APPLICATION OF MOLECULAR TECHNIQUES FOR DIAGNOSIS OF NEUROMUSCULAR DISORDERS

An Internship Report submitted

for the partial fulfilment of the Degree of Master of Science

By

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CERTIFICATE

This is to certify that this training report entitled **"Application of Molecular Techniques for Diagnosis of Neuromuscular Disorders"** was successfully carried out by Miss. Janvi Tejasbhai Pujara towards the partial fulfilment of requirements for the degree of Master of Science in Biotechnology of Atmiya University, Rajkot. It is an authentic record of her own work, carried out by her under the guidance of Dr Rashna Dastur for a period of 3 months during the academic year of 2022-23. The content of this report, in full or in parts, has not been submitted for the award of any other degree or certificate in this or any other University.

Signature
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(Head Of The Department)

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Miss Zeenia Avari
(Supervisor)

DECLARATION

I hereby declare that the work incorporated in the present dissertation report entitled "**Application of Molecular Techniques for Diagnosis of Neuromuscular Disorder**" is my own work and is original. This work in part or in full has not been submitted to any University for the award of any Degree or a Diploma.

Date

Signature Pujara

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Abstract

CAMDND is a molecular diagnostic laboratory located in Sion, Mumbai. The main objective of the lab is to perform molecular tests for diagnosis of rare neuromuscular disorders. Neuromuscular disorders (NMDs) are inherited or acquired conditions that affect the skeletal muscles, motor neurons, or neuromuscular junctions. Most of them are characterized by a progressive damage of muscle fibers with reduced muscle strength, disability, and poor health-related quality of life of affected patients. The diagnosis is done with the help of various molecular techniques such as Multiplex Polymerase Chain Reaction for Duchene Muscular Dystrophy and Becker's Muscular Dystrophy and technique such as Restriction Fragment Length Polymorphism for Spinal Muscular Atrophy. A literature survey on the neuromuscular disorders was conducted. As a result, the clinical symptoms of the various dystrophies and the genes and their variants that are responsible for the disorders could be identified. Hands on training on all the steps beginning from DNA Extraction, Polymerase Chain Reaction and Restriction Digestion to Gel Electrophoresis were conducted. Gels of different concentration and porosity are made for different samples (Muscular Dystrophies and Spinal Muscular Atrophy). During the sample collection, there was an interaction with the patients where the clinical history of the patient was collected. For getting precise results, certain samples are also outsourced for Next Generation Sequencing (NGS) as there are more than 6000 genes that are responsible for causing a neuromuscular disorder. The NGS reports includes all the gene variants (pathogenic or non pathogenic) that are responsible for the symptoms visible in the patient.

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About The Laboratory

Centre for Advanced Molecular Diagnosis in Neuromuscular Disorder (CAMDND) is molecular laboratory located in Sion (East), Mumbai, Maharashtra. The main objective of the lab is to provide genetic diagnostic services for neuromuscular disorders such as DMD and SMA to the patients and their family members at risk referred by clinicians. The genetic tests are performed with the help of Molecular Techniques such as Restriction Fragment Length Polymorphism (RFLP) and Multiplex Polymerase Chain Reaction (mPCR). Dr. Rashna Dastur, the director of CAMDND is a recognized guide for M.Sc and PhD in Applied Biology with the University of Mumbai. Ms. Zeenia Avari, is a JRF student working here and is also a supervisor to the interns. Mrs. Jayashree Thorat is the Lab Assistant.

The laboratory has instruments such as Thermal Cyclers for running PCR and restriction digestion, a Gel Documentation for observing the gel images under UV, hot plate, centrifuge spinner, micro centrifuge and a vortex machine for the DNA extraction process.

The samples mostly come from Bombay Hospital through recommendations from neurologists.

Report

Introduction

Neuromuscular disorders are a heterogeneous group of disorders that affect various elements of the nervous system, including the neuromuscular junction, nerve roots, anterior horn cells, muscles and peripheral nerves. These disorders can be genetic or acquired defects, and can be present at any age [1]. To make therapeutic decisions about these disorders, clinicians should be able to recognize their clinical presentation and characteristics [2]. There are various types of neuromuscular disorders such as infantile spinal muscular atrophy (a motor neuron disease), peripheral neuropathies, congenital muscular dystrophies, progressive muscular dystrophies, Steinert myotonic dystrophy and many more. Muscle weakness is a common symptom which is shared by all of these conditions [3].

