	1.1	0.9	0.9	0.9	1.0	0.8	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.1	1.0	1.0	0.9	1.0

Fig 5.3b

B194	Peak Area		Exons																						
Exons	Control	Patient	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	58	60	62	63	68	71	75	GSG-6
42	27447	15393	1.0	0.9	0.9	1.7	1.7	1.8	3.5	1.8	1.8	0.4	1.0	0.9	0.9	0.8	0.9	0.8	1.0	1.0	0.9	0.8	0.9	1.6	1.0
43	10125	6591	1.2	1.0	1.1	1.9	2.0	2.1	4.1	2.0	2.0	0.5	1.2	1.1	1.0	1.0	1.0	0.9	1.2	1.2	1.1	0.9	1.1	1.9	1.1
44	21563	12972	1.1	0.9	1.0	1.8	1.8	2.0	3.8	1.9	1.9	0.5	1.1	1.0	0.9	0.9	1.0	0.8	1.1	1.1	1.0	0.8	1.0	1.7	1.0
45	18950	6329	0.6	0.5	0.6	1.0	1.0	1.1	2.1	1.1	1.0	0.3	0.6	0.6	0.5	0.5	0.5	0.5	0.6	0.6	0.5	0.5	0.6	1.0	0.6
46	13097	4289	0.6	0.5	0.5	1.0	1.0	1.1	2.1	1.0	1.0	0.3	0.6	0.5	0.5	0.5	0.5	0.5	0.6	0.6	0.5	0.5	0.5	0.9	0.6
47	8743	2695	0.5	0.5	0.5	0.9	0.9	1.0	1.9	1.0	1.0	0.2	0.6	0.5	0.5	0.5	0.5	0.4	0.6	0.6	0.5	0.4	0.5	0.9	0.5
48	18084	2868	0.3	0.2	0.3	0.5	0.5	0.5	1.0	0.5	0.5	0.1	0.3	0.3	0.2	0.2	0.3	0.2	0.3	0.3	0.3	0.2	0.3	0.5	0.3
49	18444	5864	0.6	0.5	0.5	1.0	1.0	1.0	2.0	1.0	1.0	0.3	0.6	0.5	0.5	0.5	0.5	0.4	0.6	0.6	0.5	0.4	0.5	0.9	0.5
50	17420	5578	0.6	0.5	0.5	1.0	1.0	1.0	2.0	1.0	1.0	0.3	0.6	0.5	0.5	0.5	0.5	0.5	0.6	0.6	0.5	0.4	0.5	0.9	0.6
51	1084	1353	2.2	1.9	2.1	3.7	3.8	4.0	7.9	3.9	3.9	1.0	2.2	2.1	1.9	1.9	2.0	1.8	2.3	2.3	2.0	1.7	2.1	3.6	2.2
52	13240	7357	1.0	0.9	0.9	1.7	1.7	1.8	3.5	1.7	1.7	0.4	1.0	0.9	0.9	0.8	0.9	0.8	1.0	1.0	0.9	0.8	0.9	1.6	1.0
53	11714	7049	1.1	0.9	1.0	1.8	1.8	2.0	3.8	1.9	1.9	0.5	1.1	1.0	0.9	0.9	1.0	0.8	1.1	1.1	1.0	0.8	1.0	1.7	1.0
54	21332	13679	1.1	1.0	1.1	1.9	2.0	2.1	4.0	2.0	2.0	0.5	1.2	1.1	1.0	1.0	1.0	0.9	1.2	1.2	1.0	0.9	1.1	1.9	1.1
55	21645	14358	1.2	1.0	1.1	2.0	2.0	2.2	4.2	2.1	2.1	0.5	1.2	1.1	1.0	1.0	1.1	0.9	1.2	1.2	1.1	0.9	1.1	1.9	1.1
56	14320	8939	1.1	1.0	1.0	1.9	1.9	2.0	3.9	2.0	1.9	0.5	1.1	1.0	1.0	0.9	1.0	0.9	1.1	1.2	1.0	0.9	1.0	1.8	1.1
58	15095	10712	1.3	1.1	1.2	2.1	2.2	2.3	4.5	2.2	2.2	0.6	1.3	1.2	1.1	1.1	1.1	1.0	1.3	1.3	1.2	1.0	1.2	2.1	1.2
60	9310	5064	1.0	0.8	0.9	1.6	1.7	1.8	3.4	1.7	1.7	0.4	1.0	0.9	0.8	0.8	0.9	0.8	1.0	1.0	0.9	0.7	0.9	1.6	0.9
62	9097	4874	1.0	0.8	0.9	1.6	1.6	1.7	3.4	1.7	1.7	0.4	1.0	0.9	0.8	0.8	0.9	0.8	1.0	1.0	0.9	0.7	0.9	1.5	0.9
63	9306	5697	1.1	0.9	1.0	1.8	1.9	2.0	3.9	1.9	1.9	0.5	1.1	1.0	1.0	0.9	1.0	0.9	1.1	1.1	1.0	0.8	1.0	1.8	1.1
68	19185	13943	1.3	1.1	1.2	2.2	2.2	2.4	4.6	2.3	2.3	0.6	1.3	1.2	1.1	1.1	1.2	1.0	1.3	1.4	1.2	1.0	1.2	2.1	1.3
71	18082	10879	1.1	0.9	1.0	1.8	1.8	2.0	3.8	1.9	1.9	0.5	1.1	1.0	0.9	0.9	1.0	0.8	1.1	1.1	1.0	0.8	1.0	1.7	1.0
75	9801	3388	0.6	0.5	0.6	1.0	1.1	1.1	2.2	1.1	1.1	0.3	0.6	0.6	0.5	0.5	0.6	0.5	0.6	0.6	0.6	0.5	0.6	1.0	0.6
GSG-6	16207	9408	1.0	0.9	1.0	1.7	1.8	1.9	3.7	1.8	1.8	0.5	1.0	1.0	0.9	0.9	0.9	0.8	1.1	1.1	0.9	0.8	1.0	1.7	1.0

Figure 5.3: Quantitative fluorescence multiplex PCR results of 2 samples. *5.3a is result of a sample which is normal and 5.3b shows heterozygous deletion of exons 45 to 50 confirming the sample to be a carrier. (Yellow colour represents Dosage quotient (DQ) values less than 0.65 or more than 1.35)*

5.3.3 Carrier analysis

Carrier status of 149 probable carriers from 109 apparently unrelated families was tested. MLPA was used to test carrier status in 108 families where the index case mutation was deletions or duplications. For one case (B207) whose index case mutation was a point mutation in exon 51 of the DMD gene, sequencing analysis performed.

The average Dosage Quotients for deletion carriers was 0.51 (range 0.34 to 0.61) and for duplication 1.46 (range 1.34 to 1.62). Table 5.3 shows the complete list of samples, carrier status of different relations in the study. Table 5.4 shows the

consolidated results of the study. Of the 149 cases, 104 were mothers, 37 sisters, 6 maternal aunts and 2 maternal grandmothers. Among the 109 families tested, 16 showed family history, 92 cases were sporadic cases and for 1 case the family history details are not available. Fifty cases (33.6% of all cases) tested positive for carrier status, of which 42 were mothers (40% of all mothers) and 7 were sisters and 1 maternal aunt of the index cases. Of the 50 cases, carrier statuses of 49 were confirmed by MLPA and one of the cases by direct sequencing (figure 5.4). Some representative MLPA results are shown in figure 5.5 for representation.

Table 5.3: Complete carrier analysis results with the relationship and family history details

S.No.	Family no	carrier Id	Index case Id	Index case mutation	Relationship to Index Case	Carrier status	Family History
1	1	B118	B48	Exon 45 deleted	Mother	Carrier	No
2		B120		Exon 45 deleted	Maternal Grandmother	Normal	
3		B121		Exon 45 deleted	Sister	Carrier	
4		B234		Exon 45 deleted	Maternal Aunt	Normal	
5	2	B150	B149	Exons 45-53 deleted	Mother	Carrier	No
6		B151		Exons 45-53 deleted	Maternal Aunt	Carrier	
7	3	B190	B189	Exon 2 duplicated	Mother	Normal	No
8	4	B192	B174	Exon 46-50 deleted	Mother	Carrier	yes
9		B240		Exon 46-50 deleted	Maternal Aunt	Normal	
10		DBO		Exon 46-50 deleted	Maternal Aunt	Normal	
11	5	B194	B193	Exon 45-50 deleted	Mother	Carrier	No
12	6	B206	B202	Exon 9-12 deleted	Mother	Normal	No
13	7	B215	B214	Exon 48-52 deleted	Mother	Normal	No
14	8	B230	B229	Exon 46-51 deleted	Mother	Normal	No
15	9	B235	B226	Exon 45-52 deleted	Mother	Carrier	No
16	10	B242	B210	Exon 45 deleted	Mother	Normal	No
17		B243		Exon 45 deleted	Sister	Normal	
18	11	B246	B244	Exon 52-75 duplicated	Mother	Carrier	yes
19	12	B247	B95	Exon 49-53 deleted	Mother	Normal	No
20		B248		Exon 49-53 deleted	Maternal Aunt	Normal	
21	13	B249	B238	Exon 50 deleted	Mother	Normal	No
22	14	B262	B261	Exon 3-7 deleted	Mother	Carrier	yes
23	15	B264	B263	Exon 48-52 deleted	Mother	Normal	No
24	16	B267	B265, B266	Exon 8-9 deleted	Mother	Carrier	yes
25	17	B269	B268	Exon 3-11 deleted	Mother	Carrier	No
26		B270		Exon 3-11 deleted	Sister	Normal	
27		B273		Exon 3-11 deleted	Maternal Aunt	Normal	
28	18	B275	B274	Exon 44-46 deleted	Mother	Normal	No
29		B276		Exon 44-46 deleted	Sister	Normal	
30	19	B278	B277	Exon 48-50 deleted	Mother	Carrier	No
31		B279		Exon 48-50 deleted	Sister	Carrier	

32	20	B282	B281	Exon 3-7 duplicated	Mother	Carrier	No
33		B283		Exon 3-7 duplicated	Sister	Normal	
34	21	B284	B188	Exon 46-50 deleted	Mother	Normal	No
35	22	B207	B28	Exon 51-point mutation	Mother	Carrier	No
36	23	R	M	Exon 18-21 duplication	Mother	Carrier	No
37	24	B312	B305	Exons 3-8 deleted	Mother	Carrier	No
38	25	B340	B339	Exon 51 deleted	Mother	Carrier	No
39	26	B343	B336	Exons 45-50 deleted	Mother	Normal	No
40	27	B345	B344	Exons 48-53 deleted	Mother	Normal	No
41		B346		Exons 48-53 deleted	Sister	Normal	
42	28	B349	B196	Exons 45-50 deleted	Mother	Normal	No
43	29	B351	B350	Exons 45-53 deleted	Mother	Normal	No
44		B352		Exons 45-53 deleted	Sister	Normal	
45	30	B354	B148	Exons 46-52 deleted	Mother	Normal	No
46	31	B355	B181	Exons 45-48 & 53-55 duplicated	Mother	Carrier	No
47	32	B357	B356	Exons 3-8 deleted	Mother	Carrier	yes
48	33	B361	B360	Exon 45 deleted	Mother	Normal	No
49		B362		Exon 45 deleted	Sister	Normal	
50	34	B364	B358	Exons 8-34 deleted	Sister	Normal	No
51		B365		Exons 8-34 deleted	Sister	Carrier	
52	35	B390	B389	Exons 49-50 deleted	Mother	Carrier	No
53		B391	B389	Exons 49-50 Deleted	Sister	Normal	
54	36	B468	NA	Exons 51 deleted	Mother	Carrier	yes
55	37	B469	B433	Exons 45-53 deleted	Mother	Carrier	yes
56		B470		Exons 45-53 deleted	Sister	Normal	
57	38	B476	B475	Exon 53 deleted	Mother	Normal	No
58	39	B479	B478	Exons 49-52 deleted	Mother	Normal	No
59	40	B565	B564	Exons 48-50 deleted	Mother	Normal	No
60		B567		Exons 48-52 deleted	Sister	Normal	
61	41	B568	B473	Exons 48-52 deleted	Mother	Normal	No
62	42	B289	B288	Exons 45-50 Deleted	Mother	Carrier	No
63		B290		Exons 45-50 Deleted	Sister	Normal	
64	43	B292	B291	Exon 44 Deleted	Mother	Carrier	No
65	44	B297	B60	Exons 49-50 Deleted	Mother	Normal	No
66	45	B299	B298	Exons 48-52 Deleted	Mother	Normal	No
67	46	B318	B314	Exons 46-47 Deleted	Mother	Normal	No
68		B319		Exons 46-47 Deleted	Sister	Normal	
69	47	B323	B322	Exons 48-50 Deleted	Mother	Normal	No
70	48	B335	B334	Exons 2-4 duplicated	Mother	Carrier	No
71	49	B338	B337	Exon 44 Deleted	Mother	Carrier	No
72	50	B467	B458	Exons 46-48 Deleted	Mother	Normal	No
73	51	B480	B472	Exon 51 Deleted	Mother	Normal	No
74	52	B502	B485	Exon 2 Duplicated	Mother	Normal	No
75	53	B503	B486	Exons 8-11 Duplicated	Mother	Normal	No
76	54	B504	B487	Exons 8-11 Duplicated	Mother	Normal	No
77		B701		Exons 8-11 Duplicated	Sister	Normal	
78	55	B505	B488	Exons 45-54 Deleted	Mother	Normal	No
79	56	B506	B489	Exons 51-53 Deleted	Mother	Normal	No
80		B507		Exons 51-53 Deleted	Maternal Grandmother	Normal	
81	57	B508	B490	Exon 45 Deleted	Mother	Normal	No

82	58	B510	B492	Exons 48-52 Deleted	Mother	Normal	No
83	59	B511	B493	Exon 50 Deleted	Mother	Normal	No
84		DBO		Exon 50 Deleted	Sister	Normal	
85	60	B512	B494	Exons 28-44 Deleted	Mother	Normal	No
86	61	B513	B495	Exons 22-44 Deleted	Mother	Normal	No
87		B514		Exons 22-44 Deleted	Sister	Normal	
88	63	B531	B521	Exons 45-48 Deleted	Mother	Carrier	No
89	64	B532	B524	Exon 52 Deleted	Sister	Normal	yes
90		B533		Exon 52 Deleted	Sister	Normal	
91		B534		Exon 52 Deleted	Mother	Carrier	
92	65	B535	B528	Exons 46-47 Deleted	Mother	Normal	No
93		B536		Exons 46-47 Deleted	Sister	Normal	
94	66	B537	B525	Exons 17-43 Deleted	Mother	Normal	No
95	67	B538	B529	Exons 46-50 Deleted	Mother	Normal	No
96	68	B544	B368, B369	Exon 50 Deleted	Mother	Carrier	Yes
97	69	B547	B387	Exon 46-47 Deleted	Mother	Normal	No
98	70	B576	B575	Exons 45-52 Deleted	Mother	Normal	No
99	71	B594	B593	Exons 48-50 Deleted	Mother	Normal	No
100	72	B602	B601	Exons 45-50 Deleted	Mother	Normal	No
101	73	B604	B603	Exons 46-47 Deleted	Mother	Normal	No
102	74	B608	B607	Exon 45 Deleted	Mother	Normal	No
103	75	B610	B609	Exon 45 Deleted	Mother	Normal	No
104	76	B612	B611	Exons 51-53 Deleted	Mother	Normal	No
105	77	B653	B623	Exons 45-52 Deleted	Mother	Carrier	No
106	78	B654	B625	Exon 51 Deleted	Sister	Carrier	yes
107		B655		Exon 51 Deleted	Sister	Normal	
108	79	B656	B632	Exons 47-50 Deleted	Mother	Normal	No
109	80	B657	B633	Exons 46-52 Deleted	Mother	Normal	No
110	81	B658	B642	Exons 45-52 Deleted	Mother	Normal	No
111	82	B660	B650, B651	Exons 42-43 Deleted	Sister	Carrier	yes
112	83	B664	B588	Exons 8-9 Deleted	Mother	Carrier	No
113	84	B665	B587	Exons 48-52 Deleted	Mother	Carrier	No
114	85	B667	B666	Exons 46-55 Deleted	Mother	Normal	No
115	86	B672	B671	Exon 20, Exons 45-52 Deleted	Mother	Normal	No
116	87	B678	B606, B 677	Exon 51 Deleted	Mother	Carrier	yes
117	88	B680	B679	Exons 48-50 Deleted	Mother	Carrier	No
118		B681		Exons 48-50 Deleted	Sister	Carrier	
119	89	B683	B682	Exons 45-54 Deleted	Mother	Carrier	No
120	90	B685	B684	Exons 8-44 Deleted	Mother	Carrier	No
121	91	B687	B686	Exons 45- 50 Deleted	Mother	Normal	No
122		B688		Exons 45- 50 Deleted	Sister	Normal	
123	92	B691	B690	Exons 45- 50 Deleted	Mother	Normal	No
124		B692		Exons 45- 50 Deleted	Sister	Normal	
125	93	B694	B693	Exons 10-17 Deleted	Mother	Carrier	No
126		B782		Exons 10-17 Deleted	Sister	Normal	
127	94	B696	B695	Exons 49-50 Deleted	Mother	Normal	No
128	95	B698	B697	Exon 45 Deleted	Mother	Normal	No
129	96	B699	B552	Exons 46-47 Deleted	Mother	Normal	No
130		B700		Exons 46-47 Deleted	Sister	Normal	

131	97	B717	B716	Exon 45 Deleted	Mother	Carrier	yes
132	98	B719	B718	Exon 45 Deleted	Mother	Carrier	yes
133	99	B727	B726	Exons 18-26 Deleted	Mother	Normal	No
134		B728		Exons 18-26 Deleted	Sister	Normal	
135	100	B730	B729	Exons 46-48 Deleted	Mother	Normal	No
136		B731		Exons 46-48 Deleted	Sister	Normal	
137		B732		Exons 46-48 Deleted	Sister	Normal	
138	101	B737	B736	Exons 45-52 Deleted	Mother	Carrier	NA
139	102	B739	B738	Exons 49-50 Deleted	Sister	Carrier	No
140	103	B748	B747	Exons 48-52 Deleted	Mother	Carrier	yes
141	104	B750	B749	Exons 46-48 Deleted	Mother	Carrier	No
142	105	B767	B766	Exon 8-12 Deleted	Mother	Carrier	No
143	106	B771	B770	Exon 46-47 Deleted	Mother	Carrier	yes
144	107	B780	B689	Exons 46-50 Deleted	Mother	Normal	No
145		B781		Exons 46-50 Deleted	Sister	Normal	
146	108	B784	B783	Exons 46-48 Deleted	Mother	Normal	No
147	109	B801	B800	Exons 49-52 Deleted	Mother	Normal	No
148		B802		Exons 49-52 Deleted	Sister	Normal	
149	110	B471	B465	Exons 45-47 Deleted	Sister	Normal	No

All mothers from families showing history of the disease tested positive for carrier status. Among the 109 families, mother's samples were not available for 5 and only sister's samples were available. Of these 5 families, 4 sisters were carriers and 1 was not carrier. This family where the sisters were non-carriers has been left out for calculating the number of hereditary cases in the study. Therefore, of the 92 cases which were sporadic forms, 27 families (29.3%) tested positive for carrier status and the rest 66 (70.7%) tested negative and hence could be a *de novo* mutation in the index case.

Seven sisters to index cases out of the 37 tested were positive for carrier status. Of these seven, one belonged to families where there was a family history of the disorder.

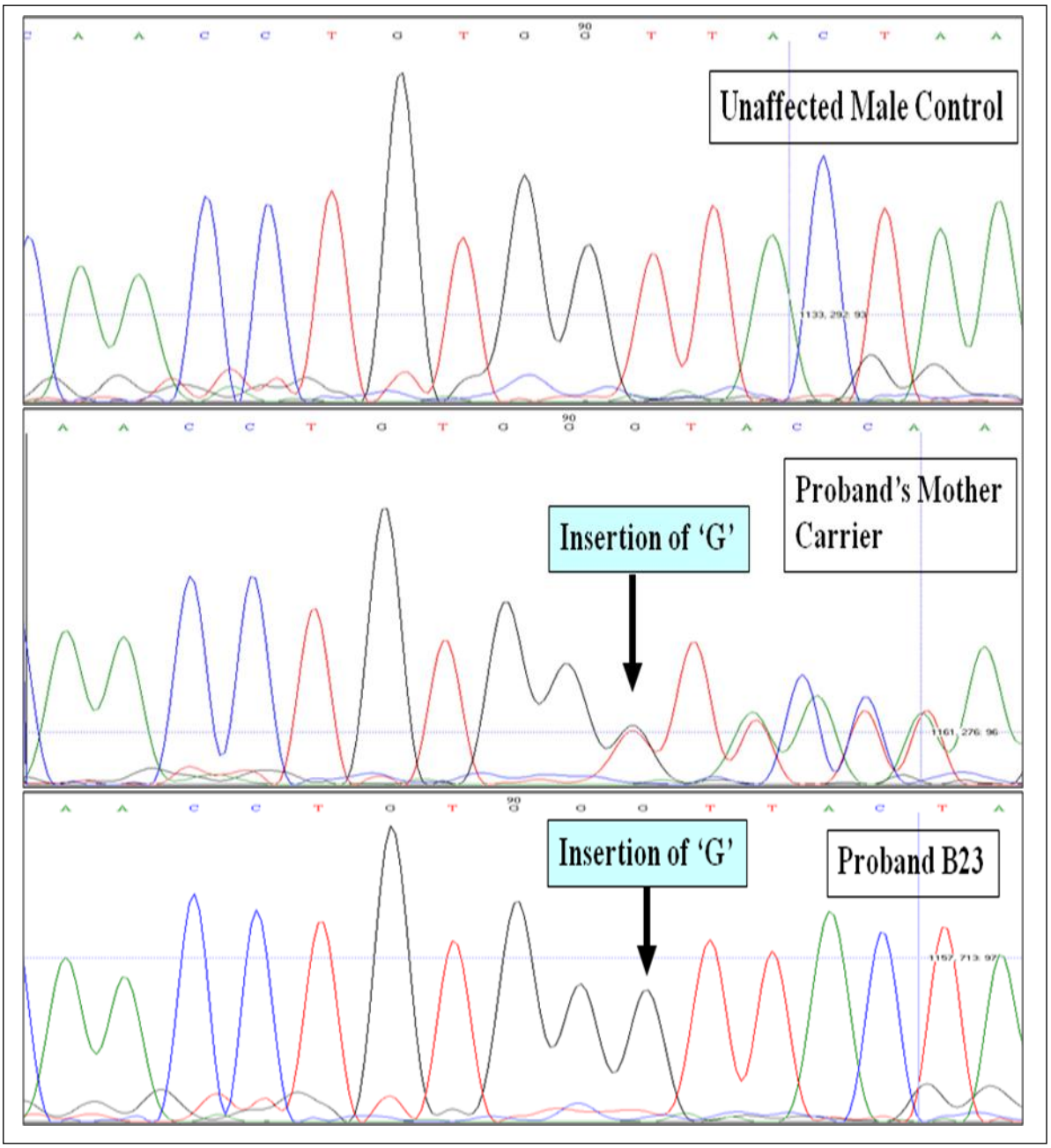


Figure 5.4: Sequencing result of a patient's mother showing point mutation in exon 51. *Insertion of G can be observed in the proband and in the mother the same mutation is observed in a heterozygous state.*

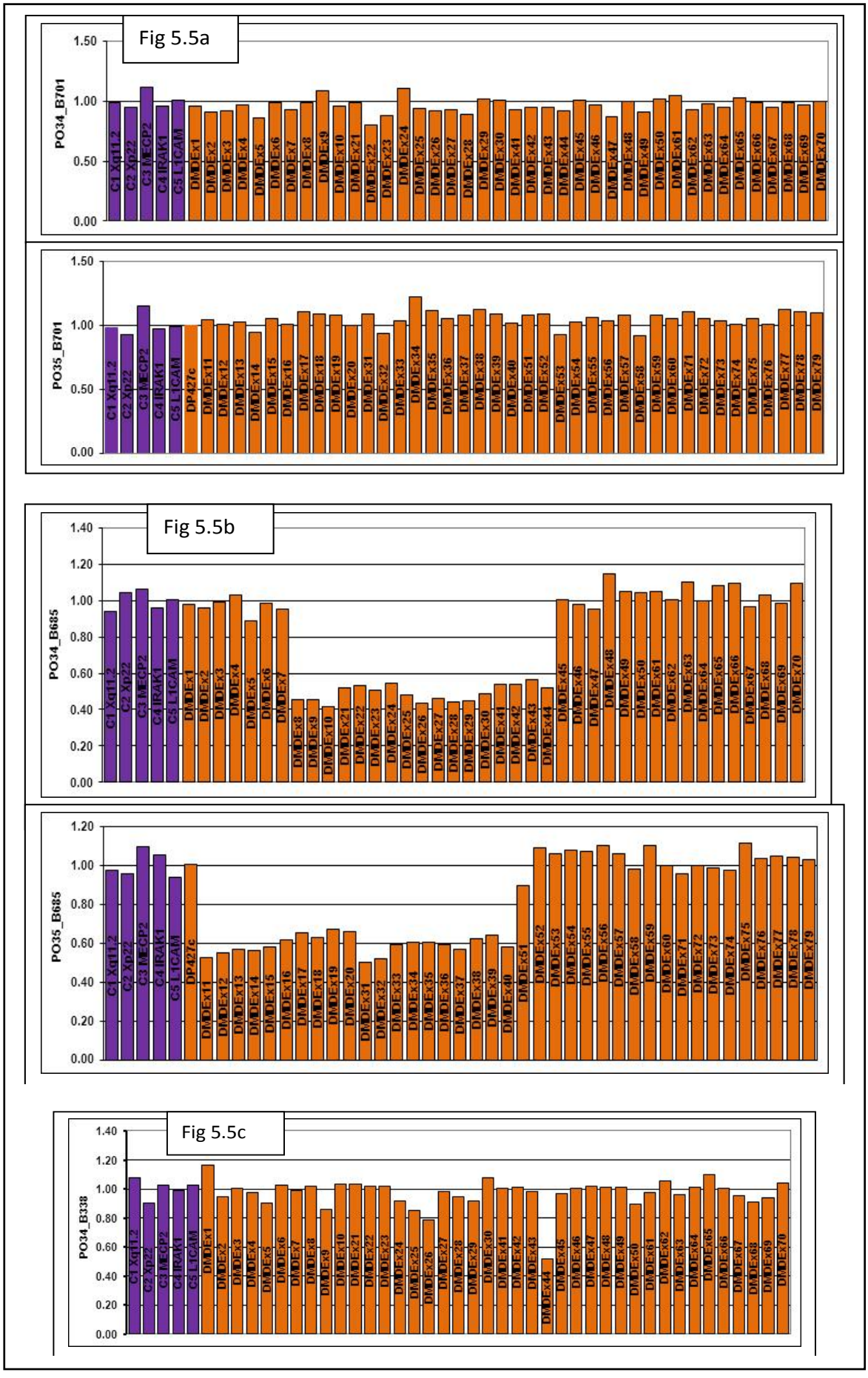


Fig 5.5a
PO34_B701
PO35_B701
Fig 5.5b
PO34_B685
PO35_B685
Fig 5.5c
PO34_B338

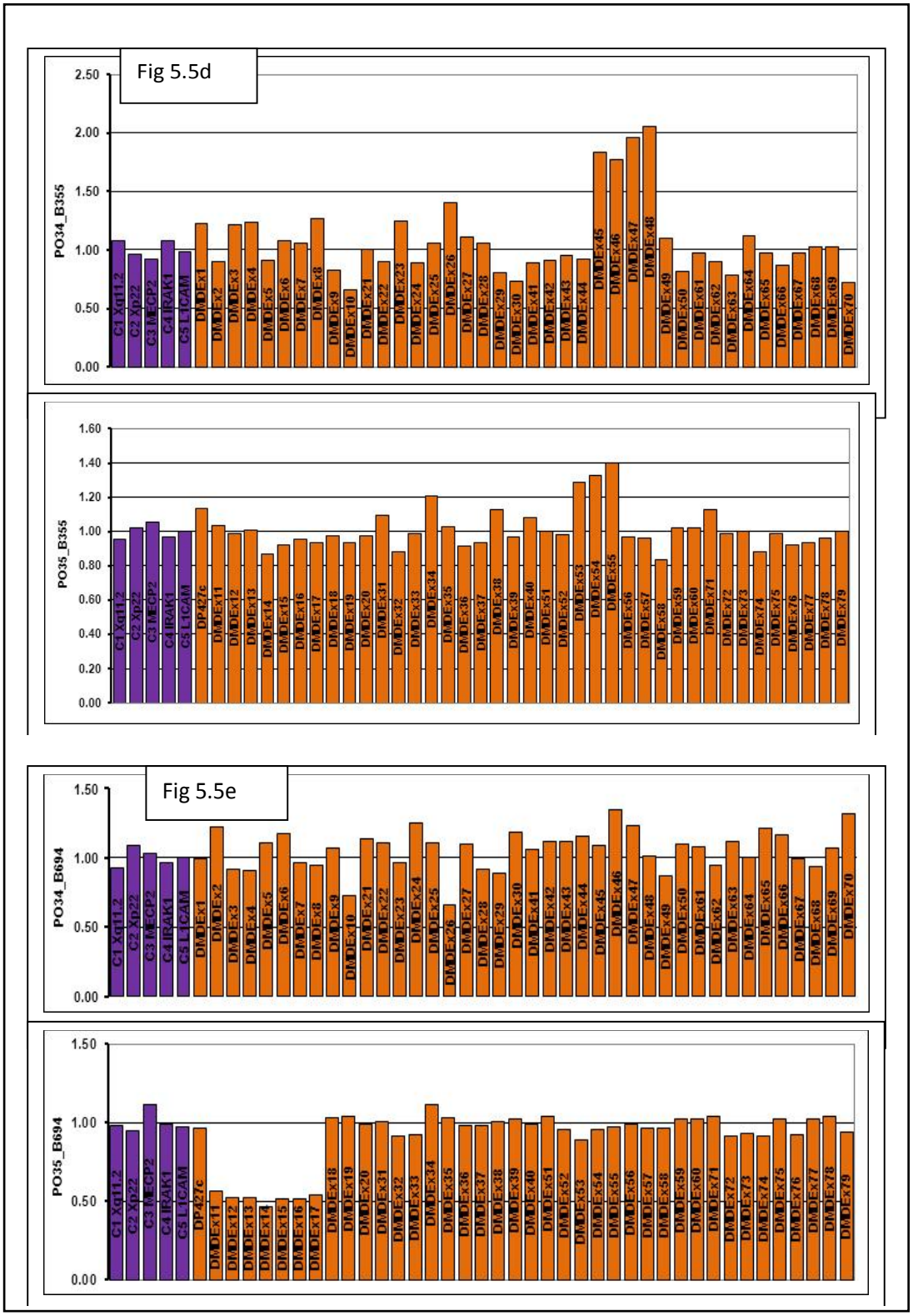


Figure 5.5: MLPA results in carrier diagnosis samples. *Blue bars represent the controls and the orange bars represent DMD exon.*

Figure 5.5a*: Sample B701 showing no deletion/duplication in both the probes, hence is normal.*

Figure 5.5b*: Sample showing heterozygous deletion of exons 8 to 44. Probe PO34 showing exons 8-10, 21-30 and 41-44 and probe PO35 showing exons 11-20 and 31-40.*

***Figure 5.5c**: Sample B338 showing heterozygous single exon deletion of exon 44 in PO34.*

***Figure 5.5d**: Sample B355 showing non contiguous duplication of exons 45-48 and 53-55 confirming a carrier of the duplication. Probe PO34 shows exon 45-48 duplication and probe PO36 shows exon 53-55 duplication.*

***Figure 5.5e**: Sample B694 showing heterozygous deletion of exons 11 to 17 confirming a carrier status.*

Hereditary nature of single and multi exon deletions was studied in this cohort and it was found that there was no difference among them when the complete data was taken into account. However, when only the mothers were taken into account it was found that multi exon deletions showed 12% more inheritance than the single exon deletions. (Table 5.5)

Table 5.4: Consolidated carrier analysis results

Relationship to proband/ No of cases	Mother	Sister	Maternal Aunt	Maternal Grandmother	TOTAL
Total cases	104	37	6	2	149
Carrier	42	7	1	0	50 (33.6%)
Normal	65	28	4	2	99 (66.4%)

Table 5.5: Inheritance of single exon and multi exon deletions for all the cases and mothers.

Inheritance of Single exon- and multi exon- deletions					
Single exon deletions			**Multi exon deletions**		
Overall cases			**Overall cases**		
n	**Carriers**	**Normals**	**n**	**Carriers**	**Normals**
31	12 (38.7%)	19 (61.3%)	109	34 (31.2%)	75 (68.8%)
Mothers			**Mothers**		
n	**Carriers**	**Normals**	**n**	**Carriers**	**Normals**
21	10 (47.6%)	11 (52.4%)	74	26 (35%)	48 (65%)

It can be observed that there is not any difference if the complete data is considered. But if only the mothers are considered single exon deletions show a higher inheritance.

5.4 DISCUSSION:

The identification of female carriers of deletions/duplications of the DMD gene is a crucial point in order to prevent the birth of children affected by DMD or BMD. Since, theoretically, about the 30% of the DMD mutations are ‘‘de novo’’, the risk of recurrence of the disease in families with a single affected male is related to the carrier or non-carrier status of the mother of the patient (Dubowitz, 1982). Although several approaches for the identification of female carriers are available, many of these cannot be readily used for routine diagnostics. In diagnostic laboratories DMD carrier has been principally based on linkage studies, initially using restriction fragment length polymorphisms , by Southern blot analysis (Bakker et al., 1986), and subsequently by PCR amplification of short tandem repeat (STR) loci (Clemens et al., 1991). Linkage studies can be used to identify deletion mutation carriers on the basis of the presence of an informative STR locus within the deletion interval, and the demonstration of either heterozygosity or apparent non-Mendelian inheritance, but are unable to determine maternal carrier status for duplication or DNA sequence mutations. Linkage results are frequently compromised by the unavailability of DNA samples, the distribution and informativeness of STRs, and the possibility of gonadal mosaicism. Hence, to resolve such cases, adjunct techniques such as fluorescence in situ hybridisation and pulsed field gel electrophoresis have been used (Den Dunnen et al., 1989; Voskova-Goldman et al., 1997; Ligon et al., 2000).

The use of MLPA for DMD carrier detection in families where the proband mutation is a deletion/duplication has been well documented (Janssen et al., 2005). There are few studies in India assessing the carrier status in DMD families using CA repeat analysis and quantitative multiplex PCR (Sinha et al., 1996; Kumari et al., 2003; Mukherjee et al., 2003; Basak et al., 2009). However, there are no studies using MLPA for carrier analysis from India. In this study, we have investigated the usefulness of the MLPA approach for the detection of female carriers of deletions/duplications of the DMD gene. We studied probable carriers from 110 families where the index case mutation was known.

The results showed that MLPA analysis is a powerful tool for the detection of female carriers in families with a DMD or BMD affected male, with known DMD gene deletion/duplication. This technique was able to pick up carrier status in cases that could not be confirmed by CA repeat analysis. Compared to qmfPCR, this technique is easier to perform with only two reactions per sample and studying all the 79 exons of the DMD gene, as opposed to the 51 exons studies by qmfPCR. It was also seen that MLPA cannot be used to pick up point mutations and direct sequencing is the only way to detect carriers in families were index case mutation is a point mutation.

Current genetic counselling practice is to cite a maternal carrier risk of two-thirds for the mother of an isolated case of DMD (Emery, 1991). This risk for an X linked disorder with early lethality assumes that there is equilibrium between mutation and selection, the mutation rates for all mutational classes observed to cause DMD are the same in the ova and the sperm, and carrier women have the same reproductive fitness as non-carrier women. Several studies have shown that the observed carrier frequency among the mothers of isolated cases in much lower that the expected theoretical value (Bakker et al., 1989; Sinha et al., 1996; Alcantara et al., 1999; Mukherjee et al., 2003). In our study it was observed that carrier frequency among the isolated cases was only 29 % and de novo mutations accounted for the rest 71%. Therefore it can be seen that the occurrence of de novo mutations among sporadic cases of DMD with is very high compared to the theoretical data (30%). Our results are consistent with other studies from India which also show a high percentage of de novo mutations among DMD cases in India (Sinha et al., 1996; Mukherjee et al., 2003). These studies from India and some other studies which showed a low carrier frequency were mainly for deletion mutations. It has been seen that the decreased carrier rates are restricted to deletion mutations and the likelihood of being a carrier for the other classes of mutation being in the theoretical range of 55-63% (Taylor et al., 2007). In our study too we observed that in deletion mutations the carrier rates were 27% as compared to the 50% among the mothers of isolated cases with duplication mutations. The mother of the isolated case with point mutation was also a carrier. Hence it can be seen that, as Peter J Taylor et al observed, the carrier risk calculations for the mother of an isolated case of DMD are not valid for deletion mutations, but do appear to be valid for other mutational classes and suggest a

basic biological difference in the effect of a deletion mutation compared with a non-deletion mutation. Or it can be related to the viability of the gametes with deletion mutations as compared to the other classes of mutations. It can also be predicted that the carrier rate in isolated cases in our study will increase if we include the other cases which did not show a deletion or duplication mutation (Table 5.6).

Table 5.6: **Inheritance of different mutation classes and carrier frequency in mothers of isolated cases.**

Mothers of isolated cases			
Mutation class	**Total cases**	**Expected carriers**	**Observed carriers**
Deletions	137	90.4	44
Duplications	11	7.3	**5**
Point mutation	1	0.7	1

It can be observed that the inheritance of deletion mutation is lesser than the expected 66%. Data on duplication and point mutation are not enough to arrive at a conclusion.

Mutation detection protocols using mPCR followed by MLPA are able to pick mutations in only 75% of DMD probands. This necessitates the importance of mutation detection protocols that could pick up all mutations in DMD probands, so that carrier detection and hence genetic counseling and prenatal diagnosis can be effective in most of the DMD affected families. Recently, MLPA assay has been suggested as the first screening test for clinically suspected DMD/BMD patients as well as for women who have a DMD/BMD family history. However, according to the best-practice guidelines for medical genetics laboratories, it is recommended that at least two independent alternative methods are available for confirmation of each genotype (Abbs et al., 2010).

5.5 CONCLUSION

- In conclusion, the MLPA approach seems to be a simple rapid reliable tool in the screening of carrier status in cases where index case mutations are deletions or duplications of the DMD gene.
- This avoids unnecessary invasive prenatal tests with the inherent miscarriage risk and emotional upheaval.
- Prenatal testing is still recommended for non-carrier mothers of an isolated male proband due to the residual chance of gonadal mosaicism.
- Confirmed carriers are able to make informed reproductive choices and undergo necessary cardiac assessments.
- Most importantly, MLPA analysis could represent a first choice method for the detection of disease-causing deletions/duplications in female relatives of affected males, especially in those cases that cannot be investigated by other approaches.

Publication:

Sakthivel Murugan S.M., Arthi C., Thilothammal N., Lakshmi B.R. Carrier Detection in Duchenne Muscular Dystrophy using molecular methods. (*In press – 2012 IJMR*)

CHAPTER 6 - DUCHENNE MUSCULAR DYSTROPHY IN FEMALES – DIAGNOSIS AND MECHANISM OF DISEASE

6.1 INTRODUCTION

DMD is expected to affect males exclusively, and autosomal recessive conditions like LGMDs are expected to affect males and females equally. **For this reason, girls presenting with a DMD-like dystrophy are diagnosed as having limb-girdle dystrophy rather than DMD.** In rare instances, females heterozygous for dystrophin mutations are also severely affected. In many cases, these females are carriers of balanced X autosome translocations that disrupt the dystrophin gene. The manifestation of DMD in these females is due to the preferential inactivation of the normal X chromosome (Boyd et al., 1986), most likely as a consequence of random X chromosome inactivation followed by selection against cells in which autosomal genes have been inactivated as a result of their proximity to inactivated X chromosome sequences. A skewed pattern of X chromosome inactivation is also seen in severely affected females with a normal appearing karyotype (Pegoraro et al., 1994). Since X chromosome inactivation occurs early in embryogenesis, this skewed pattern of X inactivation is most likely a consequence of the stochastic variation observed for a random process. It is interesting that a number of monozygotic (MZ) female twin pairs heterozygous for dystrophin gene mutations and discordant for the DMD phenotype have also been reported (Richards et al., 1990; Lupski et al., 1991; Zneimer et al., 1993). In these cases, a skewed pattern of X chromosome inactivation is observed in the affected twin, while a random or oppositely skewed pattern of X chromosome inactivation is seen in the unaffected twin. Since no cases of MZ female twin pairs concordant for the DMD phenotype have been observed, the skewed pattern of X inactivation has been proposed to be the result of random X chromosome inactivation followed by asymmetric splitting of the inner cell mass as part of the twinning process(Nance, 1990; Lupski et al., 1991). Females with Turner syndrome (45,X) are affected with DMD if they carry a dystrophin mutation on

the remaining X chromosome (Chelly et al., 1986). DMD in females has been instrumental in the mapping of the DMD gene (Lindenbaum et al., 1979; Boyd et al., 1986) and is still important since many female DMD are wrongly diagnosed as LGMD (Hoffman et al., 1992; Hoffman et al., 1996).

6.1.1 Genetic Mechanisms for Female DMD

Reported genetic mechanisms for female DMD include

(1) A skewed pattern of X-chromosome inactivation in female carriers of a DMD mutation (Azofeifa et al., 1995).
(2) X autosome translocations that disrupt the DMD gene (Cantagrel et al., 2004).
(3) Monosomy X, or Turner syndrome, associated with a DMD mutation in the remaining X chromosome (Chelly et al., 1986) and
(4) Maternal isodisomy for the X chromosome carrying a DMD mutation (Quan et al., 1997).

Katayama et al. (2006) reported a fifth mechanism in a Vietnamese child with DMD confirmed by genetic analysis (Katayama et al., 1988). Although the child was phenotypically female, the karyotype showed 46,XY, and she was found to have a mutation in the AR gene causing androgen insensitivity syndrome (AIS; 300068). The patient's sister also had the AR mutation and AIS, but did not have the DMD mutation. The unaffected mother was found to be heterozygous for the AR mutation, but did not have the DMD mutation, indicating it was de novo in the proband. Katayama et al. concluded that the co-occurrence of independent mutations in both the DMD and AR genes constituted a fifth mechanism underlying female DMD (Katayama et al., 1988).

6.1.2 Skewed X-Chromosome Inactivation:

X-inactivation (also called **lyonization**) is a process by which one of the two copies of the X chromosome present in female cell is inactivated. The inactive X chromosome is silenced by packaging into transcriptionally inactive heterochromatin. X-inactivation occurs so that the female, with two X chromosomes, does not have twice as many X chromosome gene products as the male, which only possess a single copy of the X chromosome. The choice of

which X chromosome will be inactivated is random in placental mammals such as mice and humans, but once an X chromosome is inactivated it will remain inactive throughout the lifetime of the cell and its descendants in the organism. Unlike the random X-inactivation in placental mammals, inactivation in marsupials applies exclusively to the paternally derived X chromosome. (Figure 6.1)

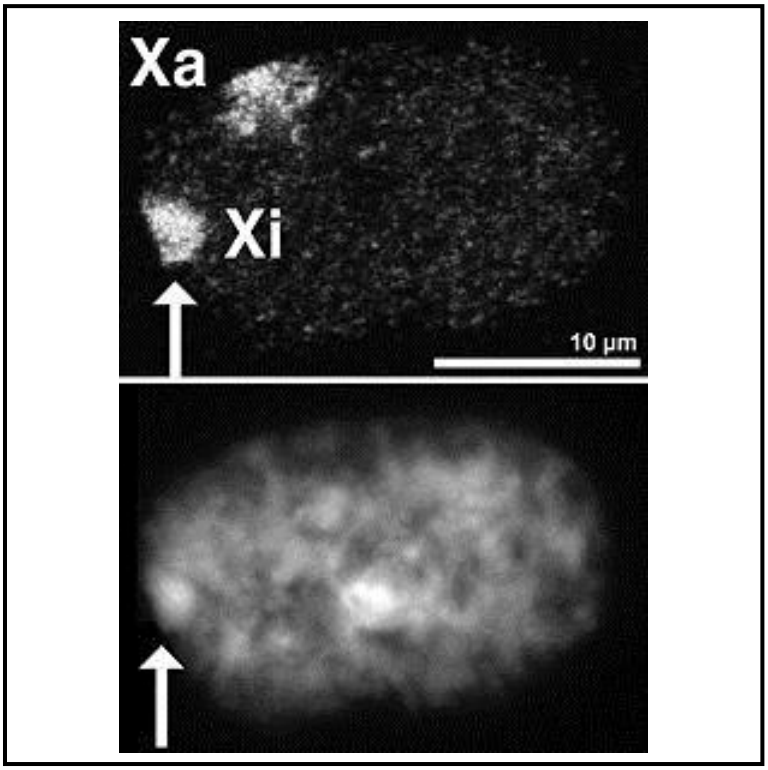


Figure 6.1: Nucleus of a human female cell. *In top panel: Both X-chromosomes are detected, by FISH. Bottom panel: The same nucleus stained with a DNA stain (DAPI). The Barr body is indicated by the arrow, it identifies the inactive X (Xi). (Adapted from R Eils et al., 1996).*

The random X-inactivation which occurs early in a developing female can result in skewed or non-random X-inactivation. This becomes important when a carrier female of an X-linked recessive condition 'randomly' inactivates the chromosome containing the unaffected gene. This can lead to mild symptoms of the disease.

Pegoraro et al. showed that more than 90% of female carriers with skewed XCI (defined as ≥ 75% of nuclei harboring the mutant *DMD* gene on the active X-chromosome) as demonstrated in blood develop moderate to severe muscular dystrophy (Pegoraro et al., 1995).

While the large majority of sex-based differential diagnoses are correct, there are expected exceptional cases. Females heterozygous for DMD have been found with "true" DMD. Many of these "manifesting carriers" have been found to have gross chromosomal rearrangements involving translocations between the X chromosome and an autosome (Boyd et al., 1986), one breakpoint invariably involving the DMD gene. While these manifesting carriers with translocations all have normal dystrophin genes on their intact X chromosomes, their normal X chromosomes appear to be preferentially inactivated.

Such a mechanism has been supported by the finding of markedly decreased levels of dystrophin in translocation-bearing manifesting carriers (Hoffman et al., 1988; Arahata et al., 1989; Arahata et al., 1989). Even in the absence of gross chromosomal rearrangements, statistical probability dictates that a very small number of females heterozygous for DMD will randomly inactivate a majority of their normal dystrophin genes and thereby manifest either typical DMD or a milder variant. Such karyotypically normal manifesting carriers are in fact observed (Nisen et al., 1986).

6.1.3 Gonadal Mosaicism and heterozygotes

Yoshioka observed unusually severely affected heterozygotes and suggested that factor(s) other than lyonization may be involved. One of the women was the product of a consanguineous mating, suggesting modification of expression by homozygosity at an autosomal locus (Yoshioka et al., 1998). Burn et al. reported monozygotic twin girls, one of whom had typical clinical features of DMD despite a normal female karyotype and the second of whom was normal. Burn et al. proposed that differences in lyonization accounted for the findings (Burn et al., 1986). Hybridization of fibroblasts from each twin with RAG-mouse cell line deficient in HPRT showed that in the affected twin it was the mother's X

chromosome that was predominantly the active one, whereas in the normal twin it was the father's. In female monozygotic twins discordant for muscular dystrophy, Richards et al. showed that there was a mutation in dystrophin in both twins. Uniparental disomy and chromosome abnormality were excluded, but on the basis of methylation differences of the paternal and maternal X chromosomes, Richards et al. concluded that uneven lyonization was the underlying mechanism for disease expression in the affected female (Richards et al., 1990).

Lupski et al. pointed out that discordance of the DMD phenotype had never been described in male monozygotic twins. Lupski et al. likewise described monozygotic twins who carried the same mutation involving duplication of exons 42 and 43 of the DMD gene (Lupski et al., 1991).One was a manifesting heterozygote, whereas the other was normal. Unlike the study of Richards et al. in which the skewed inactivation pattern was symmetrical in opposite directions, one twin being affected with DMD and the other being normal, the skew in this case involved only the affected twin, while the normal twin showed a random X-inactivation pattern (Richards et al., 1990). They suggested that the result was consistent with the model of twinning and X-inactivation proposed by Nance in that these twins probably represented asymmetric splitting of the inner cell mass (ICM), the affected twin probably arose when a small proportion of the ICM split off after lyonization had occurred (Nance, 1990). In this situation, the original ICM could have given rise to the normal twin with random lyonization, while the newly split cells would experience catch-up growth and lead to the affected twin.

Many DMD patients have rare staining dystrophin-positive fibers. The possibility of somatic mosaicism can be raised, but somatic reversion/suppression is another possibility. Indeed, the dystrophin-positive fibers have been referred to as 'revertants.' The revertants are found in both familial and non-familial cases. Klein et al. found that in patients with deletions, revertants did not stain with antibodies raised to polypeptide sequences within the deletion. These results indicated that positively stained fibers were not the result of somatic mosaicism in deletion patients (Klein et al., 1992). Klein et al. concluded that the most

likely mechanism giving rise to positively staining fibers is a second site in-frame deletion (Klein et al., 1992). Thanh et al. used exon-specific monoclonal antibodies to determine which exons are removed in order to correct the reading frame in individual revertant muscle fibers (Thanh et al., 1995). They showed that 15 revertant fibers in a DMD patient with a frameshift deletion of exon 45 had correction of the frameshift by the additional deletion of exon 44 (or perhaps exon 46 in some fibers) from the dystrophin mRNA, but not by larger deletions. This result was consistent with RT-PCR and sequencing of a minor dystrophin mRNA with an exon 43/46 junction in the biopsy. The results were consistent with somatic mutations in revertant-fiber nuclei, which result in removal of additional exons from dystrophin mRNA. However they did not clearly distinguish between additional somatic deletions and somatic effects on dystrophin mRNA splicing and both mechanisms may be operating.

Pena et al. reported an extraordinary case of DMD leading to death at age 28 years in a heterozygous monozygotic twin. Her sister was clinically normal but had an affected son (Pena et al., 1987). Eleven affected males in 3 generations and 7 separate sibships of the kindred were known. An undetected monozygotic twinning event was proposed by Glass et al. to explain a manifesting female for Becker muscular dystrophy (Glass et al., 1992). They concluded that females heterozygous for BMD have less likelihood of showing manifestations of muscular dystrophy than do females heterozygous for DMD. Abbadi et al. reported a pair of female monozygotic twins heterozygous for a deletion in the DMD gene and discordant for the clinical manifestations of the disorder (Abbadi et al., 1994). Results in lymphocytes and skin fibroblast cell lines suggested a partial mirror inactivation with the normal X chromosome preferentially active in the unaffected twin, and the maternally deleted X chromosome preferentially active in the affected twin.

Pegoraroet al. studied 13 female dystrophinopathy patients, 10 isolated cases and 3 with a positive family history for DMD in males (Pegoraro et al., 1994). All 13 had skewed X-inactivation patterns in peripheral blood DNA. Of the 9 isolated cases informative in their assay, 8 showed inheritance of the dystrophin gene mutation from the paternal germline. Only a single case showed maternal

inheritance. Pegoraro et al. estimated that the 10-fold higher incidence of paternal transmission of dystrophin gene mutations in these cases is at 30-fold variance with Bayesian predictions and gene mutation rates (Pegoraro et al., 1994). Thus they suggested that there is some mechanistic interaction between new dystrophin gene mutations, paternal inheritance, and skewed X inactivation.

Chelly et al. reported the first observation of a girl with typical DMD and typical 45,XO Turner syndrome (Chelly et al., 1986). The one X chromosome in the girl was normal by high resolution banding, but DNA analysis by Southern blotting and hybridization with 7 cloned probes mapping in the Xp21 region showed a deletion of 3 of the probes. Here, the paternal chromosome was lost and the maternal X chromosome suffered a deletion mutation in the Xp21.2 region.

6.1.4 X:autosome translocations that disrupt the DMD gene:

Female DMD patients have played a crucial role in the mapping and isolation of the DMD-gene. At a time when the localization of the DMD gene to Xp21 had not been firmly established, the identification of a range of X-autosome translocations in DMD females, (Figure 6.2, table 6.1) all disrupting the short arm of the X-chromosome at Xp21, clearly pointed to this region as harboring the gene involved in DMD (Lindenbaum et al., 1979; Boyd et al., 1986). In addition, these reports suggested that, due to the translocation, the locus on the normal X was inactivated.

Worton studied a DMD female which carried a X;21. This translocation turned out to split the large block of ribosomal RNA genes on the short arm of chromosome 21. Consequently, rRNA gene probes could be used to identify and clone the translocation junction fragment which contained both rRNA gene sequences and segments of the X-chromosome at or near the DMD locus (Ray et al., 1985). The DNA fragment derived from the X-chromosomal portion, designated XJ1.1 (DXS206), detected a TaqI-RFLP closely linked to the DMD gene and it uncovered a chromosomal deletion in a male DMD patient.

Detailed analysis of several translocation breakpoints in the DMD-gene which were characterized down to the sequence level did not reveal any clear relation with the structure or sequence of the region at or flanking the region involved (Bodrug et al., 1987; Giacalone and Francke, 1992; van Bakel et al., 1995). Frequently, the translocation seems to involve the deletion of sequences at the translocation junction. These deletions are mostly a few base pairs, Giacalone & Francke reported a 5 kb deletion in intron 16 associated with a X;4 translocation.

Table 6.1.List of Translocations in the DMD gene reported in literature

Translocation	Position in DMD gene	Cell line	Phenotype	Reference	Remark
X;8 (p11.4;q24.2)	5' of gene (754)	LOS	no DMD	(Hofker et al., 1986)	--
X;1 (p21;p34)	intron 7	WLS	DMD	Cockburn DJ (1991), thesis PhD thesis Univ. Oxford	cloned and sequenced by Cockburn (GenBank Z21689)
X;1(p21.2;q34.1-3) (invXp11.4-Xp21.2)	--	--	DMD	(Lindenbaum et al., 1979)	--
X;2 (p21;q14)	--	--	--	(Boyd et al., 1986)	--
X;2 (p21.2;q37.3)	intron 63	--	--	(Holden et al., 1986)	moderate mental retardation; cloned/ sequenced by (Bodrug et al., 1991) (GenBank M62512, M62513); paternal origin
X;3 (p21;q27)	--	--	--	(Boyd et al., 1986)	--
X;3 (p21.2;q13.3)	SfiI-GH	VSN	girl with DMD, MR and dysmorphic signs	(Boyd et al., 1986)	mother thought to be heterozygous.
X;4 (p21.1;q26)	--	--	female with DMD	(Saito et al., 1985)	--
X;4 (p21.2;q31.22)	intron 16	--	4 year girl with DMD	(Giacalone and Francke, 1992)	cloned and sequenced, includes ~5 kb deletion. De novo translocation of paternal origin
X;4 (p21;q35)	intron 51	--	--	(Bodrug et al., 1989)	cloned/sequenced by (Bodrug et al., 1991) (GenBank M62514, M62515); paternal origin
X;5 (p21.2;q31.1)	intron 51	HEM	female with DMD	(Nevin et al., 1986)	moderate MR, cloned and sequenced; cloned and sequenced by (van Bakel et al., 1995)
X;5 (p21.1;q35.3)	intron 1	LUM	female with DMD and MR	(Jacobs et al., 1981)	mapped by (Bodrug et al., 1989)

X;6 (p21;q16)	--	--	female with DMD	(Boyd et al., 1986)	--
X;6 (p21.2;q21)	SfiI-EF	EDN	female with DMD	(Zatz et al., 1981)	--
X;8 (p21.1;q24.3)	SfiI-EF	KIY	female with mild DMD	(Narazaki et al., 1985)	--
X;9 (p21;p21)	--	--	girl with DMD	(Bjerglund Nielsen and Nielsen, 1984)	Turner's syndrome, epilepsy, mental retardation
X;9 (p21.2;p22.3)	--	--	girl with DMD	(Boyd et al., 1986)	moderate mental retardation
X;11 (p21.1;q13.5)	intron 1	--	16-year-old girl	(Boyd et al., 1986)	mother not a carrier, mapped by (Bodrug et al., 1989)
X;11 (p21.2;q23.3)	SfiI-EF	LAR	female with mild DMD	(Boyd et al., 1986)	--
X;15 (p21;q26)				(Boyd et al., 1986)	parents first cousins
X;19 (p21.2;q12)	SfiI-HJ	ORI	female with DMD	(Boyd et al., 1986)	--
X;21 (p21.1;p12)	intron 7	FRA	female with mild DMD	(Verellen-Dumoulin et al., 1984) (Worton et al., 1984)	translocation splits block ribosomal RNA genes, cloned by (Ray, Belfall et al. 1985) sequenced by (Bodrug et al., 1987) (GenBank M18740 / M18023)
X;22 (p21;q13)	SfiI-EG	DEB	female with DMD	(Boyd et al., 1986)	--
X;22 (p21.2;q13.3)	3' of gene	KOG	no DMD	(Boyd et al., 1986)	--

Legend: Position in DMD gene: location of translocation breakpoint in the DMD gene; bold italics = translocation breakpoint sequenced and listed in the DMD gene mutation database. **Cell line:** as reported in Meitinger (Meitinger et al., 1988).

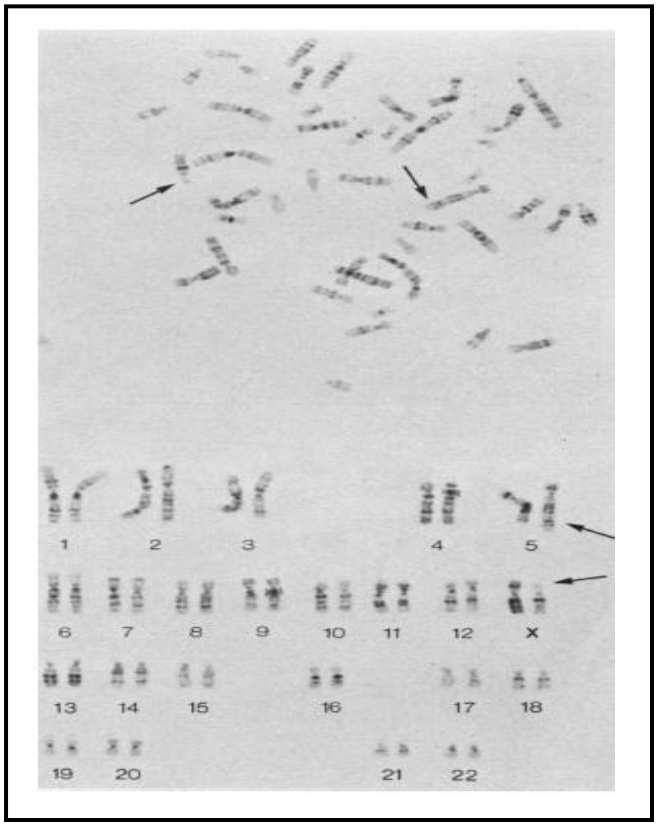


Figure 6.2: G-banded Karyotype of a patient showing X:Autosome translocation *(Adapted from Jacobs, Hunt et al. 1981) Arrows indicate X:5 translocation.*

6.1.5 Monosomy X, or Turner syndrome, associated with a DMD mutation

Turner syndrome or **Ullrich-Turner syndrome** (also known as "Gonadal dysgenesis") encompasses several conditions, of which monosomy X (absence of an entire sex chromosome) is most common. It is a chromosomal abnormality in which all or part of one of the sex chromosomes is absent (unaffected humans have 46 chromosomes, of which two are sex chromosomes). (Figure 6.3) Typical females have two X chromosomes, but in Turner syndrome, one of those sex

chromosomes is missing or has other abnormalities. In some cases, the chromosome is missing in some cells but not others, a condition referred to as mosaicism or 'Turner mosaicism'.

Occurring in 1 out of every 2500 girls, the syndrome manifests itself in a number of ways. There are characteristic physical abnormalities, such as short stature, swelling, broad chest, low hairline, low-set ears, and webbed necks. Girls with Turner syndrome typically experience gonadal dysfunction (non-working ovaries), which results in amenorrhea (absence of menstrual cycle) and sterility. Concurrent health concerns are also frequently present, including congenital heart disease, hypothyroidism, diabetes, vision problems, hearing concerns and many autoimmune diseases. Finally, a specific pattern of cognitive deficits is often observed, with particular difficulties in visuospatial, mathematical, and memory areas.

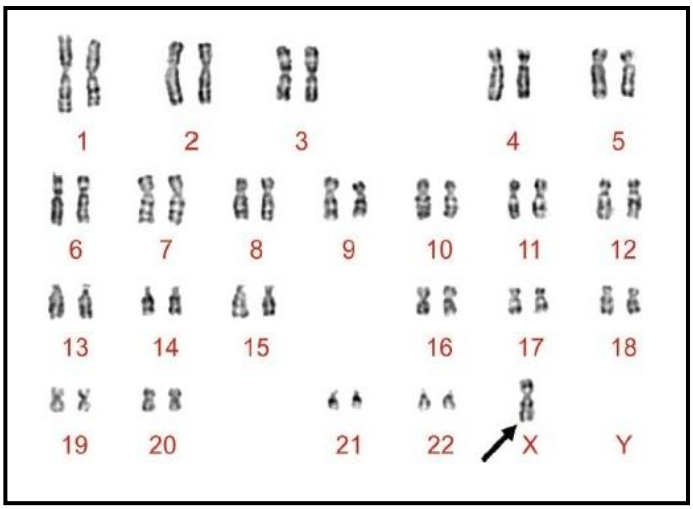


Figure 6.3: Geimsa banding of chromosome showing 45:X karyotype. *Turner's syndrome is caused due to the loss of one X chromosome in a female resulting in 45:X karyotype. (Adapted from Malini S. Suttur et al, 2009).*

Chelly et al have reported a De novo DNA microdeletion in a girl with Turner syndrome and Duchenne muscular dystrophy. There have been few other reports of DMD carrier diagnosis involving female with mosaic Turner's syndrome with a DMD gene deletion on the other X-chromosome (Chelly et al., 1986).

6.1.6 Maternal isodisomy for the X chromosome carrying a DMD mutation

Uniparental disomy (**UPD**) occurs when a person receives two copies of a chromosome, or part of a chromosome, from one parent and no copies from the other parent.

UPD can occur as a random event during the formation of egg or sperm cells or may happen in early fetal development. It can also occur during trisomic rescue. (Figure 6.4)

- When the child receives two (different) homologous chromosomes (inherited from both grandparents) from one parent, this is called a **heterodisomic** UPD. Heterodisomy (heterozygous) indicates meiosis I error.

- When the child receives two (identical) replica copies of a single homolog of a chromosome, this is called an isodisomic UPD. Isodisomy (homozygous) indicates either a meiosis II or post zygotic chromosomal duplication.

Quan et al have reported a case of a karyotypically normal female affected with DMD as a result of homozygosity for a deletion of the dystrophin gene. Homozygosity for the dystrophin gene deletion was the result of maternal isodisomy of the entire X chromosome (Quan et al., 1997).

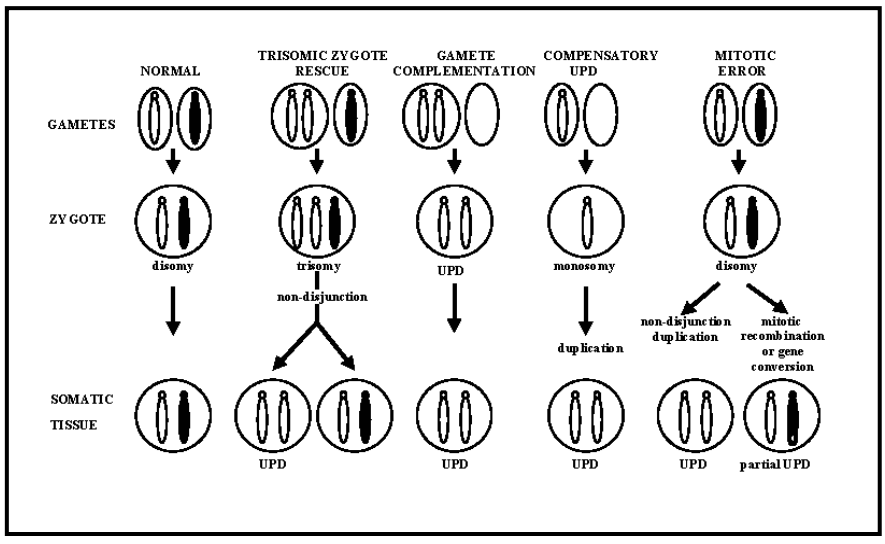


Figure 6.4: Seggregation of chromosomes seen in uniparental isodisomy. *Figure shows the mechanisms by which non-disjuction leading to uniparenatal disomy can happen. Trisomy or monosomy rescue may lead to somatic tissues having chromosomes from the same parent. (Adapted from Spence et al., 1988).*

There are no reports from India describing female cases with Duchenne muscular dystrophy. Here we describe the diagnostic strategies and methods used for identifying the mechanism in a female clinically diagnosed as Duchenne muscular dystrophy. Molecular diagnosis further led to the clinical characterization and diagnosis of the disorder in the child.

6.2 MATERIALS AND METHODS

6.2.1 Samples

An eight year old girl child was sent to our facility for DMD molecular diagnosis. Clinically she was diagnosed to have DMD or LGMD. She presented with developmental delay, mental and growth retardation along with proximal muscle weakness and calf hypertrophy. Her CPK value at the time of diagnosis was 648 IU/L. She was an isolated case born of a non-consanguineous marriage and there was no history of the disorder in the family (See figure 6.5). Her parents first saw her symptoms at the age of 3 years and she was ambulant when she visited the centre.

Blood sample was collected from her after obtaining informed consent from her parents and DNA was extracted as described in methods section and stored for further analysis.

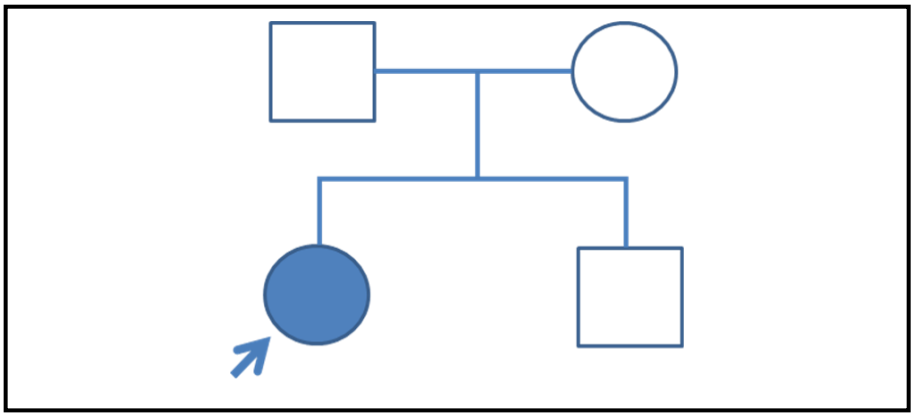


Figure 6.5: Pedigree showing the affected female as the isolated case with no family history. *The brother was unaffected at the age of 7 years.*

6.2.2 Multiplex PCR

Multiplex PCR for the 30 exons of the DMD gene was done as described in methods section.

6.2.3 Native PCR for DMD exon 62

PCR for exon 62 was conducted using the primer set given in the materials and methods and the following protocol was used. (Table 6.2)

Table 6.2: PCR reaction protocol for DMD exon 62

Reagent	**Volume (μL) for 1X Reaction**
10X PCR Buffer	2.5
$MgCl_2$ Solution	2.5
DNTP Mix	1.0
Taq Polymerase	0.1
D. Water	15.9
Working Primers (1uL each of the forward and reverse primers of each exon)	2.0
DNA (250 ng/μL)	1.0
Total	**25.0**

Table 6.3: PCR reaction conditions for DMD exon 62

Steps	**Temperature**	**Time**	**Cycles**
Initial Denaturation	94^{o}C	180 seconds	
Denaturation	93^{o}C	60 seconds	28 Cycles
Annealing	60^{o}C	45 seconds	
Elongation	65^{o}C	60 seconds	
Final Elongation	65^{o}C	600 seconds	
Hold	4^{o}C	Hold	

The products were run in 2% agarose gel and viewed in BIORAD gel documentation system.

6.2.4 Multiplex Ligation-dependent Probe Amplification was done as per manufacturers guidelines described in methods section.

6.2.5 Gene Sequencing

Gene sequencing for DMD exon 62 was done as described in methods section.

6.2.6 Karyotyping by GA banding

Cytogenetic analysis was done by outsourcing the sample. The analysis results were sent to us, which is discussed in the results.

6.2.7 Microarray based Cytogenetic analysis

Microarray based cytogenetic analysis was done using the 2.7M Affymetrix Cyto array as described in chapter 2.

6.3 RESULTS

6.3.1 Molecular genetic work up

The first step in the diagnosis of DMD in males is the use of **Multiplex PCR** to look for hot spot whole exon deletions. Multiplex PCR for this girl did not reveal any deletion of the tested 30 exons in the hot spot regions. This led us to believe that this might just be a case of LGMD. But the clinician who sent us the case believed the clinical symptoms suggested DMD and wanted further genetic analysis to be done.

MLPA, the second step in DMD diagnosis as per our algorithm, was done on this sample. MLPA showed a deletion of exon 62 of the DMD gene (Figure 6.6). The table 6.4 shows the peak height and peak area obtained for the various exons of the DMD gene and the control loci for probe set PO34 and table 6.5 for probe set PO35. The exons tested in each of the probes are arranged in the ascending order as required by the Andrew's software used for analysis, followed by the control probes. From this data a few crucial observations were made which helped in downstream analysis.

Table 6.4: Peak size, height and area of the different exons tested in MLPA probe set PO34.

Sample File Name	Marker	Size	Height	Area
P034-sample_O126.fsa	Exon1	135.03	4364	48115
P034-sample_O126.fsa	Exon2	168.32	3228	35557
P034-sample_O126.fsa	Exon3	208.24	3067	34997
P034-sample_O126.fsa	Exon4	240.39	2569	31027
P034-sample_O126.fsa	Exon5	280.85	1883	22847
P034-sample_O126.fsa	Exon6	314.39	1375	16974
P034-sample_O126.fsa	Exon7	353.26	1610	21096
P034-sample_O126.fsa	Exon8	384.46	1912	25304
P034-sample_O126.fsa	Exon9	423	738	10664
P034-sample_O126.fsa	Exon10	456.07	912	13091
P034-sample_O126.fsa	Exon21	152.7	4577	51813
P034-sample_O126.fsa	Exon22	185.04	2932	31906
P034-sample_O126.fsa	Exon23	222.81	3057	34487
P034-sample_O126.fsa	Exon24	255.49	1800	21449
P034-sample_O126.fsa	Exon25	297.77	1557	19211
P034-sample_O126.fsa	Exon26	329.64	1530	19536
P034-sample_O126.fsa	Exon27	368.8	1127	14946

P034-sample_O126.fsa	Exon28	400.75	1338	18716
P034-sample_O126.fsa	Exon29	439.38	912	12927
P034-sample_O126.fsa	Exon30	471.76	695	10211
P034-sample_O126.fsa	Exon41	142.91	3737	41497
P034-sample_O126.fsa	Exon42	177.63	3152	35458
P034-sample_O126.fsa	Exon43	215.27	2607	30018
P034-sample_O126.fsa	Exon44	248.21	2390	28089
P034-sample_O126.fsa	Exon45	288.81	2108	25906
P034-sample_O126.fsa	Exon46	322.03	1730	21246
P034-sample_O126.fsa	Exon47	359.67	1514	19382
P034-sample_O126.fsa	Exon48	392.38	1112	15267
P034-sample_O126.fsa	Exon49	430.36	1121	16300
P034-sample_O126.fsa	Exon50	462.74	529	7438
P034-sample_O126.fsa	Exon61	160.78	2590	29599
P034-sample_O126.fsa	**Exon62**	**192.75**	**256**	**3052**
P034-sample_O126.fsa	Exon63	232.68	2374	27718
P034-sample_O126.fsa	Exon64	264.19	3399	40047
P034-sample_O126.fsa	Exon65	305.36	2009	24798
P034-sample_O126.fsa	Exon66	338.98	1295	16576
P034-sample_O126.fsa	Exon67	376.19	1515	20142
P034-sample_O126.fsa	Exon68	407.25	1159	16142
P034-sample_O126.fsa	Exon69	446.58	1053	15445
P034-sample_O126.fsa	Exon70	478.62	578	8619
P034-sample_O126.fsa	XFrag	96.77	3474	39444
P034-sample_O126.fsa	XFrag	100.52	3157	32539
P034-sample_O126.fsa	Xq11.2	126.11	3406	36462
P034-sample_O126.fsa	Xp22	201.95	1640	17647
P034-sample_O126.fsa	Xq28	272.35	1820	23247
P034-sample_O126.fsa	Xq13	415.37	1384	19188
P034-sample_O126.fsa	Xq28b	484.95	661	9857
P034-sample_O126.fsa	**YFrag**	**104.93**	**320**	**2724**
P034-sample_O126.fsa	**YFrag2**	**114.84**	**206**	**2209**

The highlighted data is that of exon 62 showing small values for peak height. The data showing values for probes for Y chromosomes are also boldened.

Table 6.5: Peak size, height and area of the different exons tested in MLPA probe set PO35.

Sample File Name	Marker	Size	Height	Area
P035-sample_O126.fsa	Exon11	135.14	4308	49538
P035-sample_O126.fsa	Exon12	170.23	3961	45872
P035-sample_O126.fsa	Exon13	208.63	3758	43097
P035-sample_O126.fsa	Exon14	240.86	1606	19365
P035-sample_O126.fsa	Exon15	279.94	3628	45710
P035-sample_O126.fsa	Exon16	313.81	1452	19020
P035-sample_O126.fsa	Exon17	349.83	1320	18243
P035-sample_O126.fsa	Exon18	384.4	1316	18800
P035-sample_O126.fsa	Exon19	422.01	979	14753
P035-sample_O126.fsa	Exon20	456.09	917	13935
P035-sample_O126.fsa	Exon31	153.2	4877	54582
P035-sample_O126.fsa	Exon32	186.55	3039	34680
P035-sample_O126.fsa	Exon33	225.34	2980	35102
P035-sample_O126.fsa	Exon34	256.37	2152	26860
P035-sample_O126.fsa	Exon35	295.57	2612	33965
P035-sample_O126.fsa	Exon36	330.85	1881	25548
P035-sample_O126.fsa	Exon37	368.77	1134	15772
P035-sample_O126.fsa	Exon38	398.37	1414	20717
P035-sample_O126.fsa	Exon39	439.61	1126	17556
P035-sample_O126.fsa	Exon40	470.71	599	9386
P035-sample_O126.fsa	Exon51	143.61	4133	46792
P035-sample_O126.fsa	Exon52	178.3	2156	24859
P035-sample_O126.fsa	Exon53	217.03	2900	34732
P035-sample_O126.fsa	Exon54	248.46	2452	29301
P035-sample_O126.fsa	Exon55	288.67	2168	28233
P035-sample_O126.fsa	Exon56	321.99	1601	21393
P035-sample_O126.fsa	Exon57	358.12	1591	22306
P035-sample_O126.fsa	Exon58	392.89	1429	20827
P035-sample_O126.fsa	Exon59	430.72	1572	24086
P035-sample_O126.fsa	Exon60	463.17	1074	17148
P035-sample_O126.fsa	Exon71	162.1	3510	39861
P035-sample_O126.fsa	Exon72	194.28	1938	22161
P035-sample_O126.fsa	Exon73	232.88	2416	28883
P035-sample_O126.fsa	Exon74	264.24	1234	15463
P035-sample_O126.fsa	Exon75	305.31	2253	29449
P035-sample_O126.fsa	Exon76	339.6	1284	17388
P035-sample_O126.fsa	Exon77	376.04	901	13262
P035-sample_O126.fsa	Exon78	406.7	1241	18475
P035-sample_O126.fsa	Exon79	447.41	809	12592
P035-sample_O126.fsa	Xfrag	96.77	3675	45077
P035-sample_O126.fsa	Xfrag	100.49	2591	28595

P035-sample_O126.fsa	Xq11.2	126.1	2702	29182
P035-sample_O126.fsa	Xp22	202.04	1625	18545
P035-sample_O126.fsa	Xq28a	272.28	1790	22926
P035-sample_O126.fsa	Xq13	415.25	1358	20339
P035-sample_O126.fsa	Xq28b	484.84	592	9711
P035-sample_O126.fsa	ExonDP427C	479.45	573	9116
P035-sample_O126.fsa	**Yfrag**	**104.9**	**282**	**2884**

The data showing values for probes for Y chromosomes are boldened.

When this data was analysed using the Andrew's software, the results obtained can be seen in the figure 6.6. Results for both the probe sets passed the QC as can be seen by the even peaks obtained for the control probes. Probe set PO34 showed deletion of exon 62 and probe set PO35 showed no deletion or duplication. Following are the crucial observations from the MLPA results obtained for this sample;

1. Exon 62 of the DMD gene is deleted.
2. This deletion is not complete, as can be seen in the fig 5.5. There is a small peak suggesting a mild amplification of exon 62.
3. This can be also seen in the table 6.4 where exon 62 shows small values of peak height and peak area.

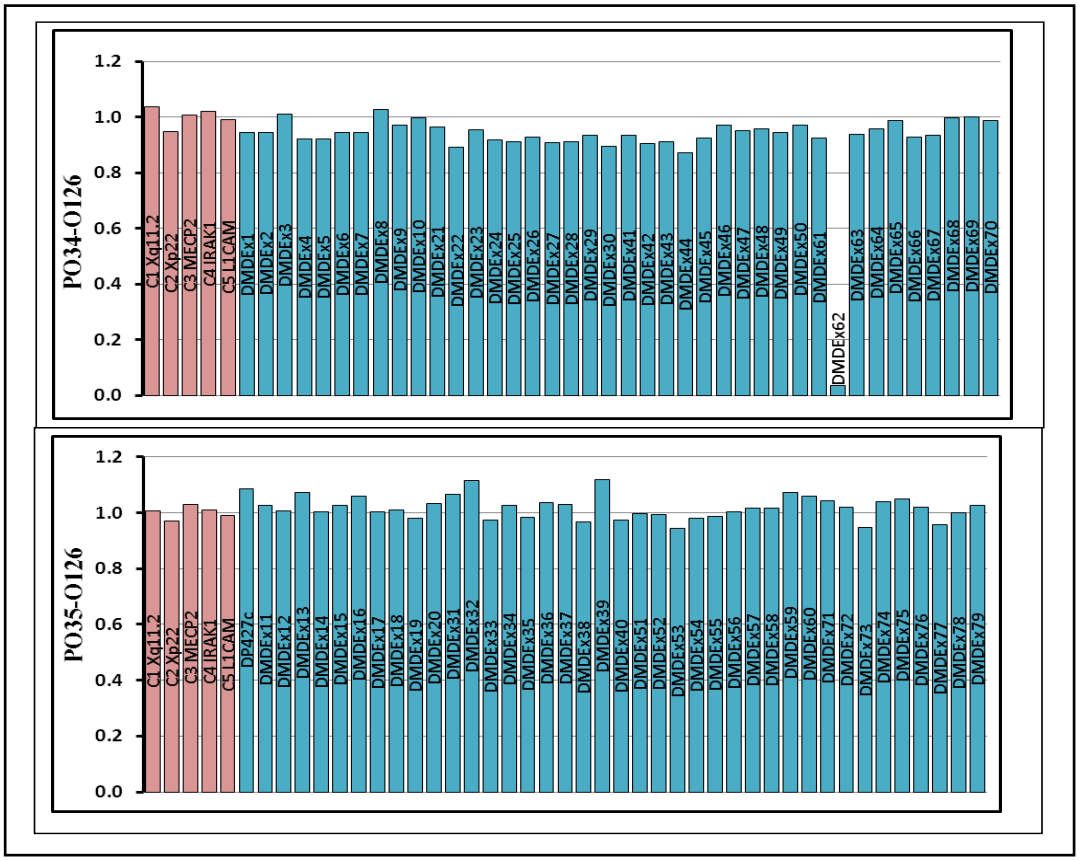


Figure 6.6: MLPA results for probes PO34 and PO35 showing a deletion of exon 62 of the DMD Gene. *It can be onserved that exon 62 is deleted but still shows a small peak height corresponding the value obtained.*

It can also be observed that the probes for Y controls also show peak height and peak area values. Samples from males will show a high peak height value for the Y control, and females should not show any amplification for this probe.

The assay was repeated twice with repeat samples to ensure the correctness of the data. The same results were seen uggesting these observations are true and not due to technical errors or contaminations.

However, since a single exon deletion in MLPA can also be due to point mutations in the probe-binding site, we did native PCR to confirm the deletion of exon 62.

6.3.2 PCR for exon 62

PCR for exon 62 of DMD gene on this sample showed no deletion (figure 6.7). However, quantitative PCR revealed that the band intensity is lesser that the control sample.

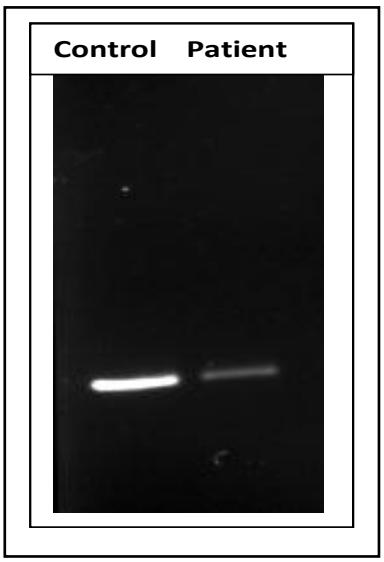


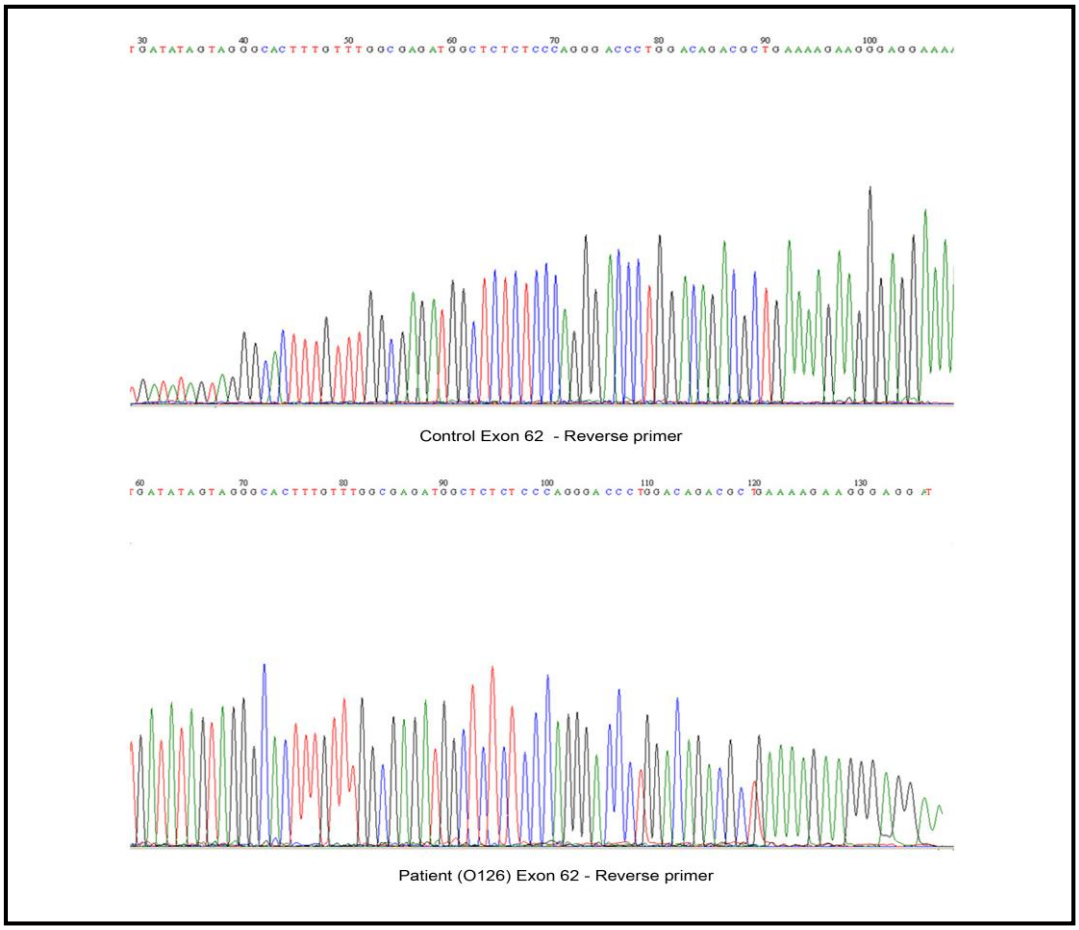
Figure 6.7: Quantitative PCR for exon 62 of DMD gene for a female control and the patient sample. *In both control (lane 1) and patient (lane 2) samples are showing bands, but the intensity of the control band is much higher than that of the patient's.* ***Lane 1 – Female control, Labe 2 – Patient.***

No deletion in native PCR and deletion in MLPA suggest a point mutation at the MLPA probe binding site. To test this, direct sequencing of the DMD exon 62 was done.

6.3.3 DMD exon 62 sequencing

The patient sample and a control sample were run for both forward and reverse primer and the data obtained was viewed using the BioEdit software, freely available online and the sequence data was compared with reference DMD gene sequence (GenBank NM_004006.1). There was no point mutation in the exon 62 of the DMD gene confirming exon 62 deletion in MLPA was not due to point mutations in the probe binding site (Figure 6.8)

At this point, the above results of MLPA and native PCR suggested that there could be a mosaicism in the sample, of normal and deleted alleles, which caused this discrepancy in mPCR and MLPA results. The small peak of exon 62 in MLPA and the faint band in mPCR also supported our views.



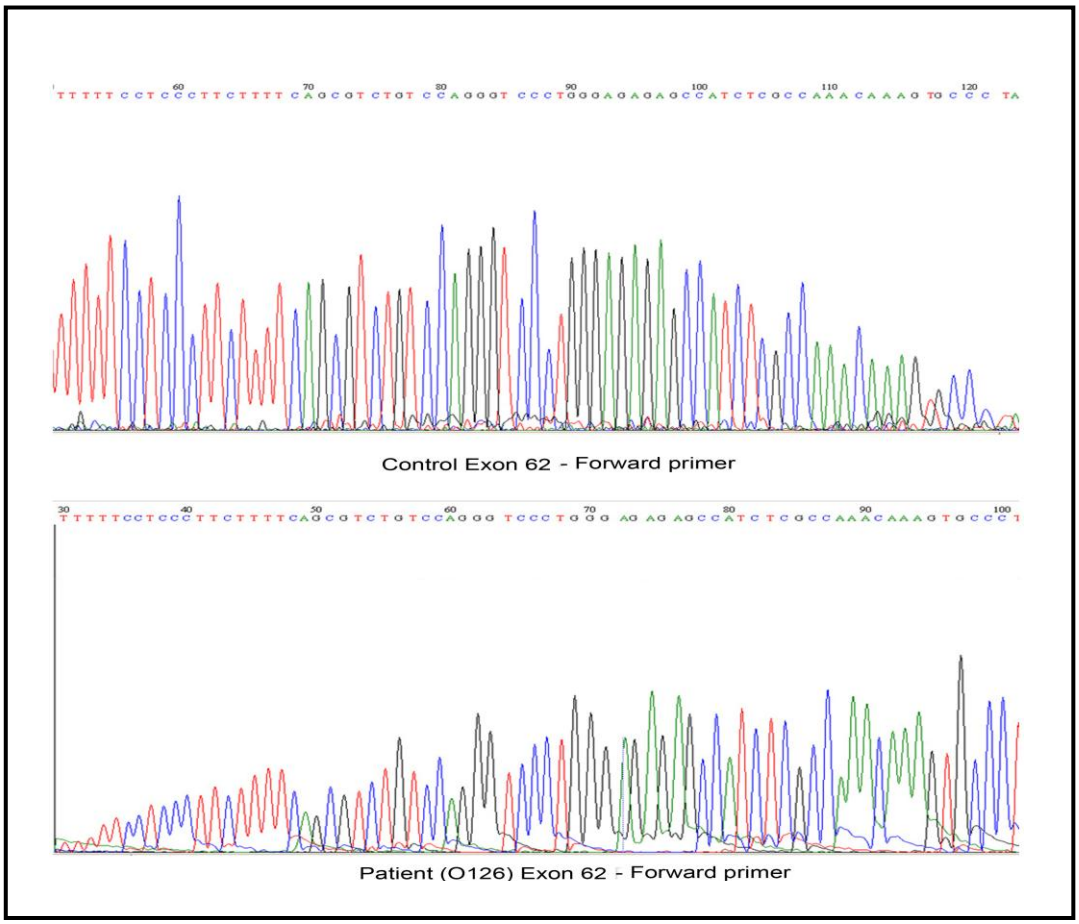


Figure 6.8: Direct sequencing of exon 62 forward and reverse primers for patient sample and control. *No mutation was seen in the patients as compared to the control and to the database.*

At this point it appeared that the molecular diagnosis for this case was complete, and the diagnosis was confirmed as DMD. However, there were a few unanswered questions like the discrepancies between the MLPA and native PCR results, and the amplification of the Y-control probes in MLPA. The mechanism of DMD was also unclear. If this was a homozygous deletion, which MLPA suggested, this could be either due to Turner's syndrome where the DMD gene in the single X-Chromosome was mutated, or Uniparental isodisomy where both the X-chromosome may harbor the same mutation. If this was a heterozygous deletion, as suggested by PCR for exon 32, the possible cause could be a X; autosome translocation or X-chromosome inactivation. To test this, karyotyping was done.

6.3.4 Chromosomal analysis by GA banding

We visited the patient again for fresh blood sample for lymphocyte culture and karyotyping. Blood was collected in Heparin and the samples were sent to a diagnostic lab for karyotyping. Karyotyping was done as given in materials and methods. The results of cytogenetic analysis are shown in figure 6.9. Chromosomal analysis showed mosaic for 45, X (85% of cells) and 46, XY (15% of cells). FISH was not done for this patient to confirm the GA banding results due to non-availability of sample.

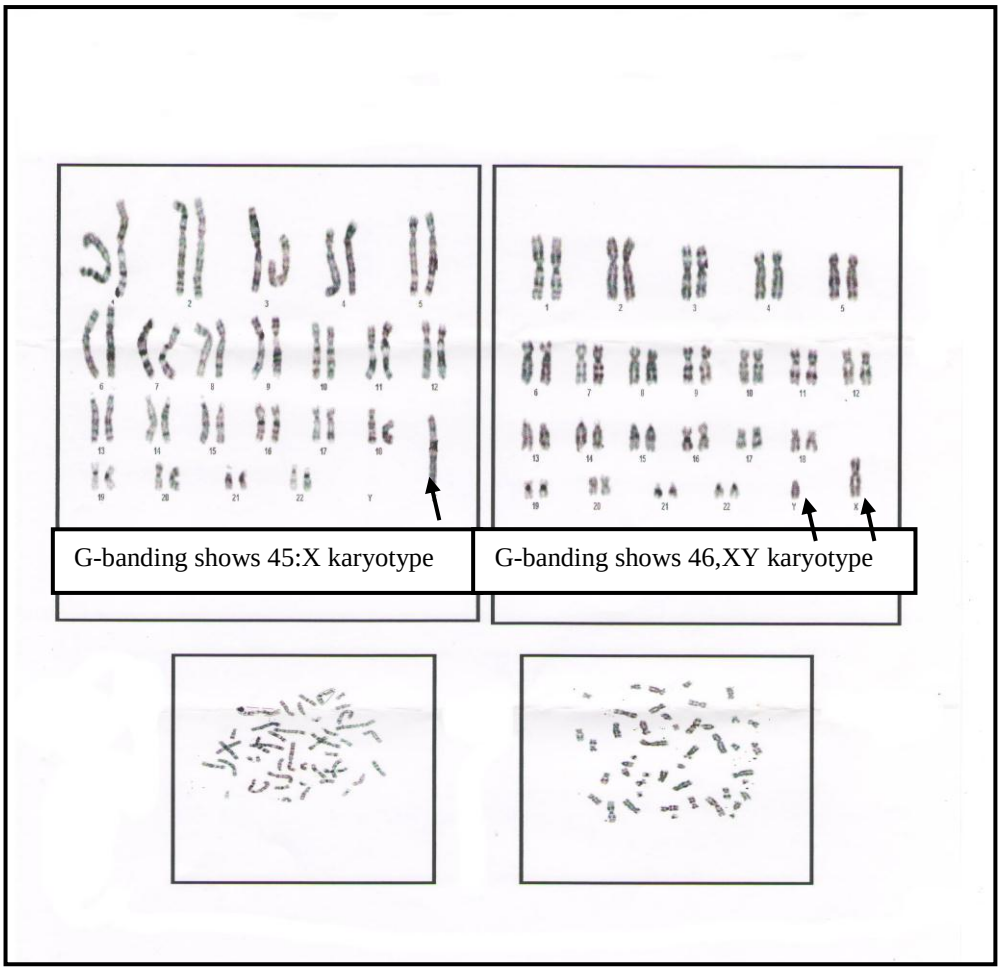


Figure 6.9: Karyotyping of the patient sample by Geimsa banding. *Two spreads, one with 45:X karyotype and one with 46:XY karyotype can be observed (shown in arrows).*

However, as mentioned earlier Y-probes in MLPA analysis showed the presence of Y chromosome. Also, even on repeated MLPA analysis, the small peak which

was not consistent with heterozygous deletion, was observed for exon 62 which was suggestive of a mosaic. The faint band in PCR for exon 62 and small peak in MLPA confirmed the presence of mosaic seen in chromosomal analysis.

Since a repeat sample for this child was not possible, we attempted to check for the mosaicism using microarray based cytogenetic analysis.

6.3.5 Microarray based Cytogenetic analysis

Cytogenetic array analysis was done using the 2.7M array from Affymetrix as described by the manufacturer, the results of which can be seen in figure 6.10. Cytoarray analysis confirmed the deletion of exon 62 and the absence of one X-Chromosome (45,X).

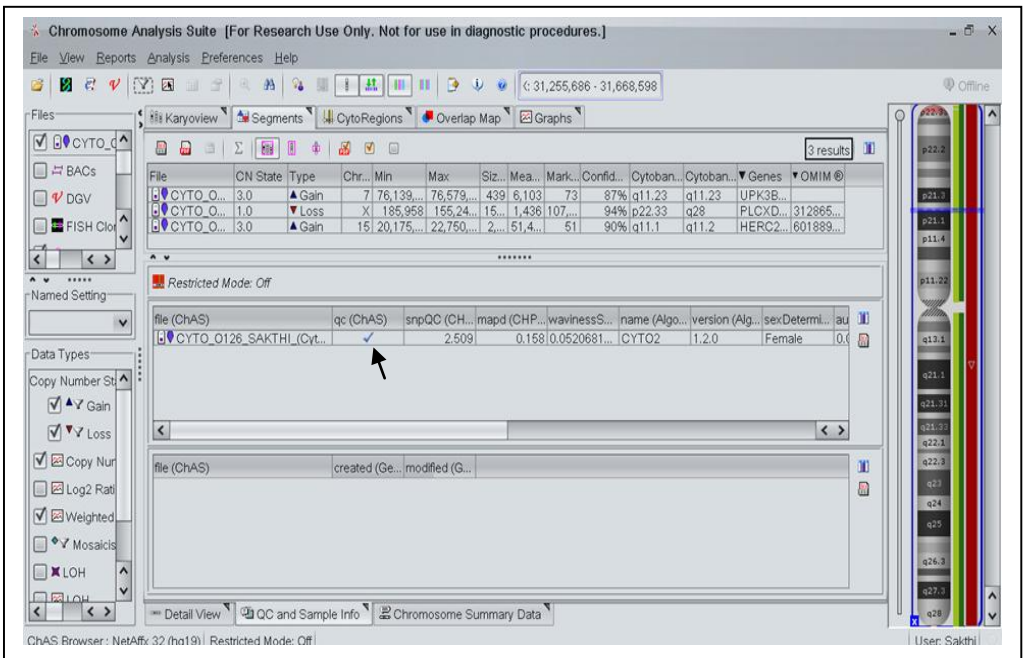


Figure 6.10a: ChAS full screen view showing segments table with lost and gained segments. *The figure shows that the analysis has passed QC (arrow) and the segment view of the ChAS software.*

But the mosaic involving 46, XY was not picked up by the array, even though probes for Y-chromosome are present. This may be due to the fact that the analysis is sensitive to mosaic of more than 20% only. (www.affymetrix.com)

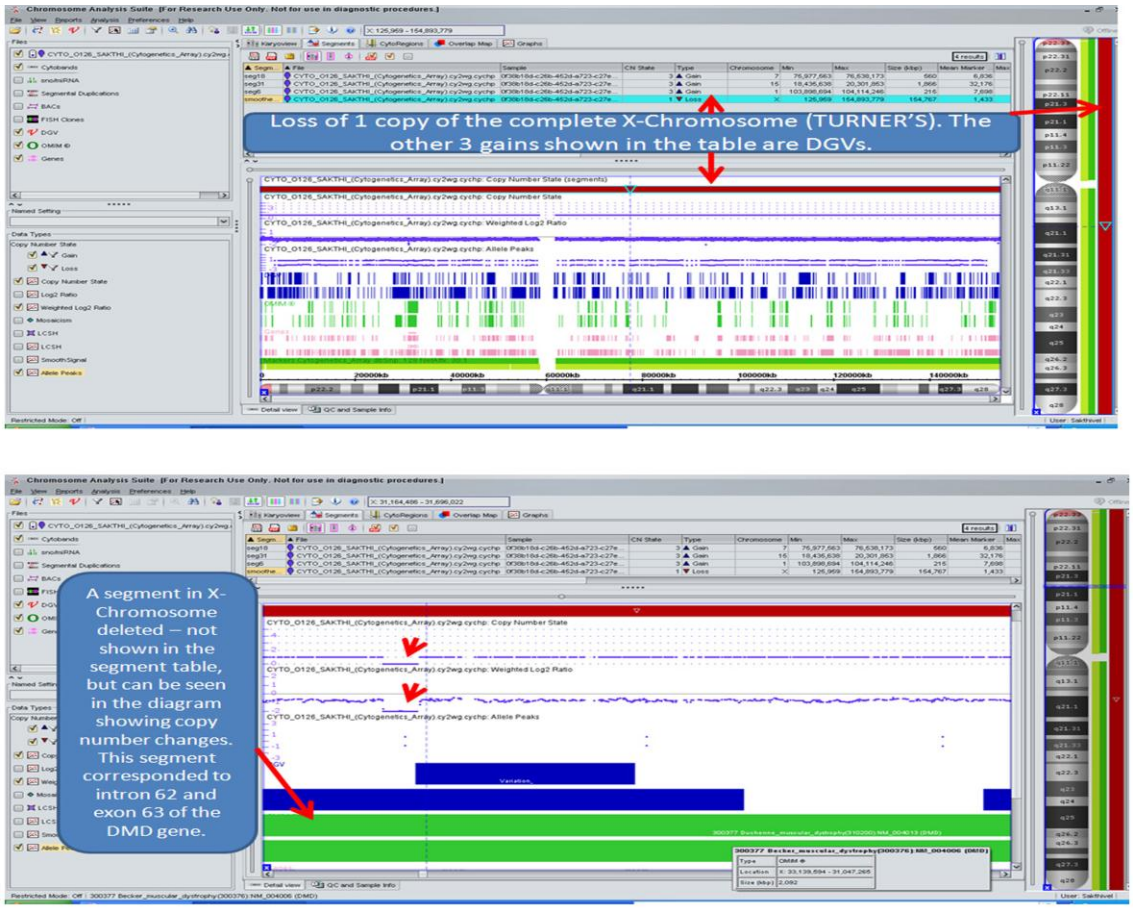


Figure 6.10 b: ChAS full screen view showing the complete loss of X-chromosome and X-Chromosome copy number to be 1. Also shown below is the deletion of the DMD gene.

The coordinates of the deleted region mapped to the exon 62 of the DMD gene. At this point of time molecular diagnosis and the mechanism of DMD for this girl was almost complete. This child was diagnosed to have DMD caused due Turner's syndrome with the DMD gene in the only X-chromosome mutated. Additionally the girl was also found to have a mosaicism involving 45,X and 46,XY.

Above results suggested that this could be a case of 45,X/46,XY mosaicism with DMD gene mutation. A wide spectrum of phenotypic manifestations of 45,X/46,XY mosaicism have been reported (Telvi, Lebbar et al. 1999; Canning 2000; Fernandez-Garcia, Garcia-Doval et al. 2000; Nishi, Domenice et al. 2002),

this being the first report associated with DMD. Hence, a complete clinical analysis for the child was done further to confirm our molecular results.

6.3.6 Clinical work up

At the time of initial diagnosis the child was eight years old. She presented with developmental delay, mental and growth retardation, proximal muscle weakness, muscle hypertrophy (Calf, pectoral). Her CPK at the time of diagnosis was 648 IU/L. Thyroid function tests were normal. However when the complete molecular diagnosis was done including SNP microarray, the child was 11 years old. She underwent complete clinical work up pertaining to Duchenne muscular dystrophy and Turner's syndrome (www.genereviews.com). A complete multidisciplinary evaluation by neurologists, orthopedecian, physiatrist, pulmonologist and gynecologist was done. At the time of evaluation, she had not attained menarche.

The following are the salient points of her clinical examination;

General examination:

- Family and sibling history nil.
- Bowels and micturition normal.
- No anemia, no edema.
- Height – 99 cms. Weight – 16.8 Kg.
- **Breasts – Areola with nipple budding were seen.**
- Soft divarication of recti was seen.
- **Pseudohypertrophy of calf seen.**
- **No secondary sexual characters.**
- L/E of external genetalia – Normal.

General pediatric examination:

- **Development – All motor milestones delayed, speech delayed, bladder and bowel delayed.**
- **Frequent falls since 7 years age.**
- Delayed dentition seen.

- Dependent on care taker.
- **Difficulty in – Climbing stairs, getting up from sitting position, walking, reaching for objects overhead, in mixing food, bringing food to mouth, buttoning and unbuttoning.**
- **Short stature, pectus excavation.**
- **Waddling gait.**
- **Gower's sign positive.**
- Late ambulatory stage.

Physiatry:

- **Low muscle strengths – Upper and lower limbs. Muscle score of 3 and less than 3 in shoulder abductors and adductors, elbow, wrist, and finger flexors and extensors.**
- ROM – upper and lower limb normal.

Orthopedics:

- Sitting and standing balance adequate.
- No limb deformities.
- Spine normal.

Respiratory:

- History of frequent and prolonged colds, no wheezing.
- No sleep disturbance.
- Inadequate cough efforts for spirometry.

Cardiology:

- **Pectus evacuation.**

- Femnal pulse felt.
- Xray – CTR 40%
- Chambers normal.
- No RWMA.
- No COA/PS.
- Liver ECHO normal.
- HBsAg, HCV – Negative.

Ultrasound of the ovaries – **Small underdeveloped ovaries.**

Eye and ear examination – Normal.

All the above clinical symptoms pointed towards Turner's syndrome and DMD. There was no evidence of symptoms related to the mosaicism.

6.4 DISCUSSION

The proband was a female child with the clinical manifestations of muscular dystrophy, which is likely to be confused with LGMD due to the sex of the proband. However, since the clinician firmly believed that this could be DMD, molecular diagnosis was initiated and the DMD gene mutation was identified. This underlines the importance of suspecting DMD in females too, if the clinical manifestations are pointing towards it. This suspicion laid the foundation for accurate diagnosis leading to proper management in the child. The fact that this was a homozygous deletion, found in both the copies of the dystrophin gene, indicates that skewed lionization or X; autosome translocation are not a cause of her clinical symptoms.

There is no algorithm or guidelines for the diagnosis of DMD in females. The usual first step in diagnosis of female muscular dystrophy is muscle biopsy study and immunohistochemistry. As the study aims to avoid invasive methods, muscle biopsy was not attempted and our work was based on less-invasive techniques and diagnosis from peripheral blood lymphocyte DNA. However, best practice guidelines are in place for the diagnosis of DMD in male children, which also aims at having muscle biopsy as the last step, when the other DNA based methods fail (Abbs et al., 2010). As per these guidelines, the screening test of choice is the multiplex PCR for the hot spot exons. When this test showed no deletion for hot spot exons in this girl, the immediate conclusion could have been that this is a case of LGMD, and not DMD. But on the persistence of the clinician, MLPA was done, which showed a homozygous deletion of exon 62 of the DMD gene. This underlines the usefulness of MLPA in the diagnosis of DMD. In males, where the number of cases suspected to have DMD is high, and the clinical symptoms are usually direct mainly due to the age at onset of the disease and the sex of the affected, mPCR may be considered the most economic screening test for DMD gene mutation detection. Whereas in females, we suggest MLPA may be a more useful method, which picks up almost 75% of DMD gene mutations (Murugan et al., 2010), as the first step in diagnosis. This is more important when they are isolated cases in the family. MLPA has also proved to be a method which could pick up mosaics. There are no reports where MLPA has been showed to pick mosaics in DMD cases and this is first such report. This

study also showed the usefulness of checking the single exon deletions in MLPA by native PCR. This not only helped in picking up point mutations in the gene which may cause the MLPA deletion, in this case it also helped in confirming the mosaic picked in MLPA.

Though molecular diagnosis was complete here with the identification of mutation in the DMD gene, the mechanism of the homozygous mutation was still unclear. The usual causes for homozygous deletion in females are likely to be either Turner's syndrome (Sybert and McCauley, 2004) or uniparental isodisomy (Lebre et al., 2009).Cytogenetic and molecular cytogenetic analysis confirmed the cause to be Turner's syndrome. Cytogenetic analysis gave an additional information that of a mosaic involving 45,X (85%) and 46,XY (15%). This explained the mosaic pattern observed in MLPA and PCR. As mentioned earlier, mosaics of less than 20% will not be picked by the molecular cytogenetic analysis. However, for higher percentages this would be an ideal method to confirm mosaicism without the necessity of a fresh repeat sample, which is required for FISH analysis.

The clinical manifestations were consistent with Turner's syndrome (Short stature, underdeveloped ovaries, widely spaced nipples etc.) and Duchenne muscular dystrophy (progressive muscle wasting, elevated CPK, calf muscle pseudohypertrophy, Gower's sign etc.). Turner Syndrome is characterized cytogenetically by X chromosome monosomy, the presence of an abnormal X chromosome, or mosaicism of a 45,X cell line with another cell line, which might be 46,XX, 46,XY or have an abnormal sex chromosome rearrangement (Jacobs et al., 1997). The incidence of Turner syndrome is approximately 1 in 5000 newborn girls (Jacobs et al., 1997) 97% of the TS conceptions are spontaneously aborted (Jacobs et al., 1997). On chromosomal analysis, the percentage occurrences of the various karyotypes observed in TS are 45,X (50%), 45,X/46,XX (20%), 46,X,i(Xq)(15%), 46,X,r(X) or 46,X,del(X)(10%), and others (5%) (Visootsak and Graham, 2006). Sybert and McCauley observed occurrences of 46,X,i(Xq) (7%) (Sybert and McCauley 2004), 45,X/46,X,i(Xq) (8%), 45,X/46,X,+ring (6%), 45,X/46,X,+mar (1%), 45,X/46,XY or 46,X,Yvar/Ydel (7%), 45,X/46,XX/47,XXX (3%), 45,X/46,XX (13%), 46,X,Xp (short-arm deletions) (2%), 46,X,Xq (interstitial long-arm deletions) (2%); and others (6%).

Mosaicism is the presence of 2 or more cell lines with different chromosomal constitutions in the affected individuals. The cell lines are derived mostly due to post zygotic mitotic nondisjunction; they are represented as, for example, 45,X/47,XXX/46,XX/46,XY. The chromosome constitution is also clinically significant in this syndrome. Individuals with i(Xq) show characteristics similar to individuals with classical 45,X. However, patients with a deletion of Xp have short stature and congenital malformations, and those with a deletion of Xq often display only gonadal dysfunction.

In our case, though 15% of the cells carried the Y-chromosome, the phenotype was a female and no male characters were seen. The most important factor in determining the formation of male phenotype is the SRY gene on Y chromosome, but the 15% XY cell fraction we observed in our patient was not sufficient to create male phenotype. This observation was consistent with other studies showing different proportions of XY mosaicism. Derbent and colleagues stated that 45,X/46,XY mosaic karyotype produces a wide range of phenotypes, from normal female to TS to male (Derbent, 2010). Our case, as reported by others, suggest that a low degree of XY mosaicism may produce a mild Turner's Syndrome phenotype (Akbas et al., 2009). Although the SRY gene is necessary for the formation of male phenotype, in our case the presence of SRY did not produce a male phenotype (Akbas et al., 2009).

6.5 CONCLUSION

- In conclusion, Duchenne Muscular Dystrophy in females is rare, but cannot be ruled out. Muscular dystrophy in females is commonly diagnosed as LGMD, but tests to rule out DMD have to be done.
- One of the mechanisms discussed above may cause symptomatic DMD in the females and they must be considered as a possible cause in females with unexplained severe manifestations of X-linked conditions.
- In this study we have used various techniques like mPCR, MLPA, conventional PCR, karyotyping, and molecular cytogenetics in a step wise process to diagnose DMD and to understand the mechanism of DMD.
- However, FISH analysis on a fresh sample from the patient would have helped in further confirming the mosaicism.

CHAPTER 7 - GENOME WIDE COPY NUMBER VARIATIONS IN DUCHENNE MUSCULAR DYSTROPHY – THEIR EFFECT ON MOLECULAR PATHOGENESIS

7.1 INTRODUCTION

Geneticists have long recognized the role of genomic imbalances (eg, deletions or duplications of chromosomal material) in the pathogenesis of human disorders. Numerous methods have been developed to detect genomic alterations since the discovery of the correct chromosome number in human cells in 1956. In 1959, Lejeune et al discovered that an extra copy of chromosome 21 (trisomy 21) caused Down syndrome, the first evidence linking genomic imbalances with human disease (Lejeune and Turpin, 1961). Soon after, new clinical syndromes were delineated on the basis of the identification of multiple patients with the same cytogenetic abnormality, such as trisomy 13 in Patau syndrome and trisomy 18 in Edwards syndrome. The identification of the Philadelphia chromosome, which was later showed to be caused by a trans-location between chromosomes 9 and 22, and its association with chronic myelocytic leukemia in 1960 marked the beginning of cancer cytogenetics (Rudkin et al., 1964). The invention of chromosome banding techniques in 1970 led to the discovery of numerous structural chromosome aberrations and their association with human diseases (Caspersson et al., 1970). By optimizing culture conditions to arrest cellular division at prometaphase, high-resolution banding could detect chromosomal changes to a resolution of 3 to 5 Mb. The next breakthrough in cytogenetics was the development of fluorescent in situ hybridization (FISH) technology, which laid the foundation for molecular cytogenetics (Lichter and Ward, 1990). The technology not only allows the detection of small genomic alterations of 50 Kb to 100 Kb, but also permits the direct visualization of these alterations in uncultured cells. These features made FISH testing ideal not only in detecting microdeletion/microduplication syndromes, but also for prenatal aneuploidy screens, where a fast turnaround time is highly desirable, and for cancer genetics studies, where metaphase chromosomes may not be obtainable. Although FISH allows the detection of genomic imbalances with great accuracy, it can only

probe specific sequences that are known and suspected to be associated with known syndromes. Microarray-based technology, developed in the last decade, affords the capacity to examine the whole human genome on a single chip with a resolution as high as a few hundred base pairs, a process also known as microarray-based cytogenetics (Pinkel et al., 1998). This resolution is at least 10-fold greater than the best prometaphase chromosome analysis, heretofore the most sensitive whole-genome screen for genomic deletions and duplications (Lee et al., 2007). Microarray technology represents the technical convergence of molecular genetics and cytogenetics and is rapidly revolutionizing modern cytogenetics. Submicroscopic chromosome copy number variations (CNVs), including 0, 1, or 3 copies, defined as deletions or duplications involving >1 Kb DNA, are detected in patients with mental retardation (MR), autism spectrum disorders (ASD), developmental delay (DD), and multiple congenital anomalies (MCA) of unknown causes. New syndromes begin to emerge on the basis of findings of similar genomic alterations. More than 5000 CNVs have been collected in the Toronto database (http://projects.tcag.ca/variation) so far, and many are associated with human diseases (Feuk et al., 2006).

7.1.1 Microarray-Based Cytogenetic Technology

Two major groups of microarray-based platforms are currently used in clinical cytogenetics: microarray-based comparative genomic hybridization (aCGH), and single nucleotide poly-morphism (SNP) genotyping-based arrays.

Array CGH

aCGH, which directly measures genomic copy number differences between the patient DNA and a normal reference DNA, allows the construction of a high resolution map of genome-wide copy number alterations. aCGH arrays contain thousands of bacterial artificial chromosome (BAC)/P1–derived artificial chromosome (PAC) clones or in situ–synthesized oligonucleotide probes. These probes may either be enriched for known genes or specific chromosomal regions for known syndromes, or distributed relatively evenly across the whole genome.

SNP-based array

SNP-based arrays probe thousands of SNPs and provide data about both copy number and genotype; the latter can be used to study copy-neutral genomic alterations, such as uniparental disomy (UPD) seen in imprinting disorders. Although SNP-based arrays have the advantage of detecting UPD and consanguinity, they offer a poor representation of genomic regions with low SNP incidences (SNP deserts). Additionally, SNP-based plat-forms do not use intra experimental control; rather, they compare patient data with a pre-established laboratory standard. A comparison of the 2 major microarray platforms is summarized in Table 7.1.

Table 7.1: Comparison of aCGH and SNP-based arrays

	aCGH	**SNP-based arrays**
Probes	BAC/PAC, oligos	oligos
SNP genotyping	No	Yes
SNP desert coverage	Yes	No
UPD detection	No	Yes

As microarray-based technologies continue to improve, many new platforms are being developed. These platforms will provide a combination of high probe density and optimal probe distribution across the genome, including SNP deserts and known chromosome regions that contain repetitive DNA sequences, and allow detections of both CNVs and loss of heterozygosity, including UPD. Better platforms that offer microarray-based cytogenetics with much higher resolution and significantly lower cost are likely to continue to emerge as the technology matures.

7.1.2 Clinical Application in Genetic Disorders

The prevalence of MR, ASD, and DD are reported to be 1%to 3%, 0.67%, and 3.7%, respectively, for which a cause is unknown in as many as 60% to 70% of patients (Shevell et al., 2003). With conventional cytogenetics, the diagnostic yield (ie, proportion of positive results) is about 3% to 4%; with subtelomere FISH, the yield is 5% to 7% (Ravnan et al., 2006). The positive yield for clinically relevant CNVs with microarray-based cytogenetics is 15% to 20% (Lu

et al., 2008). The diagnostic yield for isolated MR and ASD may be slightly lower than 15%, but much higher than that of FISH testing. CNVs may occur within so-called genomic ‘‘hotspot’’ regions leading to recurrent micro deletion/microduplication syndromes such as DiGeorge/velocardiofacial syndrome (DGS/VCFS) and its reciprocal 22q11.2 duplication syndrome (Ou et al., 2008).

More often, CNVs are randomly distributed outside hotspot regions with higher incidences in the subtelomeric regions. The hotspot-associated CNVs, which have been postulated to be the result of nonallelic homologous recombination (Lupski, 1998), often present as simple deletions or duplications. Alternatively, CNVs outside hotspot regions often originate from non-homologous end-joining (Korbel et al., 2007), some of which occur at the breakpoints of apparently balanced chromosomal translocations or inversions or as subtle unbalanced rearrangements of the subtelomere regions (Li et al., 2008). Because of the wide distribution and heterogeneity of CNVs in the human genome, whole-genome microarrays are the most useful method for detection of unpredictable, clinically-relevant genomic alterations. The diagnostic yield of whole-genome microarrays is largely dependent on the resolution (average inter-marker distance) of the arrays.

7.1.3 Delineation of Genotype-Phenotype Correlations of known Syndromes

Phenotypic expression among patients with well-recognized micro deletion or micro duplication syndromes varies considerably at least partially because of the size differences of the genomic alterations. FISH analysis, which is still the primary method in many cytogenetics laboratories for identifying deletions/duplications, does not delineate the specific size of the deletion or duplication. Microarray based cytogenetic testing characterizes CNV size and genomic location, which facilitates genotype-phenotype correlations.

Phenotypic variability between individuals may be due to differences in the makeup of the rest of the genome other than CNV size. The phenotypes of patients with similar genomic alterations may range from apparently normal presentation to profound mental retardation. For example, in DGS/VCFS, intra familial phenotypic variation is a common phenomenon even when the deletions

have been shown to be identical in different individuals.These other genomic differences, which are often subtle and variable, can be characterized by use of a CGH- or SNP-based arrays, which can detect the allelic differences between individuals. Patients with the same genomic alteration but variable phenotypic expression are an important cohort for further study because they offer clues to the pathophysiologic study of syndromes with CNVs. As more information from other genomic variations is correlated with the effects of CNVs and other factors such as epigenetic and environmental factors, a clearer picture of the role of CNVs in the pathogenesis of genetic disorders will emerge.

7.1.4 Identification of Genes Responsible for Known Syndromes

Microdeletion syndromes may be the phenotypic effects of haploinsufficiency of single genes. Pertinent examples include the UBE3A gene in Angelman syndrome (Kishino et al., 1997), RAI1 gene in Smith-Magenis syndrome (Slager et al., 2003), and NSD1 gene in Sotos syndrome (Kurotaki et al., 2003). Additionally, many monogenic diseases with MR and DD are due to genomic deletions. Relevant examples include a micro deletion at 11p13 where the PAX6 gene resides in Aniridia type II, and a 7p21 deletion including the TWIST gene in Saethre-Chotzen syndrome (Crolla and van Heyningen, 2002). Microdeletions/ microduplications are estimated to comprise up to 15% of all disease-causing mutations underlying monogenic diseases (Vissers et al., 2005). Microarray-based cytogenetics provides a powerful strategy for dosage-sensitive disease gene identification. A prime example of such application is the identification of the CHD7 gene as the cause of CHARGE syndrome (Vissers et al., 2005). CHARGE syndrome is characterized by some combination of coloboma of the eye, heart anomaly, atresia of choanae, retardation of mental and somatic development, genitourinary abnormalities, and ear abnormalities or deafnessn (Davenport et al., 1986). Using a genome-wide BAC array with 1-Mb resolution, Vissers et al identified a 4.8 Mb deletion at 8q12 in a patient with CHARGE syndrome (Vissers et al., 2004). In vitro studies from another patient with CHARGE syndrome and an apparently balanced trans-location involving chromosome 8 revealed 2 micro deletions overlapping with the deletion identified in the first patient. Using data from these 2 individuals, they defined the ‘‘shortest region of overlap’’ encompassing 2.3 Mb of genomic sequence on 8q12. By

sequencing candidate genes within or just outside the shortest region of overlap in an additional 17 patients, they identified 10 heterozygous mutations in the CHD7 gene. The CHD7 gene codes for a protein of the chromodomain family, which affects chromatin structure and gene expression and is widely expressed in undifferentiated neuroepithelium and in mesenchyme of neural crest origin.
Therefore it likely plays an important role in regulating early embryonic development. Other examples of new disease gene identification with microarray-based cytogenetics include the STXP1 gene (deletion of 9q33.3-34.11) in early infantile epileptic encephalopathy (Saitsu et al., 2008), the B3GALTL gene (deletion of 13q12.3-13.1) in Peters-Plus syndrome (Lesnik Oberstein et al., 2006), the PORCN gene (deletion of Xp11.23) in focal dermal hypoplasia (Goltz syndrome) (Lesnik Oberstein et al., 2006), and the FAM58A gene (deletion of Xq28) in Star syndrome (Unger et al., 2008). As microarray-based cytogenetics becomes the primary diagnostic method in cytogenetic laboratories, more disease-causing genes, especially dosage-sensitive genes, will be identified.

7.1.5 Discovery of New Genetic or Genomic Syndromes or Conditions

In recent years, many new syndromes or conditions have been defined on the basis of detection of associated genomic alterations with microarray-based cytogenetics. These genomic alterations are often smaller than those of more well-defined syndromes (eg, 15q11.2 deletion in Prader-Willi/Angelman syndromes). The phenotypic features caused by these micro genomic alterations may be mild, nonspecific, or substantially variable (Slavotinek, 2008). The rarity of these syndromes and the incomplete phenotypic penetrance of the micro genomic alterations further complicate the characterization of these syndromes (Sharp et al., 2008). Microarray-based cytogenetics allows the discovery of novel syndromes by the identification of causative genomic alterations preceding the definition of the corresponding clinical phenotype. Table 7.2 summarizes a list of recently characterized new genetic or genomic syndromes or conditions with microarray-based cytogenetics. The list will continue to grow as the technology becomes more widely used in clinical cytogenetics laboratories.

Table 7.2 - Summary of genomic alterations and main clinical features of novel microdeletion and microduplication syndromes

Genomic aberration	Genomic location (NCBI Build 36.1)	Main phenotype	References
Del. 1q21.1	1.35 Mb Chr1: 145-146.35	Mild to moderate MR, DD, microcephaly, dysmorphic features, cardiac anomalies, and cataracts	(Mefford et al., 2008)
Dup. 1q21.1	1.35 Mb Chr1: 145-146.35	Mild to moderate MR, DD, microcephaly, ASD	(Mefford et al., 2008)
Del. 1q41-42	2.7 - 9 Mb Chr1: 219.49-220.66	Moderate to severe MR, coarse facies, deep-set eyes, broad nasal bridge, full lips, cleft palate, diaphragmatic hernia	(Shaffer et al., 2007)
Del. 2p15-16.1	0.5 – 5.7 Mb Chr2: 61.1-61.7	Moderate to severe MR, ASD, microcephaly, dysmorphic features, high nasal bridge, low-set ears, high palate, optic nerve hypoplasia	(Rajcan-Separovic et al., 2007)
Dup. 3q29	1.61 – 1.76 Mb Chr3: 197.22-198.83	Mild to moderate MR and DD, microcephaly, round face, long arched eyebrows, broad nasal bridge, large eyes, ears and mouth, and obesity	(Lisi et al., 2008)
Dup. 7q11.23	1.55 Mb	ASD, speech delay, and variable DD	(Somerville et al., 2005)
Del. 8q21.3-22.1	4 Mb Chr8: 93.21-97.94	Normal to mild DD, microcephaly, tight shiny facial skin, blepharophimosis, contracture of large joints, camptodactyly, hypoplastic genitalia	(Shieh et al., 2006)
Del. 9q22.3	6.5 Mb Chr9: 94.42-99.1	Severe MR, DD, trigonocephaly, dysmorphic facial features, overgrowth, ventriculomegaly, cerebral atrophy, advanced bone age, and hyperactivity	(Redon et al., 2006)
Del. 15q13.3	1.5 Mb Chr15: 28.06-30.79	Mild to moderate MR and DD, dysmorphic facial features, and seizures	(Sharp et al., 2008)
Del. 15q24	1.7 – 3.9 Mb Chr15: 72.15-73.85	Mild to moderate DD, autistic trait, dysmorphic facial features, and seizures	(Sharp et al., 2007)
Del. 16p11-12.1	0.6 – 8.7 Mb Chr16: 21.4-30.25	Mild to severe MR, DD, seizures, dysmorphic features, palate defect, aortic valve and other cardiac anomalies, and digital anomalies	(Ballif et al., 2007)
Dup. 17p11.2	1.3 – 15.2 Mb Chr17: 16.47-18.03	Infantile hypotonia, failure to thrive, mental retardation, autistic features, sleep apnea, and structural cardiovascular anomalies	(Potocki et al., 2007)
Del. 17q11.2-12	4.4 – 4.6 Mb Chr17: 26.28-31.03	Mild DD, coarse facial features, digital and skeletal anomalies	(Brunetti-Pierri et al., 2007)
Del. 17q12	1.5 - 2.1 Mb Chr17: 31.83-33.35	Renal cystic dysplasia, renal hypoplasia, abnormal renal function, cryptorchidism, elevated hepatic enzymes, and MODY5	(Mefford et al., 2007)

Del. 17q21.31	0.5 – 0.65 Mb Chr17: 41.03-41.52	Mild to moderate MR, severe hypotonia, ptosis, dysmorphic facial features, eye and ear anomalies	(Koolen et al., 2006)
Del. 20q13.13-13.2	4 Mb Chr20: 49.76-50.84	DD, autistic tendencies, dysmorphic features, Duane anomaly, hearing loss, choanal atresia, renal and cardiac anomalies	(Borozdin et al., 2007)
Del. 21q22.12	0.7 Mb Chr21: 33.83-35.64	Growth restriction, dysmorphic features, DD, chronic thrombocytopenia, and predisposition to AML	(Shinawi et al., 2008)
Dup. 22q11.2	1 - 3 Mb Chr22: 17.2-22.1	Highly variable phenotype including MR, DD, learning difficulties, growth retardation, hypotonia, and dysmorphic facial features	(Ou et al., 2008)
Dup. Xq28	0.4 – 2.2 Mb ChrX: 152.5-152.9	Severe MR, progressive spasticity, proneness to infections, absent or limited speech	(Van Esch et al., 2005)

7.1.6 Clinical Applications in Cancer

Genetic mutations that alter cellular division are key features of cancer. Aberrations implicated in tumorigenesis include point mutations, balanced rearrangements such as translocations and inversions, and deletions or duplications. Specific information associated with these genomic aberrations has been used for cancer diagnosis and prognosis, disease classification, risk stratification, and treatment selection. Microarray-based cytogenetics in cancer research has produced a wealth of useful information about CNVs and their implications with regard to cancer classification, disease progression, therapy response, and patient outcome. For example, in breast cancer, CNVs in certain genomic regions are linked to specific clinical and pathologic characteristics, including tumor grade, estrogen receptor status, p53 mutation status, and overall survival (Bergamaschi et al., 2006). CNV profiles can also be used to define distinct therapeutic response subgroups for a given type of tumor. Clustering of a CGH data was able to distinguish multiple myeloma cases into subclasses with different clinical outcomes (Carrasco et al., 2006). Certain CNVs, such as deletions at 17q11.2 or 5q31.1, are associated with high genomic instability and confer an unfavorable outcome on patients with acute myeloid leukemia (AML), even those otherwise classified in the favor-able or intermediate cytogenetic prognostic groups (Suela et al., 2007). The application of microarray-based cytogenetics in cancer has also led to the identification of putative oncogenes and tumor suppressor genes. A CGH analysis of mantle cell lymphoma identified

three recurrent homozygous deletions which led to the identification of the proapoptotic gene BIM as a novel candidate tumor suppressor gene in mantle cell lymphoma (Tagawa et al., 2005). Analysis of CNVs in ovarian cancer resulted in the discovery of the FGF-1 gene that was amplified and overexpressed in ovarian cancer cells as a putative oncogene (Birrer et al., 2007). The discovery of cancer-specific CNVs and the genes involved provides the basis for a better understanding of cancer initiation and progression. More importantly, these putative genes and their associated pathways could be potential therapeutic targets. Internal tandem duplication of FLT3 gene (FLT/ITD) occurs in more than 15% of patients with pediatric AML (Meshinchi et al., 2006). FLT/ITD constitutively activates the FLT3 receptor tyrosine kinase and causes autonomous, cytokine-independent proliferation in vitro. A few FLT3-inhibitors, such as PKC412 (Stone et al., 2005), are currently being tested in clinical trials in adults and show great potential for treatment of pediatric AML. Genomic alterations have also been found to confer in-creased cancer susceptibility. With a CGH that targeted 6 known cancer-associated genes and their flanking sequences, Staaf et al revealed germline deletions or duplications in BRCA1, BRCA2, MSH2,and MLH1 genes in patients with breast cancer or hereditary nonpolyposis colorectal cancer, indicating a cancer predisposition. Using SNP-based microarrays in 2 independent studies, analysis of patients from non-familial adenomatous polyposis (FAP)/non-hereditary nonpolyposis colorectal cancer colorectal cancer families un-covered a novel colorectal cancer susceptibility locus on chromosome 3q (Picelli et al., 2008). These findings will potentially aid in the early identification of at-risk individuals and allow earlier detection of cancer, optimizing prognosis and the chance for cure.

7.1.7 Clinical Application in Other Complex Disorders

The power of microarray-based technologies allows modern cytogenetics to expand its testing capacity to disorders that were, until recently, the concern of other medical disciplines. CNVs have been found to be associated with many complex traits or diseases either as a single underlying factor, or more often, as part of a complex etiology. Duplication of the chromosome 21q21 region that contains the amyloid precursor protein gene accounts for as many as 8% of the patients with autosomal dominant early-onset Alzheimer diseases with cerebral

amyloid angiopathy (Rovelet-Lecrux et al., 2006). Duplication of the APP locus has also been reported in patients with sporadic Alzheimer disease (Rovelet-Lecrux et al., 2006). CNVs additionally explain phenotypic variability in familial autosomal dominant Parkinson disease (PD). The phenotype of patients with a duplication of the α-synuclein (SNCA) locus is indistinguishable from that of patients with idiopathic PD, which progresses slowly and has a late onset with no apparent cognitive decline or dementia. In contrast, patients with a triplication of the SNCA locus display hereditary early-onset PD with cognitive decline and dementia (Nishioka et al., 2006). Other examples of characterization of complex diseases using microarray-based technologies include the following: a lower copy number (<4 copies) of the HBD-2 (human beta-defensin) gene predisposing carriers to colonic Crohn's disease (Fellermann et al., 2006), and a CCL3L1 (CC chemokine ligand 3-like 1) gene copy number lower than the population average, which is associated with markedly enhanced susceptibility to human immunodeficiency virus/acquired immunodeficiency syndrome (Gonzalez et al., 2005).

Another promising area for the clinical application of microarray-based technology in the near future is the diagnosis and monitoring of infectious diseases. High or low density (from <100 to 60000 probes per array) Gene Chips are avail-able for identifying as many as 9000 species of bacteria, or as low as one single species. The latter can be easily converted into affordable point of care testing devices or field systems (Mikhailovich et al., 2008).

7.1.8 Limitations of Microarray-Based Cytogenetics

Microarray-based genetic testing has both technical and post-analytical limitations. The most apparent intrinsic limitation of currently used microarray-based cytogenetic techniques is the inability to detect balanced genetic rearrangements, such as balanced translocations, and whole-genome ploidy changes, such as triploidy, although the vast majority of real balanced chromosome rearrangements are phenotypically benign. The ability to detect cellular mosaicism is also limited. Other limitations include the challenge of interpreting CNVs of unknown significance and distinguishing disease-causing CNVs from normal CNV polymorphisms. After detection of a child with a CNV,

every effort should be made to obtain and evaluate parental DNA samples to aid in the interpretation of novel CNVs. In certain situations, if parent samples are not available, interpretation of the significance of the child's CNV may not be possible. Micro-array-based cytogenetics is beginning to be applied for prenatal diagnosis (Van den Veyver and Beaudet, 2006).

Although this would increase the diagnostic yield in the identification of fetuses with known microdeletion or microduplication syndromes, it could similarly lead to finding fetuses with CNVs of uncertain significance. This would lead to parental anxiety and the need for pediatricians to more closely follow the long-term growth and development of those children born with CNVs of uncertain significance. In an effort to address many of these interpretive limitations, large collaborative databases are being populated with genomic data together with clinical information from different laboratories, such as the Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources. (http://www.sanger.ac.uk/Software/analysis/decipher/database.shtml).

Empowered with microarray-based technology, modern molecular cytogenetics begins to characterize CNVs responsible for MR, ASD, DD, and MCA of unknown causes, identify causal genes for known genetic conditions, and define new genomic syndromes on the basis of common genomic aberrations. The technology is changing the practice of cancer genetics and other traditionally nongenetic diseases as well. Microarray-based cytogenetics is revealing the tremendous fluidity and complexity of the human genome, and is starting to illustrate the implications of genomic variability with respect to human health and disease. Most patients with MR, ASD, DD, or MCA present in childhood and are cared for by pediatricians. Microarray-based cytogenetics should be the first line test for these patients, except those suspected of having classic whole chromosome aneuploidy, such as Down syndrome or Turner syndrome. Because microarray-based cytogenetic tests are now available in many clinical cytogenetics laboratories, pediatricians can now order the test as a replacement for or an adjunct to conventional chromosomal analysis. Pediatricians can also consult with clinical geneticists and clinical cytogeneticists to decide the best testing strategy for their patients.

CNVs in muscular dystrophies: There are no reports of genome wide CNV associations in muscular dystrophies. However, deletions and duplications of whole exons are a common cause of muscle disorders like DMD and SMA. FSHD also is known to be caused due to increase in number of CA repeats.

Studies on CNVs in India: Microarray-based cytogenetics is not yet popular in India as a research or diagnostic tool. However, there have been a few studies on the CNV spectrum involvement in phenotypic diversity (Gautam et al., 2010), which was a genome wide scan, and another study using real time PCR studying the CNVs in CYP2C19 (Devendran et al., 2012).

DMD is a lethal childhood muscular disease, is due to dystrophin gene mutations resulting in loss of dystrophin protein at muscle plasma membrane. To date the only proven palliative treatment is chronic glucocorticoids treatment and the natural history of DMD is also heterogeneous, with inter-patient variability in disease progression, motor, respiratory and cardiac involvement making it a difficult disease to treat. As most patients show complete loss of dystrophin in muscle it is believed that genetic modifiers (multigenic polymorphisms remote from the dystrophin gene), and/or environmental factors influence variability in disease progression and response to steroids.

Identification of genetic modifiers has significance at multiple levels. First, the ability to stratify patients according to their genotype at specific loci may help selecting clinically homogeneous subsets of patients, and increasing the probability of reaching significant results in upcoming clinical trials. Second, genetic modifiers may point to factors that are important in molecular physiology, and potential novel drug targets.

In this study, our aim is perform a genome wide scan for CNVs in Duchenne Muscular dystrophy patients to scan for CNVs that affect the severity and progression of the disorder.

7.2 MATERIALS AND METHODS

7.2.1 Samples

Samples of Duchenne/Becker muscular dystrophy patients with confirmed Dystrophin gene mutations were selected for the study. The cases were selected from the database available at our facility. Eighteen samples, 7 DMD and 11 BMD, were selected from the database based on the mutations, removed from the DNA bank, quantified and used for the assay. List of the samples selected for the study are shown in table 7.3.

Twenty two male control DNA samples were selected, quantified and used for the assay. Informed consent was obtained from the patients and controls and this study was approved by the Institutional review board.

Table 7.3: List of samples selected for CNV analysis in the study

S.No	Patient ID	Clinical Diagnosis	DMD gene mutation	Framedness	Age of Onset of symptoms (in years)
1	B159	DMD	Exon 45 Deleted	Out of frame	9
2	B325	DMD	Exon 45 Deleted	Out of frame	2
3	B474	DMD	Exon 45 Deleted	Out of frame	10
4	B490	DMD	Exon 45 Deleted	Out of frame	3
5	B609	DMD	Exon 45 Deleted	Out of frame	11
6	B615	DMD	Exon 45 Deleted	Out of frame	3
7	B813	DMD	Exon 45 - 47 Deleted	In frame	6
8	B145	BMD	Exon 45 - 47 Deleted	In frame	23
9	B306	BMD	Exon 45 - 47 Deleted	In frame	5
10	B317	BMD	Exon 45 - 47 Deleted	In frame	16
11	B465	BMD	Exon 45 - 47 Deleted	In frame	9
12	B644	BMD	Exon 45 - 47 Deleted	In frame	19
13	B418	BMD	Exon 45 - 48 Deleted	In frame	16
14	B521	BMD	Exon 45 - 48 Deleted	In frame	22
15	B550	BMD	Exon 45 - 48 Deleted	In frame	8
16	B624	BMD	Exon 45 - 48 Deleted	In frame	8
17	B450	BMD	Exon 45 - 49 Deleted	In frame	5
18	B164	BMD	Exon 45 - 49 Deleted	In frame	12

7.2.2 Genome wide SNP analysis

The genome-wide copy number screening was performed using the Affymetrix GeneChip® Genome-Wide Human SNP Array 6.0, Part#901182, (Affy 6.0) following the procedure described in Affymetrix® Cytogenetics Copy Number User Guide. The arrays were scanned by the Gene Chip Scanner3000 7G, controlled by the AGCC software. The array data were analyzed by the Affymetrix Genotyping Console™ version 3.0.1. The Contrast quality control (QC) was set to higher than 0.4 and the Median Absolute Pair wise Difference (MAPD) threshold was set to 0.30 or less for each sample. CNV analysis was performed by comparing the files from the D/BMD patients with a reference file of 14 healthy male controls. CNV were further evaluated in the Affymetrix® Genotyping Console Browser version 1.0.11 and the Affymetrix Chromosome Analysis Suite version 1.0.1. Sixteen of the 18 patient samples passed QC, and the other two (B317 and B550) were removed from further analysis. The CNVs were flagged using a cut off filter of minimum 20 markers per 100 Kbp segment. Flagged CNVs were checked against the Database of Genomic Variants (DGV) (http://projects.tcag.ca/variation/)©

7.2.3 Statistical Analysis

Fisher's Exact test was applied for comparing the frequencies of CNVs of the DMD patients and controls, using the statistical work package STATA. The data was considered to be significant when $P < 0.05$. The t-test was used to evaluate patient–control differences in the average sizes of CNVs between in the 2 populations.

7.3 RESULTS

7.3.1 Quality control of the Affy 6.0 gene chips

All 18 arrays in the patient group and 14 of 20 controls were in accordance with Affymetrix' recommendations, with MAPD values ranging from 0.194-0.299 and QC call rate ranging from 88.2-99.0%.

7.3.2 Size difference in CNVs between DMD and controls

Of the 18 patients with D/BMD and 20 healthy controls that were analyzed with the Affymetrix 6.0 microarrays, 16 cases and 14 controls survived the filtering for quality control. A total of 810 CNVs larger than 100 kb, called by at least 20 probes, were identified; 391 were among the patients and 419 were in the control group (Table 7.4). The overall CNV rate per person was not statistically different (t-test, p = 0.20) between patients (12.763.7 SD) and controls (12.563.7 SD). The deletion/duplication ratio was different in the 2 sample populations (cases, 0.45; controls, 0.22; x2, p= 0.029) (Table 7.4).

Table 7.4: Distribution of CNVs in patients and controls

Samples	No. of Subjects	No. of CNVs	CNVs/Person	t-test p-value	No. of deletions	No. of Duplications	Deletions/ Duplications
Patients	16	391	24.03	0.20	122	269	0.45
Controls	14	419	29.92	-	76	343	0.22
Total	30	810	-	-	198	612	

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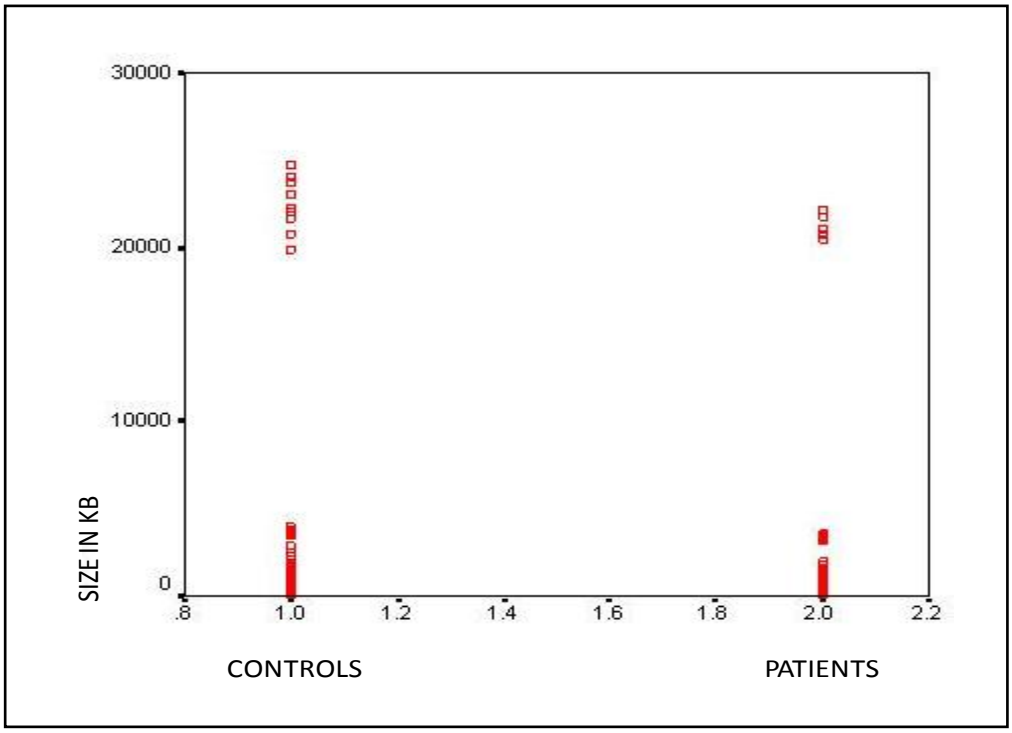

Figure 7.1: Scatter plot of rare CNV size. *Figure shows scatter plot of the distribution of rare CNVs as a function of their dimension in controls and patients.The median size for controls is 316 and for patient is 279 (p value – 0.419)*

The CNVs obtained for patients and controls were then pooled, so that CNVs corresponding to the same regions were considered only once for further analysis and all repetitions were removed. Though they in the same region, there were differences in the sizes, but overall the regions covered the same genes. This data was used for further analysis and calculations.

After this data modification, we had a total of 286 individual CNVs, 137 in patients and 149 in controls. Among the patients, 81 of the 137 cases (59.1%) were duplications and the rest were deletions. Among the controls, 111 of 149 cases (74.4%) were duplications and the rest were deletions.

The mean size of the CNVs was 1074 (range 102 – 22137 kbp) and 1354 kb (range 100 – 24750) for the patients and controls, respectively. The largest CNV, 24750 kb, was found in a control; the largest CNV among the patients was 22137 kb. The scatter plot (figure 7.1) shows the difference in sizes between CNVs of patients and controls. It can be observed that there is a specific pattern for the CNV size distribution both in controls and patients. Most of the CNVs are less than 5000 kb for both the groups, after which there are a few large CNVs, more

than 20000 kb in size. The size difference of CNVs between patients and controls do not show significant difference, and the number of large CNVs of more than 20000kb is 8 in controls as compared to 6 in patients.

7.3.3 CNVs associated with DMD

The number of patients and controls for each of the CNVs were calculated and Fisher's exact test was done to understand the significance of the difference. Four CNVs were observed, 3 of which were observed more in patients and one was more in controls. The gene regions with most/less frequent CNVs in DMD patients are given in Table 7.5 all of which are reported as normal CN polymorphisms in the DGV. (http://projects.tcag.ca/variation/)©

Table 7.5: List of all relevant CNVs (≥ 100 Kbp), chromosomal location, relevant genes and copy number frequency identified in the study.

Chromosome/ Locus	Copy number	Copy number state	Genes	CN Frequency (%)		P-Value
				DMD (N=16)	CON (N=14)	
16p11.2	1	Loss	TP53TG3B, TP53TG3, LOC390705	31.25	0	0.045
7q35	3	Gain	OR2A42, OR2A1, OR2A9P, OR2A20P, OR2A7, CTAGE4, LOC728377	43.75	7.14	0.039
9q21.1	3	Gain	PGM5P2, LOC440896, FOXD4L6, CBWD6, ANKRD20A4	6.25	57.14	0.004
14q32.33	4	Gain	NCRNA00226	56.2	14.2	0.048

The exact size and localisation of the CNVs varied slightly between patients, but in every case encompassed the listed genes.

No OMIM genes, which are associated with a disease, were observed in the CNV regions. Table 7.6 gives the list of genes known to be present in the CNV regions and their speculated functions.

Table 7.6: List of genes present in the CNVs identified in the study with functions and expression in muscle.

S.No	Gene	Descriptions	Functions	Expression in Muscle (www.genecards.org)
1	**TP53TG3B, TP53TG3**	TP53-inducible gene 3 protein, TP53-target gene 3 protein (Bechtel, Rosenfelder et al. 2007)	May play a significant role in p53/TP53-mediating signaling pathway	YES
2	**LOC390705**	protein phosphatase 2, regulatory subunit B", beta pseudogene (Strausberg, Feingold et al. 2002)	Not known	YES
3	**OR2A42**	olfactory receptor, family 2, subfamily A, member 42 (Malnic, Godfrey et al. 2004)	Odorant receptor (Potential)	NO
4	**OR2A1**	olfactory receptor, family 2, subfamily A, member 1 (Malnic, Godfrey et al. 2004)	Odorant receptor (Potential)	NO
5	**OR2A9P**	olfactory receptor, family 2, subfamily A, member 9 pseudogene(Malnic, Godfrey et al. 2004)	Odorant receptor (Potential)	NO
6	**OR2A20P**	olfactory receptor, family 2, subfamily A, member 20 pseudogene(Malnic, Godfrey et al. 2004)	Odorant receptor (Potential)	NO
7	**OR2A7**	olfactory receptor, family 2, subfamily A, member 7 (Malnic, Godfrey et al. 2004)	Odorant receptor (Potential)	NO
8	**CTAGE4**	cutaneous T-cell lymphoma-associated antigen 4 (Usener, Schadendorf et al. 2003)	Tumor-associated antigen	YES
9	**LOC728377**	Rho guanine nucleotide exchange factor (GEF) 5 pseudogene (Strausberg, Feingold et al. 2002)	Not known	YES
10	**PGM5P2**	phosphoglucomutase 5 pseudogene 2 (Wong, Vallender et al. 2004)	Not known	NO
11	**LOC440896**	uncharacterized **LOC440896 (Strausberg, Feingold et al. 2002)**	Not known	YES
12	**FOXD4L6**	forkhead box D4-like 6 (Barbe, Lundberg et al. 2008)	Not known	NO
13	**CBWD6**	Cobalamin synthase W domain-containing protein 6 (Strausberg, Feingold et al. 2002)	Not known	YES
14	**ANKRD20A4**	ankyrin repeat domain 20 family, member A4 (Humphray, Oliver et al. 2004)	Not known	NO
15	**NCRNA00226**	long intergenic non-protein coding RNA 226	Not known	No

Totally 16 genes were identified in the CNVs, of which 4 were potential olfactory receptors. Functions of none of the genes/proteins have been identified and some, like NCRNA00226, were identified to be long non-coding RNA.

CNVs between DMD and BMD samples were tested to find if there are any CNVs associated with any particular phenotype, and also between in-frame and out-of-frame deletions, but no statistically significant CNV was identified. Size differences of CNVs between BMD and DMD also did not show statistically significant results.

7.3.4 Large CNVs in patients

Large CNVs of sizes above 20,000 kb were identified in 6 cases in the study and 8 controls. Though all these are previously reported as normal CN polymorphisms in the DGV, we wanted to study the genes in the regions elaborately, mainly due to size of the CNVs. All these CNVs, except for one control were in the 9p11.2 regions and were gains. (see table 7.7).

Table 7.7: List of large CNVs in the patients. Also shown in the table are the copy number state, and the associated genes in the region.

S No	File	CN State	Type	Chr No	Size (kbp)	Marker Count	Cyto band Start	Cyto band End	Genes
1	B164	3.0	Gain	9	22137.0	100	p11.2	q13	FAM27C, FAM27A, KGFLP1, FAM74A2, FAM74A4, FAM75A5, FAM75A7, LOC442421
2	B325	3.0	Gain	9	20486.5	22	p11.2	q13	KGFLP1, FAM74A2, FAM74A4, FAM75A5, FAM75A7, LOC442421
3	B465	3.0	Gain	9	21708.1	78	p11.2	q13	FAM27C, FAM27A, KGFLP1, FAM74A2, FAM74A4, FAM75A5, FAM75A7
4	B521	3.0	Gain	9	20777.3	32	p11.2	q13	KGFLP1, FAM74A2, FAM74A4, FAM75A5, FAM75A7, LOC442421
5	B615	3.0	Gain	9	21062.3	40	p11.2	q13	FAM27A, KGFLP1, FAM74A4, FAM74A2, FAM75A5, FAM75A7, LOC442421
6	B644	3.0	Gain	9	21055.0	39	p11.2	q13	FAM27A, KGFLP1, FAM74A4, FAM74A2, FAM75A5, FAM75A7, LOC442421

Chr No denotes chromosome number.

It was observed that all of the samples showed several common genes in the region all of which were not fully characterized and functions not known.

7.4 DISCUSSION

The present study is the first effort to search for common CNVs as a source for genetic susceptibility in DMD. We found four CNVs, three of which were gains and one loss. Three of the four CNVs were seen more in patients compared to the controls and one was seen in less number of patients. All have previously been reported as regions with copy number polymorphisms in Database of Genomic Variants (http://projects.tcag.ca/variation/).

There are several different ways in which CNVs may contribute to human disease. Like many other genetic variants, a CNV could act by directly affecting gene dosage and gene expression. Recently, it was seen that variation in the copy number of the gene encoding the cytokine CCL3L1 leads to altered susceptibility to HIV infection (Gonzalez et al., 2005). However, CNVs may be part of normal variation in the human genome, and their contribution to human disease can only be identified by large case–control studies to determine whether specific variants are associated with clearly identified phenotypes. It is unlikely that they will be associated with severe early onset Mendelian disease, if they are having an effect on gene dosage. It is more likely that they may play a role in late onset disease, or more complex common diseases. CNVs appear to be enriched within genes that are important in molecular–environmental interactions and may influence immune defense and disease resistance or susceptibility of humans (Barber et al., 2005).

Specific efforts are underway to uncover genomic changes that are involved in cases of clinical abnormalities. Although copy number changes were initially documented through the study of inherited diseases, we now know that CNVs cover approximately 12% of the human genome, potentially altering gene dosage, disrupting genes or perturbing regulation of their expression, even at long-range distances; thus, a considerable number of apparently Mendelian disorders might be due to CNVs. And just as CNVs can affect monogenic traits (including monogenic forms of common disorders,(Singleton et al., 2003; Le Marechal et al., 2006; Rovelet-Lecrux et al., 2006), CNVs are also likely to underlie the aetiology of common disorders as a result of variability in gene dosage (Knight, 2005; Stranger and Dermitzakis, 2006).

In this study, though we were able to identify CNVs more frequently seen in DMD patients, we were unable to address the functional effects of these CNVs on the disease. Most of the genes identified do not have already known functions, and further study is required to understand the importance of these. The number of cases and controls taken for this study was limited by the cost of the assays. But, further study with large number of cases and controls may throw light onto more regions which may have an effect on the phenotypic variability in these disorders.

7.5 CONCLUSION

- To conclude, this is the first such study to understand the association of CNVs on Duchenne Muscular dystrophy. D/BMD are disorders caused due to Dystrophin gene mutations, but a lot of phenotypic variability has been observed between patients with similar DMD gene mutations.
- Though the basic difference between the two is framedness of dystrophin gene mutation, exceptions to that rule contribute 10% of the cases.
- Cause for these exceptions is not yet clear.
- However, SNPs associated with progression of the disorder have been published.
- A detailed study of CNVs may throw more light in understanding the molecular pathophysiology of Duchenne/Becker muscular dystrophies.

CHAPTER 8 - DIAGNOSIS OF NEUROMUSCULAR DISORDERS BY GENOME WIDE SNP BASED HOMOZYGOSITY MAPPING

8.1 INTRODUCTION

Neuromuscular disorders affect the peripheral nervous system and muscle. Their principle effect therefore is on the ability to perform voluntary movements, with resulting significant incapacity including, at the most extreme, complete paralysis. Many neuromuscular disorders were recognised in the nineteenth century, particularly by the great French neurologists (Emery, 2002). Neuromuscular disorders include some of the most devastating diseases that afflict mankind including amyotrophic lateral sclerosis (ALS), congenital muscular dystrophies and myopathies, Duchenne muscular Dystrophy and spinal muscular atrophy (SMA).

Neuromuscular disorders range in onset from *in utero* (Ravenscroft et al., 2011), to old age, but to a large extent affect infants, children and teenagers. Some neuromuscular disorders are acquired, such as botulism, idiopathic inflammatory myopathies (dermatomyositis, inclusion body myositis and polymyositis), Lambert-Eaton syndrome and myasthenia gravis, pharmaceutical induced myopathies such as steroid or statin myopathy, snake bite, and tetanus, but the vast majority are genetic or have genetic susceptibility (Emery, 2002) including in fact statin myopathy (Krivosic-Horber et al., 2004; Link et al., 2008; Supala-Berger et al., 2009). The greatest advances in neuromuscular disorders in the last 25 years have been in the identification of many of the genes mutated in the disorders and the improvement in management of symptoms and complications through the use of cough assist machines, orthotics, physiotherapy and ventilation. The improvements in symptomatic treatment and of complications, has significantly increased the life expectancy and quality of life of neuromuscular disease patients (Eagle et al., 2002; Wallgren-Pettersson et al., 2004; Sejerson and Bushby, 2009).

A list of known genes and known linked loci with genes not yet identified, for neuromuscular disorders is published annually in the Journal “Neuromuscular

Disorders", and has an on-line version (http://www.musclegenetable.org/). The latest table includes 16 categories of neuromuscular disorders and 519 genes / loci, with one of the categories being cardiomyopathy genes since so many muscle diseases / genes also affect the heart. Diagnosis of neuromuscular disorders today involves three principle arms, the clinician, the pathologist and the molecular diagnostic laboratory, with a major role also for biochemistry (for example creatine kinase (CK) and other enzyme studies). Diagnosis before molecular genetics used to be based solely on clinical expertise, electromyography (EMG), and muscle and nerve biopsy to try and determine what disease the patient had, whether, for example, at the gross level, it was myogenic (caused by a primary defect in muscle) or neurogenic (caused by a primary defect in the innervating nerve).

Despite the skill of the clinician and pathologist and increasing understanding of the different diseases, it can remain, even today, difficult to determine precisely which neuromuscular disorder a patient has. For example, clinical acumen and muscle biopsy can identify that a patient has a probable autosomal recessive limb girdle muscular dystrophy or the congenital myopathy nemaline myopathy, but which autosomal recessive limb girdle muscular dystrophy, which nemaline myopathy? Currently, there are 15 different known autosomal recessive limb girdle muscular dystrophy genes and 7 known different nemaline myopathy genes with other genes for both disorders still to be found.

Thus, discovery of neuromuscular disease genes has clarified subtypes of the diseases. Through discovery of each disease gene, the mutated or missing protein in each disease is identified. This, in turn, permits the development of specific antibodies to the proteins involved in the diseases, which the pathologist can use in immunohistochemistry to help separate the disease entities and guide molecular analysis.

Similarly, muscle imaging with magnetic resonance imaging (MRI), X-ray computed tomography (CT) or ultrasound is increasingly being used to differentiate clinically very similar muscle diseases from each other, since the pattern of affected muscles correlates with the mutated gene and can therefore guide the molecular analysis of the patient (Jungbluth et al., 2004; Mercuri et al., 2005; Fischer et al., 2008; Klein et al., 2011). However, only the identification of the precise disease-causing mutation in the patient in the correct gene can

accurately determine which neuromuscular disorder a patient has. **Therefore, molecular diagnosis is the gold standard for diagnosis of neuromuscular disorders.**

8.1.1 Diagnosis of neuromuscular disorders prior to molecular diagnosis

Before molecular diagnosis was possible, when the disease genes for neuromuscular disorders had not been discovered, the clinician was largely working in the dark. Members of families at risk of having children with genetic neuromuscular disorders were faced with very difficult reproductive choices. They could elect not to have children, adopt a child or could take a chance and play genetic roulette. Counseling on recurrence risk for subsequent pregnancies was based on guesses as to the pattern of inheritance of the disease in the family, and mathematical probabilities (Bayesian analysis) of the likelihood of carrier status in families. This was difficult to use accurately, resulting in wildly variant calculations of the risk of having an affected child (Bundey, 1978).

Using positional cloning strategies to find disease genes (Collins, 1995) the first information that is obtained is linkage of the disease to a region on one chromosome. When a disease is linked to a chromosome region, but the disease gene is not yet identified, one can do linkage analysis to follow the "disease" haplotype through the family. One complication is the possibility of recombination between the disease gene and the linked marker (Wirth et al., 1995), or double recombination between the disease gene and linked flanking markers (Abbs et al., 1990), which can make diagnosis based on linkage inaccurate. Linkage analysis was also done for DMD families, but again there was the additional problem of at what point in the family the mutation had arisen (Bakker et al., 1985; Dorkins et al., 1985).

Thus, until neuromuscular disease genes were identified, molecular diagnostics retained a level of inaccuracy, though linked markers give much more useful information, when there was no information.

8.1.2 Benefits of accurate molecular diagnosis

Accurate diagnosis and screening for carrier status of probable carriers within the families was made possible due to the identification of causative genes for several of the neuromuscular disorders. For example, in DMD affected families,

this allowed accurate prenatal diagnosis for the families, where male fetuses with the family mutation could be differentiated from male fetuses without the family mutation and families were no longer faced with terminating unaffected boys. One of the effects of accurately determining the carrier status of women in DMD families was that sometimes, by showing that the DMD patient had a *de novo* mutation and that the mother could have done nothing to avoid this, removed years, on occasion, decades of guilt from the mother. This accurate diagnosis also allowed correction of clinical and pathological diagnosis. One of the problems of differentiating myogenic from neurogenic diseases is that slow, chronic myogenic disorders can ultimately affect the innervating nerve leading to a mixed myogenic/ neurogenic picture, which can be hard to differentiate. Having accurate molecular diagnosis for DMD and the allelic Becker muscular dystrophy (Kunkel et al., 1986) allowed differentiation of the milder (Kugelberg-Welander) forms of spinal muscular atrophy from Becker muscular dystrophy (Laing et al., 1990).

8.1.3 Neuromuscular disorders molecular diagnosis – present

Today, the major difficulties faced by molecular neurogenetic diagnostic laboratories are;

- The large number of genes for neuromuscular diseases that have been identified.
- The high level of genetic heterogeneity, with large numbers of genes associated with many of the diseases.
- Multiple diverse diseases associated with many of the individual genes.
- The fact that many of the proteins associated with neuromuscular diseases are the largest human proteins.
- A large number of different types of mutations cause neuromuscular disorders and require a large number of techniques to detect them
- Not all neuromuscular disease genes have been identified

8.1.4 Bioinformatics

A major part of the work of a molecular diagnostic laboratory is determining whether a variant identified in a patient is disease-causing or simply a non-disease causing polymorphism. Results obtained for patients may be compared with multiple databases to clarify the significance of any result. If a variant identified in a patient is listed in a database as disease-causing or as a polymorphism, then that simplifies the interpretation of the result, though judgment and caution still need to be exercised, since variants listed in databases as polymorphisms may in fact be disease-causing and *vice versa*.

8.1.5 Neuromuscular disorders molecular diagnosis – immediate future

Many of the proteins associated with neuromuscular disorders are extremely large, resulting in them being expensive to fully analyse using Sanger sequencing. Therefore, diagnostic molecular neurogenetics laboratories, with current technologies and limited budgets available, one cannot analyse all the neuromuscular disease genes that need to be analysed in order to give all patients a molecular diagnosis. Neither can clinical services afford to buy all the molecular diagnostic testing required.

The impact of next generation, massively parallel, DNA sequencing (NGS)

Next generation sequencing technologies produce orders of magnitude more sequencing data than Sanger sequencing at little cost per base pair (Metzker, 2010). In the last few years, NGS has had a remarkable impact on disease gene discovery, since one no longer needs large family resources to find a disease gene. One can find disease genes with as few as two to three patients with the same disease (Lalonde et al., 2010). One may either, capture and sequence the entire exome, all the coding regions of the genome (Clark et al., 2011), to identify disease genes (Johnson et al., 2010; Ng et al., 2010; Weedon et al., 2011), or sequence the entire genome to identify disease genes (Lupski et al., 2010). NGS has accelerated the pace of human disease gene discovery to such an extent that it can be expected that in the next few years all human disease genes, including all neuromuscular disease genes, will be found.

8.1.6 History of SNP array

SNP array technology was developed in 1998 for genotyping (Wang et al., 1998). Since then, the technique has been improved dramatically and has become one of

the most powerful genomic analysis tools. The first SNP array contained 558 loci, and SNPs present within a sample amplified by multiplex polymerase chain reaction, in which primer pairs from many different loci were combined in a single reaction (Wang et al., 1998). Amplified DNA was then hybridized on the SNP array to detect the genotyping of the 558 SNPs in the sample. A large amount of primers were required to amplify these multiple SNPs in a sample in the multiplex PCR approach for array analysis. However, primer dimer formation limited the number of primer pairs that could be included in a single PCR reaction. Therefore, the sample preparation using this protocol was still labour extensive for high density SNP array analysis. The microarray company, Affymetrix, has improved the confidence of SNP array genotyping by interrogating on the array additional offset probes for each SNP locus (Mei et al., 2000). Several approaches have been used to improve the capacity of multiplex PCR. Among them, the GoldenGate assay (Fan, et al., 2003) is one of the successful highly multiplexed PCR-based SNP genotyping method which the company Illumina has adopted for their commercial SNP array chips. Although these modifications improved the number of SNPs that can be analyzed, the multiplex PCR approach still limits the member of SNPs that can be analyzed.

In 2003, researchers at Affymetrix developed the whole genome sampling method for SNP genotyping (Kennedy et al., 2003; Matsuzaki et al., 2004). This approach amplified genomic DNA pre-cut by a restriction enzyme. After digestion of the genomic DNA and ligation of primers, the amplification step was specifically designed to amplify DNA fragments between 400 to 800 base pairs. Using this approach, thousands of SNPs could be analyzed simultaneously. Using XbaI digestion, commercial SNP arrays containing 10,000 SNPs and accompanied by a sample preparation kit were produced. This development made the spot density and the genomic resolution of SNP array analysis higher than that of the 1Mb bacterial artificial chromosome array and cDNA arrays used commonly. The application of whole-genome DNA amplification techniques in combination with SNP array genotyping (Matsuzaki et al., 2004; Wong et al., 2004; Zhou et al., 2005) and genomic copy number analysis have been reported (Bignell et al., 2004). The combined genotyping and genomic copy number analysis has made the SNP array a unique technique in genomic research, and revealed many new genetic features in cancer cells such as acquired uniparental

disomy (UPD) (Raghavan et al., 2005; Teh et al., 2005) . In the last couple of years, Affymetrix has improved the coverage of their SNP array chips further into 100K and then 500K by selecting different enzymes to fragment genomic DNA. The 500K SNP array achieved a genomic resolution of average 5Kb per SNP. Recent additions from Affymetrix to this list of arrays are the SNP5.0 and SNP6.0 arrays. Data generated from these arrays can be used for several applications like association studies, LOH determination, Linkage, copy number variations, homozygosity mapping etc.

8.1.7 Affymetrix Genome wide SNP array 6.0 (www.affymetrix.com)

The new Affymetrix® Genome-Wide Human SNP Array 6.0 contains more than 906,600 single nucleotide polymorphisms (SNPs) and more than 946,000 probes for the detection of copy number variation. SNPs on the array are present on 200 to 1,100 base pairs (bp) Nsp I or Sty I digested fragments in the human genome, are amplified using the Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0. This assay, which is also compatible with the SNP Array 5.0, now combines the Nsp and Sty fractions previously assayed on two separate arrays. Figure 8.1 shows an schematic diagram giving an overview of genome wide SNP assay.

SNPs on the Genome-Wide Human SNP Array 6.0 were screened in more than 500 distinct samples, including 270 HapMap samples and separate diversity samples. Approximately 482,000

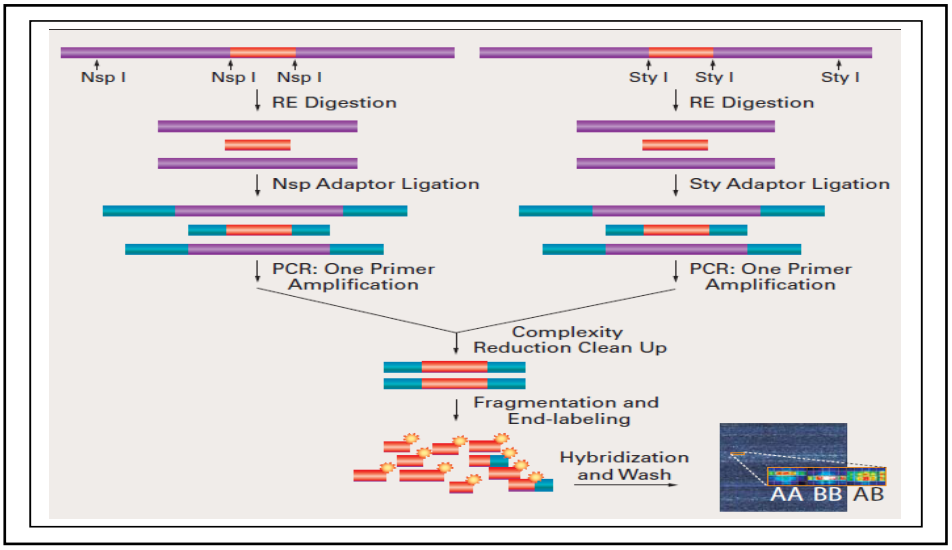

Figure 8.1: Overview of the Genome-Wide Human SNP Assay 5.0/6.0 (Adapted from www.affymetrix.com)

SNPs are derived from the previous-generation mapping 500K and SNP 5.0 Arrays. The remaining 424,000 SNPs include tag SNP markers derived from the International HapMap Project, better representation of SNPs on chromosomes X and Y, mitochondrial SNPs, SNPs in recombination hotspots and new SNPs added to the dbSNP database after completion of the Mapping 500K Array.
The array also contains 202,000 probes targeting 5,677 known regions of copy number variation from the Toronto Database of Genomic Variants. These regions resolve into 3,182 distinct, non-overlapping segments, each interrogated with an average of 61 probes. In addition to the interrogation of these regions of known copy number polymorphism, more than 744,000 probes were included, evenly spaced along the genome, to enable the detection of novel copy number variation. The median inter-marker distance taken over all 1.8 million SNP and copy number markers combined is less than700 bases.

8.1.8 SNP array homozygosity mapping

Homozygosity mapping is a method for mapping the human genome, used to detect genes that cause disease only when both copies in an individual are mutated (i.e. the genes are **homozygous**, or *the same*). This technique works for genetic disorders that are inherited from both parents, since inheriting a pair of **heterozygous** (*different*) genes results in expression of a non-mutated version from one parent, and the absence of disease symptoms.

Homozygosity mapping, also called autozygosity mapping, is a common method for mapping recessive traits in consanguineous families. It is powerful because it does not require DNA of other family members than the affected offspring (Lander and Botstein, 1987). The normal workflow consists of a genome-wide linkage analysis with microsatellites or SNPs (Gibbs and Singleton, 2006). Especially for SNP markers, owing to their low informativity and hence the usually small number of informative meioses, this is mostly carried out with multipoint linkage analysis with software such as GENEHUNTER (Kruglyak et al., 1996) or derivatives (Kong and Cox, 1997; Dietter et al., 2007), Allegro (Gudbjartsson et al., 2005), SIMWALK2 (Sobel et al., 2002) or Merlin (Abecasis et al., 2002) under a recessive disease model. This is followed by the preparation of haplotypes either manually or by the software used for the analysis.

Haplotypes are then manually inspected and searched for homozygous regions shared by all affected individuals who are homozygous by descent (if genotypes from ancestors are available) and are not homozygous in unaffected family members.

Computation of multipoint LOD scores and generation of haplotypes pose high demands on computational resources, time consuming and largely depend on correct allele frequencies (Kruglyak et al., 1995). The proper assessment of haplotypes becomes even more error-prone when no DNA from relatives is available because phase information is unknown and type I errors cannot be corrected (Kirk and Cardon, 2002). (Figure 8.2)

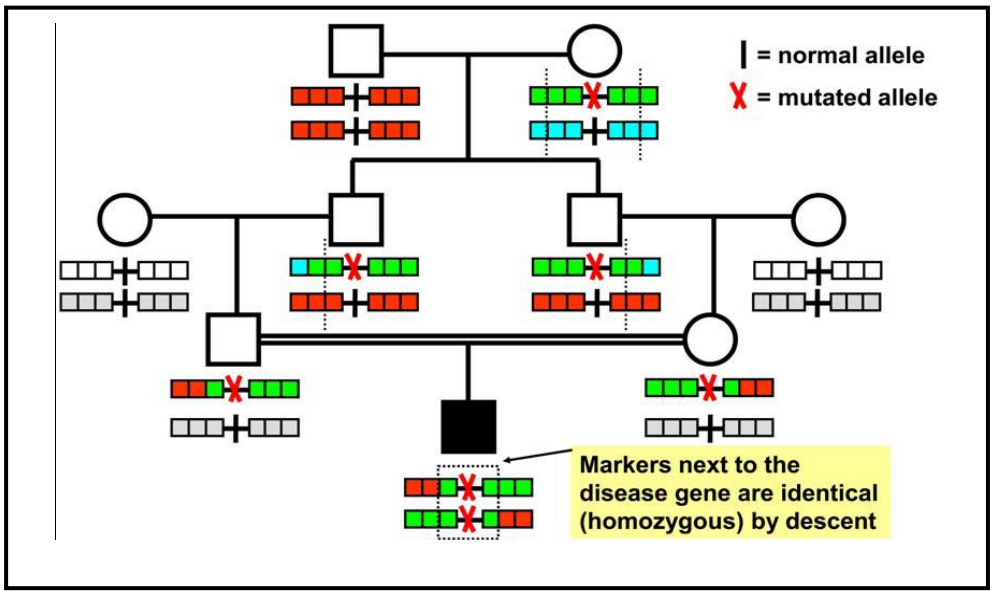

Figure 8.2. Homozygosity mapping of recessive disease genes *(Adapted from Hildebrandt, Heeringa et al. 2009)*

Beside heuristic approaches, most multipoint linkage applications use the Lander–Green algorithm (Lander and Green, 1987) which scales linearly with the number of markers analysed and due to time constraints often only a subset (i.e. about

10000) of the total SNPs is studied. Another drawback is that large families, especially those with a high level of inbreeding, have to be split because computational time increases drastically (Omran et al., 2000) or because the family size may simply become too large for the computational resources. Splitting pedigrees can however significantly reduce the information obtainable from them (Goedken et al., 2000). If studies comprise unrelated families with

only one or few affected individuals, it may occur that they do not share a disease haplotype rare or long enough to be detectable even if they carry the same founder mutation (Konrad et al., 2006). However, in consanguineous families with a rare recessive disease, it is very likely that the same disease allele has been inherited from both parents (Lander and Botstein, 1987). As long as the same locus is responsible for the phenotype, the proportion of homozygosity in the mutation's vicinity among individuals from different families should still be substantially higher than expected by chance. This is even the case when there is no common haplotype among different families due to ancient or even different mutations at the same locus. Especially in populations in which consanguinity is common, apparently unrelated individuals with the same phenotype are often found to be distantly related (Mueller and Bishop, 1993) and might hence share the same founder mutation albeit with only short shared disease haplotypes between families. Additionally to its presence in consanguineous families, autozygosity occurs also by chance and without known inbreeding (Broman and Weber, 1999; Gibbs and Singleton, 2006; Woods et al., 2006; McQuillan et al., 2008) but the use of many nonrelated families makes it very unlikely that affected individuals from different families share the same autozygous region accidentally. It might hence even be possible to find disease genes in families with a more distant inbreeding background (Hildebrandt et al., 2009).

Several researchers have suggested methods to circumvent the problems posed by using linkage analysis software for autozygosity mappings. A simple approach is the genotyping of pooled DNA samples from affected individuals and the search for markers where only one allele is present or at least predominant (Nystuen et al., 1996). However, this method will fail in case of genetic heterogeneity (i.e. different homozygous genotypes), because no correlation between a single sample and a genotype is possible.

None of the alternatives to classic linkage analysis has yet become the common choice for homozygosity mappings. We believe that this is partly due to the researchers' unwillingness to 'risk' the use of novel methods which might possibly be challenged by conservative reviewers. On the other hand, the software approaches mentioned above require at least some effort concerning installation, data preparation and familiarization. The web-based homozygosity mapping overcomes the restraints posed by linkage software and the present

applications for homozygosity mapping described above. In tests with real and simulated genotypes, it always identified the same genomic regions as conventional linkage analyses. It does not require any installation or data preparation at all. All interfaces are well known HTML pages, so it is very easy and intuitive to use. Most of all, it is by orders of magnitudes faster than conventional linkage analysis. Data upload into our database and analysis of a typical project with six affected individuals with 50 000 genotypes each is completed in less than 5 min.

A similar project on a 1M array takes less than 30 min to upload and analyse the data. Benchmarks can be found on the website http://www.homozygositymapper.org/documentation.html).

HomozygosityMapper is a web server that can analyze data from Affymetrix or Illumina genotyping platforms to identify homozygous regions. Genes in those candidate regions can be automatically identified through its interaction with GeneDistiller.

In this study we show the utility of Affymetrix SNP array 6.0 based homozygosity mapping in the diagnosis of hereditary neuromuscular disorders in consanguineous and non-consanguineous families with more than one affected member.

8.2 MATERIALS AND METHODS

8.2.1 Patients and samples

The study included two families with a clinical diagnosis of Limb Girdle Muscular Dystrophy (LGMD) with the type not known.

Family 1: This was a non-consanguineous family with 3 out of 6 siblings affected by muscular dystrophy. There was no evidence of biological consanguinity also. Of the 6 siblings, 2 of 3 males were affected and 1 of the 3 females were affected as seen in the pedigree (Figure 8.3).

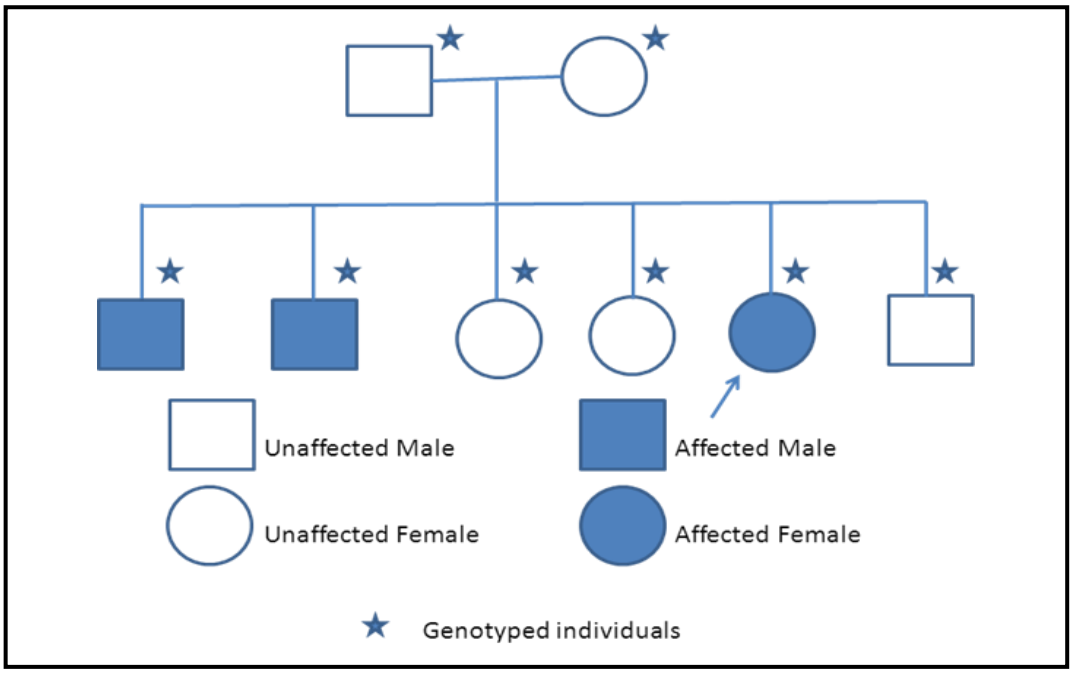

Figure 8.3: Pedigree of the family 1 affected by LGMD

The proband was a female who presented with symptoms of muscular dystrophy. She started showing symptoms at the age of 13 years. When she came for diagnosis to us, she was 35 years of age, and was ambulant. Her muscle biopsy tests were inconclusive on the type of muscular dystrophy, though immunohistochemistry for a few proteins were done.

Family 2: This was again a non-consanguineous family where the clinical diagnosis was LGMD, but definitive differential diagnosis was not made. The initial clinical symptoms of the proband included weakness of limbs, difficulty in walking and climbing stairs, difficulty in getting up from supine position, and

difficulty in lifting things. The age of onset of symptoms was 22 years, the disease being progressive with the proband becoming non-ambulant at 32 years. His elder sister, currently aged 45 years, also was affected and is non-ambulant at the age of 45 years. He also has one unaffected younger brother (Pedigree in Figure 8.4).

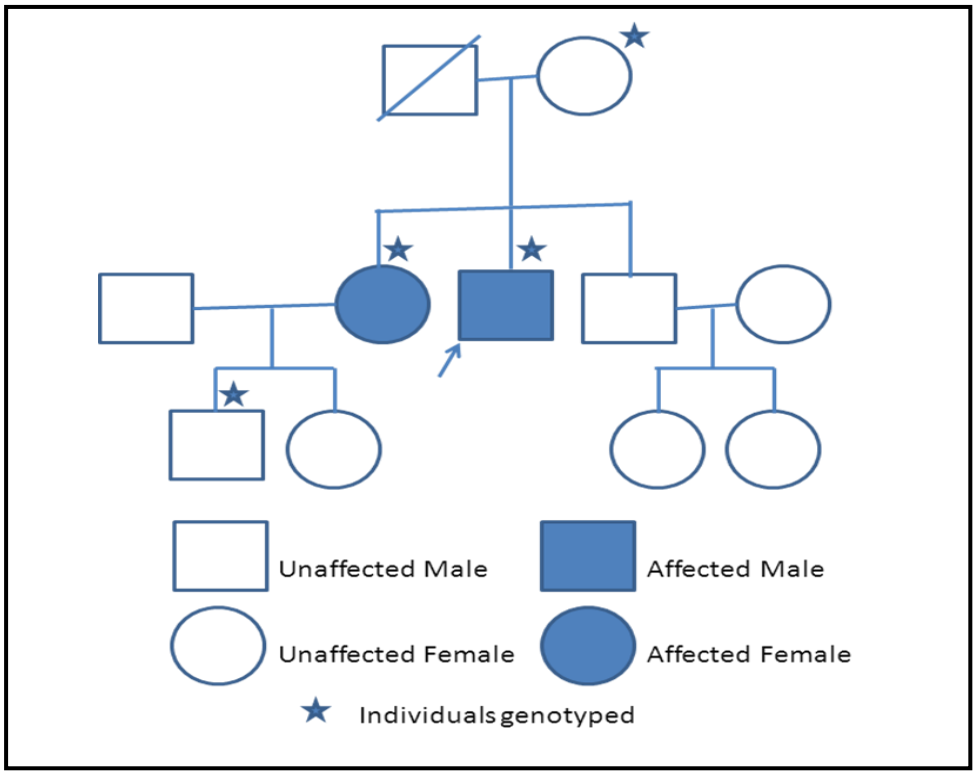

Figure 8.4: Pedigree of family 2, with two affected siblings of a non-consanguineous marriage. *Genotyping was done only for 4 members in the family*

Blood was collected in EDTA from the members of both the family after obtaining informed consent. DNA was extracted as mentioned in methods section and stored at -20°C for further analysis.

8.2.2 Whole genome SNP genotyping

Whole genome SNP genotyping was done on the Affymetrix platform using Affymetrix SNP array 6.0 as described in chapter 3. (Page 85) The SNP data was extracted in text format using the Genotyping Console (GTC) software

(Affymetrix, USA) and was modified to the format required for homozygosity mapper software.

8.2.3 Data Analysis

The whole genome SNP data was analysed using the online software homozygosity mapper (www.homozygositymapper.org). This is free online software and an account can be created by anyone who wants to use the software for analysis. This website also has other software like Gene Distiller and Mutation Taster.

Clicking on the HomozygosityMapper takes us to the Homozygosity mapper interface, where the login to the site can be done. If we are already registered to this site we can login to the site by clicking on the login icon.

Once we log in we can click on upload genotypes, to upload the data that needs to be analysed. Once uploaded this data will be stored in our account, and the analysis can be done or modified whenever we want to. Access to this data can be made user specific or available for all who use this software by clicking on the access restriction option.

8.3 RESULTS

8.3.1 Homozygosity analysis in Family 1

The results obtained from homozygosity mapper are shown in the screenshot below (figure 8.5).

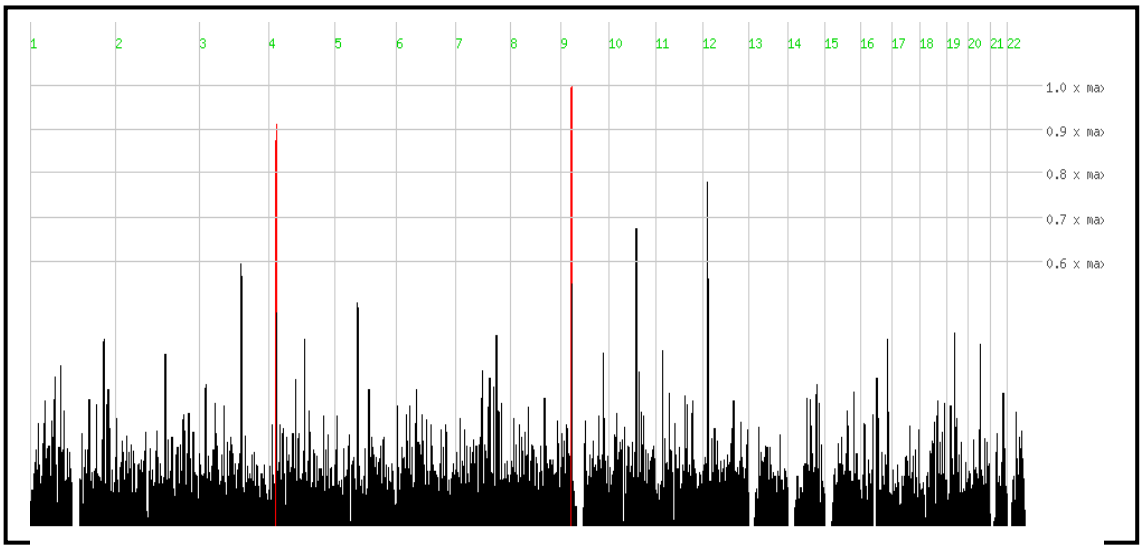

Figure 8.5: Screen shot of the genome-wide homozygosity scores produced by HomozygosityMapper for family one. *These are plotted as a bar chart with red bars indicating the most promising genomic regions. Clicking on a bar will zoom into the chromosome harbouring the score.*

Below the figure, direct links to the most interesting regions were given. These regions were divided into broad and narrow regions and can be used based on the basis of expected genetic heterogeneity. The regions obtained for family 1 is given in table 8.1.

Table 8.1: List of most interesting regions derived from homozygosity mapping for family 1

score	chr	from (bp)	to (bp)	from SNP	to SNP		
broad *- use this when you expect some genetic heterogeneity*							
2070	9	28509275	32484826	rs7856980	rs944583	region	genotypes
1886	4	22187503	24768157	rs16872464	rs978612	region	genotypes
narrow *- use this when all patients are in the same family*							
2070	9	28509275	32484826	rs7856980	rs944583	region	genotypes
1886	4	22187503	24768157	rs16872464	rs978612	region	genotypes

The above results show two loci with high scores of homozygosity, which are likely to be promising regions. The locus in chromosome 9 shows the highest score of 1, and is likely to be the region of the mutated gene. The other locus is in chromosome 4, with a score of 0.9. As the next step in diagnosis, the genomic regions with high homozygosity score were identified by giving the start and end regions in ensemble (www.ensembl.org). The locus identified were 9p21.1 and 4p16.2. Since 9p21.1 region showed the highest homozygosity score, we searched for known muscular dystrophy genes mapping to this locus. Two genes, GNE and VCP, mutations of which were known to cause Inclusion body myopathy and Inclusion body myopathy (IBM) with Paget's disease of the bone with or without frontotemporal dementia (IDMPBD) respectively, were identified. There was no known muscular dystrophy gene identified in the 4p16.2 region. We decided that 9p21.1 region and the genes at that position, GNE and VCP, are more likely to be the ones causing the disorder in this family.

8.3.2 Homozygosity analysis in Family 2

The homozygosity mapper results are shown below in figure 8.6.

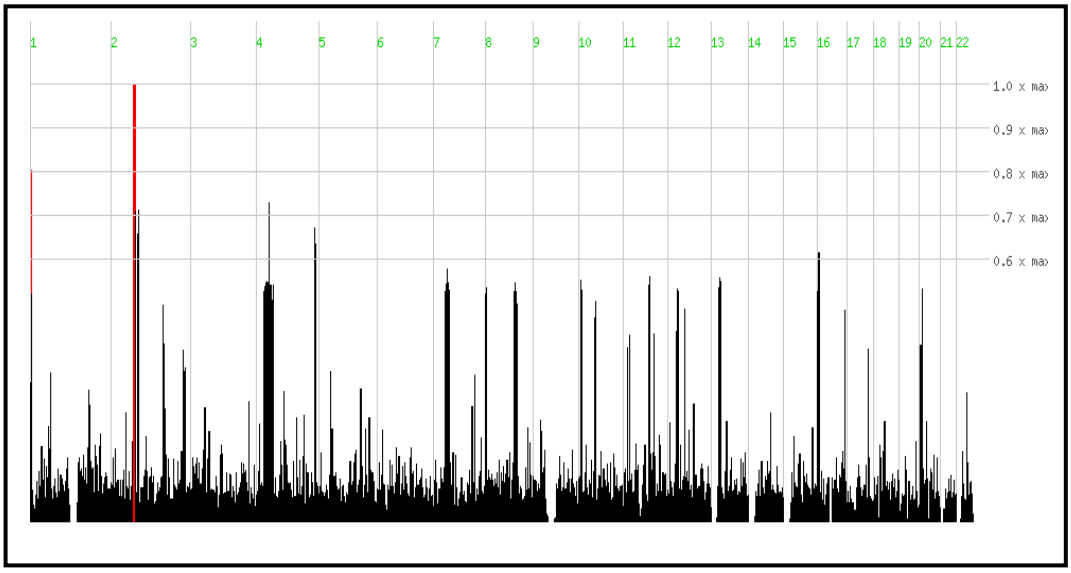

Figure 8.6: Genome-wide homozygosity scores produced by Homozygosity Mapper for family two. *This shows only one region of interest with a high score.*

Table 8.2 shows the broad and narrow homozygosity regions in family two. Unlike family one, which showed several regions in the same chromosomal region, this family showed only two narrow regions of interest, which when blasted in Ensembl, showed that the regions to be 2p14-p12 and 1p regions. The locus 2p14-p12 showed a maximum homozygosity score of 1.0 and the 1p locus showed a homozygosity score of 0.8.

Table 8.2: Broad and Narrow homozygosity regions in family 2 by homozygosity mapping.

score	chr	from (bp)	to (bp)	from SNP	to SNP		
broad *- use this when you expect some genetic heterogeneity*							
2000	2	68196994	73406828	rs17034758	rs2099194	region	genotypes
1601	1	3576197	4385179	rs10910004	rs351610	region	genotypes
narrow *- use this when all patients are in the same family*							
2000	2	68196994	73406828	rs17034758	rs2099194	region	genotypes
1601	1	3576197	4385179	rs10910004	rs351610	region	genotypes

Dysferlin gene, mutations of which are known to cause dysferlinopathy or miyoshi myopathy, is a known gene present at the 2p14-p12 locus. There are no known muscular dystrophy genes at 1p region.

8.4 DISCUSSION

Individual diagnosis and classification of the neuromuscular disorders, especially the muscular dystrophies, is challenging due to intra- and inter-familial clinical variability, overlapping clinical symptoms, genetic diversity, genetic allelic- and non-allelic heterogeneity and large number of techniques required to diagnose them. The usual diagnostic work up requires muscle biopsy and immunohistochemistry as the starting technique. Muscle biopsy is an invasive procedure disliked by the affected patients and family. Moreover, the number of proteins to be tested is huge, and will require a multistep process where the common ones are ruled out before arriving at the actual deficient protein, making it a labor intensive and expensive approach. We in this study show the successful application of SNP array based whole genome homozygosity mapping in molecular genetic diagnosis of LGMD. We emphasize this form of indirect, less invasive DNA analysis as the first step in the approach to diagnose patients from families with known or suspected NMDs, because it is fast, inexpensive, and amenable to high-throughput setting.

Though homozygosity mapping is a recommended method for inbred populations and consanguineous families, here we have used it for two outbred, non-consanguineous families with success. Another study has demonstrated that high-density SNP homozygosity mapping can also successfully be applied to non-consanguineous single individuals with rare autosomal recessive diseases, such as LGMD2, considering the hypothesis that the disease is caused by a homozygous mutation located within a homozygous haplotype, being identical by descent from an unknown common ancestor (Hildebrandt et al., 2009). Homozygous disease-causing mutations could be detected in single cases of outbred populations within homozygous areas often larger than 2 Mb in size (Hildebrandt et al., 2009). On the other hand, homozygous genomic segments measuring up to 4 Mb were not uncommon in individuals from outbred populations (McQuillan et al., 2008). Therefore, higher density SNP arrays, like in our study, might allow the detection of the disease related homozygous segments in cases of more distant or suspected parental relationship.

Family 1, in our study, showed maximum homozygosity score of 1 at 9p21.1 region, where the VCP and GNE genes are present. IBMPFD caused by VCP gene mutations are very rare and are not reported in India till date. But hereditary inclusion body myopathy (IBM) caused by GNE gene mutation are common and have been reported in India (Purushottam et al., 2008). Inclusion body myopathy (IBM) is an autosomal recessive disorder characterized by slowly progressive distal muscle weakness that begins in the late teens to early adult years with gait disturbance and foot drop secondary to anterior tibialis muscle weakness. Weakness eventually includes the hand and thigh muscles but commonly spares the quadriceps muscles, even in advanced disease. Affected individuals are usually wheelchair bound about 20 years after onset. If quadriceps sparing is incomplete, loss of ambulation tends to occur earlier. The diagnosis of IBM is based on clinical, histopathologic, and ethnic criteria. Muscle histopathology typically shows rimmed vacuoles and characteristic filamentous inclusions. *GNE* gene, which encodes the bifunctional enzyme UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase and is the only gene associated with IBM2.

Family 2 showed maximum homozygosity score at 2p14-12 region. Mutations of the Dysferlin (DYSF) gene cause dysferlinopathies which include 2 distinct clinical entities, Miyoshi myopathy (MM) (OMIM# 254130) and Limb Girdle muscular dystrophy type 2B (LGMD2B) (OMIM# 253601). Dysferlin is a sarcolemmal protein that plays an important role in patching defects in skeletal membrane by regulating vesicle fusion with the sarcolemma. Affected individuals usually present with early involvement of the posterior calf muscles (Miyoshi myopathy) in their teens or early twenties, but can present with proximal greater than distal weakness similar to other limb-girdle muscular dystrophies (LGMD2B), with anterior tibial weakness, an axial myopathy (e.g., rigid spine syndrome or hyperkyphosis resembling bent spine syndrome), or any combination of the above. Muscle biopsies may be quite inflammatory, often resulting in a misdiagnosis as polymyositis.

In both the families, final diagnosis will be achieved only with mutation screening of the respective gene and mutation detection. Lack of labs in India that do mutation screening of these two genes and the cost towards it has limited our work to just risk locus identification. However, common methods of diagnosis of

these disorders in India are only through a complete clinical work up including muscle MRI (Purushottam et al., 2008). Muscle biopsy followed by immunohistochemistry has been successfully used for dysferlinopathy in a few centres like NIMHANS, Bangalore and AIIMS, New Delhi. But dysferlin is only one of the few proteins tested for in immunohistochemistry in India, the other proteins being merosin, alpha-, beta-, gamma-, and delta-sarcoglycan, calpain and dystrophin. Most of the other LGMDs including IBM are not tested.

8.5 CONCLUSION

- To conclude, SNP based whole genome homozygosity mapping is a very useful indirect technique to screen for risk locus in affected consanguineous and non-consanguineous families, though it comes with some disadvantages like requirement of more than one affected member in the family.
- This method would direct us to the gene of interest that needs to be screened for mutations, hence taking out of equation the invasive and cumbersome method of muscle biopsy testing.
- Therefore, we recommend here that this method can be used as the first step in the molecular diagnosis of NMDs.

SUMMARY AND CONCLUSION

- We tested our less invasive algorithm of mPCR followed by MLPA for the genetic diagnosis of Duchenne/Becker muscular dystrophy on a total of 571 cases (514 query DMD and 57 query BMD). We were successful in picking mutations, mostly whole exon deletions and duplications, in 75% of all cases using this approach. MLPA was also useful in picking small insertions, deletions and substitutions, when they were present in the probe binding sites of the exons. By direct sequencing we also identified 3 more mutations. **This is the first such extensive study using MLPA for DMD in India.**

- We were unable to identify dystrophin mutations in the rest 25% of our cases, which may be small insertions, deletions and substitutions, which require whole gene sequencing method. Best practice guidelines suggest the use of muscle biopsy immunohistochemistry to confirm dystrophin deficiency, followed by cDNA sequencing of the 14 kb cDNA. This method is less tedious and less expensive than direct sequencing the genomic DNA for dystrophin gene mutations. However, muscle biopsy process is invasive and mostly disliked by the patients and their family.

- Carrier diagnosis my MLPA was done for 149 cases from 109 families. Most of these were mothers and sisters of the proband. Carrier diagnosis was done only for families in which the dystrophin gene mutation was known. Using our D/BMD diagnostic approach we were able to find out only whole exon deletions and duplications of the Dystrophin gene in the index cases. Hence, to identify copy number changes like heterozygous deletions and duplications in the probable carriers, a quantitative method was required and MLPA was considered the technique of choice. We were able to identify a total of 50 carriers in our study, one by direct sequencing, most of which were mothers and siblings of the proband. This

data helped us in further genetic counseling and also prenatal diagnosis, when required.

- Other families for which carrier diagnosis was requested and samples were available, we were unable to perform the test with this approach because the proband mutation was not known. MLPA also had the limitation of not able to pick up mosaicism, which is estimated to be seen in 9% of DMD carriers (Helderman-van den Enden, de Jong et al. 2009). Hence, only around 75% of all the families would be benefitted by this approach. The rest 25% of the families can be diagnosed by using the STR-(CA) segregation analysis with markers flanking the DMD gene. This approach has been found successful in diagnosing families without a proband diagnosis and also in picking up mosaics.

- We report on an interesting female case with symptoms of muscular dystrophy, growth and mental retardation for DMD testing. mPCR was negative and we thought that it could a case Limb girdle muscular dystrophy. On the clinician's persistence, we performed MLPA and identified exon 62 mutation. Further PCR analysis and repeated analysis of MLPA suggested this could be a mosaic of normal and mutated allele. Karyotyping on the this case revealed a 45,X / 46, XY mosaic at 85%/15%. Turner's syndrome and the mutation was also confirmed by molecular cytogenetics. This is the first case to be reported with 45,X / 46, XY mosaicism and DMD gene mutation. Further clinical analysis showed symptoms relevant to DMD and Turner's syndrome. In this study we also project a stepwise protocol to diagnose female muscular dystrophy cases. We also suggest that females with clinical symptoms of DMD should not be overlooked as LGMD but should be first tested to rule out DMD.

- As an attempt to understand the role of CNVs in the pathophysiology of D/BMD, we studied genome wide copy number variations between D/BMD patients and controls. The study showed four CNVs lodging 16 genes to be significantly more/less frequent in patients compared to the

controls. All the four regions have been reported as variations in the DGV (Database for Genomic Variants) and all the genes do not have any functional implications in DMD pathogenesis. However, further studies with more cases and controls might yield significant results to support this hypothesis. Six other very large CNVs in chromosome 9p11.2 were also seen with several genes in this region. This is the first study comparing genome wide CNVs of DMD patients with controls and further detailed study is necessary to arrive at significant results. No earlier studies also mean that there are no reported CNVs that we could specifically look into in this study.

- Diagnosis of neuromuscular disorders is hampered by the large number of genes for neuromuscular diseases that have been identified, the high level of genetic heterogeneity, and multiple diverse diseases associated with many of the individual genes. Also the fact that many of the proteins associated with neuromuscular diseases are the largest human proteins, a large number of different types of mutations that cause neuromuscular disorders which requires a large number of techniques to detect them, and that not all neuromuscular disease genes have been identified adds to the challenge in diagnosing NMDs. Common methods include muscle biopsy followed by immunohistochemistry/Western blot for individual proteins to identify the deficient protein followed by DNA sequencing. Owing to the invasiveness of this procedure, we studied the usefulness of genome wide SNP based homozygosity mapping in diagnosing LGMDs in families where more than affected member is available. We studies 2 families, both without consanguinity, and we were able to identify the affected locus in both. One of the loci, 9p21.1, lodges the GNE gene, which is known to cause Inclusion body myopathy and for the other family on 2p14-p12, where the dysferlin gene is positioned. GNE and Dysferlin gene sequencing to confirm the diagnosis was not done since it was beyond the scope of the study.

- Studying the approach with more families is necessary to confirm the usefulness of it. This approach is simple, though not a direct method, and

is less invasive with the requirement of only genomic DNA from blood cells. This is the first such approach from India using homozygosity mapper, and worldwide there is only one report using genome wide SNP basded homozygosity mapping.

- In conclusion, this thesis aimed at diagnosis, prevention and studying the molecular pathophysiology of D/BMD and molecular diagnosis of other NMDs. We were able to successfully devise an approach for the less invasive and less expensive diagnosis of D/BMD proband mutations in 75% of the cases. Carrier diagnosis aimed at prevention through genetic counseling was successfully done for families where proband mutation was known. Diagnostic approach for female DMD which was also less invasive was devised which also shed light into the mechanism of DMD in females. The effect of genome wide CNVs on D/BMD pathophysiology was studies, though with a lower number of cases. Finally, a non-invasive but effective approach using genome wide homozygosity mapping was successfully used to diagnose familial muscular dystrophies.

FUTURE SCOPE OF WORK

- Diagnosis is complete only after the rest 25% of cases without a diagnosis are diagnosed. Aiming at non-invasive diagnosis, we want to study the usefulness of Next generation sequencing (NGS) methods in screening the whole gene for mutations. Many of the genes associated with neuromuscular disorders are extremely large, resulting in them being expensive to fully analyse using Sanger sequencing. Therefore, diagnostic molecular neurogenetics laboratories, with current technologies and limited budgets available cannot analyse all the neuromuscular disease genes that need to be analysed in order to give all patients a molecular diagnosis. Neither can clinical services afford to buy all the molecular diagnostic testing required. NGS simplifies diagnosis by multiplexing several samples and screening several genes in one run. If standardized, we can not only sequence the DMD gene, but all the NMD genes in one assay, thereby reducing the cost and time of assay.

- Carrier analysis for mutation negative families can be attempted using CA-repeat based linkage analysis. This will offer diagnosis for the 25% of the cases where proband mutation is small insertions, deletions and substitutions.

- A detailed study with large sample size to understand the association of genome wide CNVs with D/BMD is necessary to understand more about genetic modifiers in DMD.

- Though DMD gene was discovered more than two decades back, proper understanding of its natural history and genotype phenotype correlations is lacking. To address this, we plan to do multidisciplinary evaluation of DMD affected kids periodically with neurologists, cardiologists, orthopedician, pulmonologists, psychiatrists and physiatrists. We have already started doing this evaluation on kids in our database, where every week children are evaluated by our panel of clinicians.

- Peripheral blood lymphocyte expression analysis of carrier mothers and normal mothers of DMD affected children to look for any biomarker predisposing the birth of DMD kids.

- Assessing the role of Ayurveda in treating children affected with muscular dystrophy.

APPENDIX 1

APPENDIX 2

APPENDIX 3

S. No	CONSUMABLES	COMPANY
1	EDTA	Merck
2	NH_4Cl	Merck
3	$KHCO_3$	Merck
4	Sodium dodecyl sulphate	Merck
5	Proteinase K	Bangalore Genei
6	Agarose	SRL
7	10X PCR buffer	Applied Biosystems, USA
8	$MgCl_2$	Applied Biosystems, USA
9	dNTP mixture – 2.5 mM	Applied Biosystems, USA
10	Sterile distilled water	Invitrogen BioServices, USA
11	Tris EDTA Buffer	Invitrogen BioServices, USA
12	Taq Polymerase	Applied Biosystems, USA
13	Primers 100 µM	Intron Bio
14	Ethidium Bromide Solution	Bangalore Genei
15	SALSA MLPA-KIT PO 34, PO 35 for DMD-BMD diagnostics	MRC, Holland
16	QFMPCR-Multiplex 1 and 2 - Primers	Applied Biosystems
19	Gene-Scan-500 LIZ size standard	Applied Biosystems
20	Sodium acetate	Ambion
21	Hi-di formamide	Applied Biosystems

LIST OF PUBLICATIONS

Articles published:

1. Sakthivel Murugan S.M., Arthi C., Thilothammal N., Lakshmi B.R. Carrier Detection in Duchenne Muscular Dystrophy using molecular methods. (*In press – IJMR*)

2. Murugan S, Chandramohan A, Lakshmi BR. Use of multiplex ligation-dependent probe amplification (MLPA) for Duchenne muscular dystrophy (DMD) gene mutation analysis. *Indian J Med Res. 2010 Sep; 132:303-11*.

Articles under review:

1. Sakthivel Murugan SM, Thilothammal N, Lakshmi BR. Duchenne muscular dystrophy in a girl with 45,x/46,xy karyotype. (*Neuromuscular Disorders)*

Articles under preparation:

1. Sakthivel Murugan SM, Lakshmi BR. Diagnosis of LGMDs using SNP array based Homozygosity mapping in consanguineous families.

2. Sakthivel Murugan SM, Lakshmi BR. Effect of whole genome copy number variations in DMD severity.

LIST OF PRESENTATIONS AND AWARDS

Papers presented at conferences

INTERNATIONAL

1. Sakthivel Murugan SM, Lakshmi BR. Uniparental Isodisomy and Turner's syndrome in two cases of Female Duchenne Muscular Dystrophy – Use of Microarray based Cytogenetic analysis to Assess Mechanism of Disease - Poster presentation in the International Congress for Human Genetics held at Montreal in October 2011
2. Sakthivel M, Lakshmi R, Thilothammal N, Viswanathan V. Female With Duchenne Muscular Dystrophy Possibly Due To Uniparental Disomy – A Case Report – Poster presentation in the 3rd International Congress of Myology held in Marsielle, France from May 26 to 30, 2008.
3. Sakthivel M, Lakshmi R, Viswanathan V, Arthi C. Carrier Analysis In Duchenne Muscular Dystrophy By Multiplex Ligation-Dependant Probe Amplification – Poster presentation in the 3rd International Congress of Myology held in Marsielle, France from May 26 to 30, 2008.
4. S C Nair, MSM Sakthivel, J J Mammen, D Sukumaran, S Singh, A Viswabandya, A Srivastava. APTT Clot Curve Analysis in FIX deficiency. Haemophilia (2006), 12, (Suppl.2) 06 PO 179 - poster presented in the Haemophilia Congress, 2006 held at Vancouver
5. MSM Sakthivel, R Prasad, S C Nair, J J Mammen, S Singh, S Baidya, B George, V Mathews, M Chandy, A Srivastava. Inhibitors to FVIII in patients with Haemophilia A in India. Haemophilia (2006), 12, (Suppl.2) 14 PO 417 - poster presented in the Haemophilia Congress, 2006 held at Vancouver.

6. Nair, SC, Sakthivel, MSM, Mammen, JJ, Singh, S, Viswabandya, A, Kavitha, ML, Mathews, V, Srivastava, A. Data from APTT clot curves of automated photo-optical coagulometers correlates better with heterogeneity of clinical presentation in severe Hemophilia A than FVIII:C levels. Journal of Thrombosis and Haemostasis, Vol 3, Supplement 1: P2004 - Poster presented in The International Society on Thrombosis & Haemostasis (ISTH) XXth congress and 51st Annual SSC Meeting, 6-12 August 2005, Sydney, Australia.
7. Sakthivel, MSM, Nair, SC, Mammen, JJ, Singh, S, Sukumaran, D, Srivastava, A. Ristocetin cofactor (VWF:RCo) Assay – A comparison of a rapid automated method on ACL 10000 coagulometer and conventional aggregometry. Journal of Thrombosis and Haemostasis, Vol 3, Supplement 1: P0868 - Posters presented in The International Society on Thrombosis & Haemostasis (ISTH) XXth congress and 51st Annual SSC Meeting, 6-12 August 2005, Sydney, Australia.

NATIONAL

1. Sakthivel Murugan S.M., Lakshmi B.R., Arthi C. Prevention of hereditary DMD by carrier diagnosis and genetic counseling - Poster presentation in 4th International Conference on Birth Defects and disabilities in the Developing World held from 4th – 7th October 2009 at Sir Ganga Ram Hospital, New Delhi, India.
2. Sakthivel Murugan S.M., Arthi C., Viswanathan V., Lakshmi B.R. Proband And Carrier Analysis In Duchenne Muscular Dystrophy Using MLPA - Poster presented in Indian Society Of Human Genetics Held In February 2008 In Vishagapatnam.
3. Sakthivel Murugan S.M., Arthi C., Viswanathan.V., Lakshmi B.R. Dystrophin gene mutations in D/BMD patients - Use of mPCR and Multiplex Ligation-dependent Probe Amplification in diagnosis – Poster presented in 9TH National Conference of Indian Society of Prenatal Diagnosis and Therapy (ISPAT 2007)

conference held from 23rd - 25th November 2007, in Chennai, India

AWARDS:

1. **Travel Grant** to participate in the 7th Summer School of Myology organized by the Institut de Myologie, Paris from June 19th to June 27th 2008.

2. **Travel grant** to attend the 3rd International Congress of Myology held in Marsielle, France from May 26 to 30, 2008 and the 5th International Congress of Rehabilitation in Neuromuscular Disease held in Marsielle, France from May 30 to June 1, 2008. The travel grant covered travel and accommodation expenses.

3. Received Reach the **World Travel Grant of AUS$2000.00** to assist me with my travel expenses to the XXth Congress of the International Society on Thrombosis and Haemostasis (ISTH), held in Sydney, Australia from the 6-12 August 2005. In addition, my Registration fee for the Congress was also waived.

4. **Dr. H.N. Madhavan Endowment Award** having been adjudged as the BEST OUTGOING STUDENT on successful completion of the Master of Science in Medical Laboratory Technology.

5. **Best performance awards** in the examinations on CLINICAL GENETICS, BIOCHEMISTRY, CLINICAL IMMUNOLOGY, DIAGNOSTIC MICROBIOLOGY, CLINICAL MICROBIOLOGY, and HUMAN ANATOMY AND HUMAN PHYSIOLOGY on successful completion of the Master of Science in Medical Laboratory Technology.

BRIEF BIOGRAPHY OF THE CANDIDATE

Mr. Sakthivel Murugan SM obtained his B.Sc (Medical Laboratory Technology) degree from Christian Medical College, Vellore, under Dr.MGR Medical University, Chennai in 1998. He obtained his M.S. (Medical Laboratory Technology) Degree from Birla Institute of Technology and Science, Pilani in 2004 with the course work at Medical Research Foundation, Chennai. He was the Best Outgoing Student in his Masters. Then he joined the Department of Clinical Pathology and Blood Bank, Christian Medical College & Hospital, Vellore as Senior Demonstrator, where he worked on physiology of Coagulation. In 2006, he joined Molecular Diagnostic Facility,, Sundaram Medical Foundation, Chennai as Senior Technologist. He registered for Ph. D in January 2007 in Birla Institute of technology & Science, Pilani. He has made 10 presentations including National and International conferences, all of which were poster presentations. He has 4 publications out of which 2 are on his thesis work and 10 abstracts presented in various national and international conferences. Four papers are under preparation. He has participated in the 7th Summer School of Myology organized by the Institut de Myologie, Paris from June 19th to June 27th 2008, with a travel grant to attend the workshop. He was Involved in conducting Workshop on "Newer Methods in Haemostasis" in the Indian Society of Haematology and Transfusion Medicine (ISHTM) Conference. He has attended short courses in health data management and statistical software in biostatistics, clinical trials, diagnostic test & cluster designs, logistic regression and survival analysis held in the biostatistics resource and training centre, department of biostatistics, Christian Medical College, Vellore. He has won 3 travel grants to participate in International conferences and workshops. With a liking towards to methods standardization and diagnostics, he has standardized a lot of tests including automated RiCof assay in ACL 10000, being the first to automate the assay in India. He has also standardized mPCR and MLPA, his lab being one of the first to perform MLPA in India for the diagnosis of DMD. He also firmly believes that science has to be taken to the community.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. Bremadesam Raman Lakshmi, is currently the Head and PI, Molecular Diagnostic Facility, obtained her Ph.D in 1995 from Indian Institute of Technology, Madras in the field of Microbial Biochemistry. She had received the;

- Gold medal for the best student award for the year 1985 –1987 honoring all round achievement.
- Best seminar presentation at the Association of Microbiologists-India 1992
- Best poster presentation at the Association of Microbiologists-India 1992
- Best Thesis Award of the year, for the M.Sc. project 1989.
- Recipient of the Junior Research Fellowship (1989) to work towards the Ph.D.
- Awarded the Young Woman Scientist Award by the Talwar Research Foundation – year 1994
- Best outgoing employee award- 2001,2003.
- Award to R&D on cost saving with alternate resources, 2003.

Her job experience has been all through in Industry in the areas of product Development and business management. Her position as Head - Research and Development for one of the leading US based firms has further honed her R&D and managerial skills. As functional head, She was responsible for product development, Quality assurance and regulatory affairs with added roles on overall production, HR and finance. In this capacity she has interacted on a day-to-day basis with her peers in Singapore, EU and USA and have had a direct responsibility on business growth.

With the passion to move to community work, she started with initiatives on a collaborative project with WHO on Malabsorption in children fed under the noon meal scheme, under the ICDS (Integrated Child developmental scheme) programme. Funded by the state government the proposal was accepted and when

ready to enter the execution stage got aborted due to priority shift of funds to Tsunami relief (December 2004).

Currently heading the molecular diagnostic facility responsible for the goals under Stitching Porticus grant. Working on the molecular aspects of Duchenne muscular dystrophy with execution of goals on diagnostics, standard of care and a work on holistic approach toward the Duchenne community is provided. Efforts on prevention strategies of carrier analysis, counseling, prenatal diagnosis and QoL aspects are taken up towards the DMD community.

She is currently the principal investigator for a major project funded by the NRHM and the Tamilnadu Government for understanding the prevalence and confirming DMD and SMA in Tamilnadu.

She has 5 publications and 4 manuscripts in preparation. She has attended and presented her work in several conferences, meets and workshops. She was part of the committee which decided on the best practice guidelines for the diagnosis of DMD.

With a keen eye towards the community, Duchenne families in particular, she is motivated and confident in taking her scientific knowledge in human genetics to the well being of the affected families.

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