The main tests performed in CAMDND lab are for muscular dystrophies such as Duchenne Muscular Dystrophy (DMD) and Becker's Muscular Dystrophy (BMD) with the help of Multiplex Polymerase Chain Reaction (mPCR), and muscular atrophies such as Spinal Muscular Atrophy (SMA) with the help of Restriction Fragment Length Polymorphism (RFLP). There are more than 6000 genes responsible for neuromuscular disorders. For getting more precise results, certain samples are also outsourced for Multiple Ligation based Probe Analysis (MLPA). Mostly, the samples of DMD are sent for MLPA testing. The samples for Limb Girdle Muscular Dystrophy (LGMD) are outsourced for Next Generation Sequencing (NGS) as there are many genes responsible for causing LGMD and more than one gene variant is responsible for giving a common symptom.

The first task assigned was a literature survey to be performed on neuromuscular disorders which included the clinical symptoms of the disorder, gene responsible for the disorder and the variant responsible for the disorder.

1. Literature Review

1.1 Duchenne Muscular Dystrophy (DMD)

Duchenne Muscular Dystrophy affects 1 in every 3500 males. It is a X-linked recessive disorder caused by mutation in Dystrophin gene. Dystrophin links the internal cytoskeleton to the extracellular matrix in the cell. (Fig.1) The amino-terminus of dystrophin binds to F-actin and the carboxyl terminus to the dystrophin-associated protein complex (DAPC) at the sarcolemma (plasma membrane of muscular cell).[4]

DMD is classified as the most severe form of dystrophinopathy as there is complete lack of the dystrophin protein. [5]

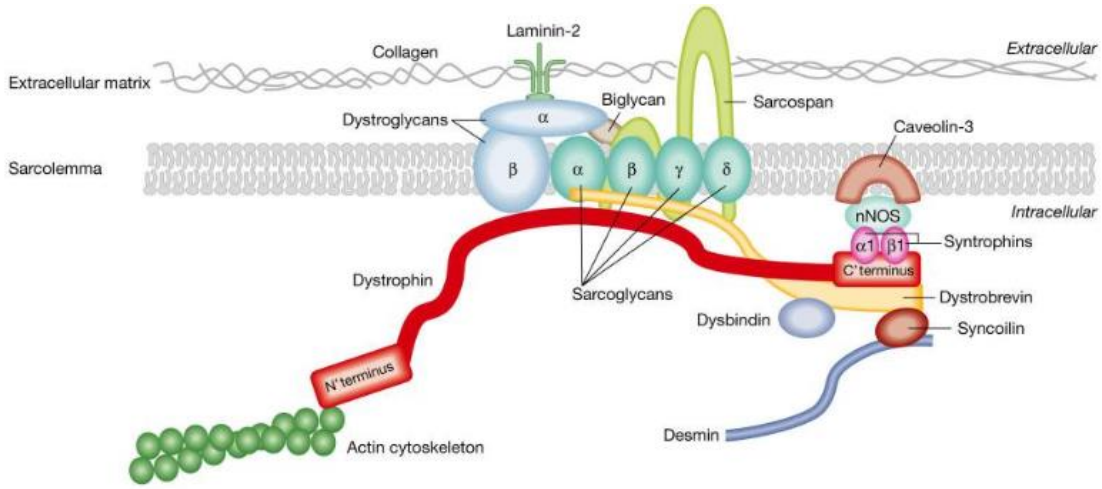


Fig1. Molecular organization of the dystrophin–glycoprotein complex (DGC) in sarcolemma. α-Dystroglycan links to the extracellular components with β-dystroglycan, which is a transmembrane glycoprotein and binds to dystrophin and sarcoglycan. Dystrophin binds to cytoskeletal actin at its N-terminus and to syntrophins and α-dystrobrevin at its C-terminus. [4]

1.1.2 Clinical Symptoms

For children suffering with DMD, initial findings may include delays in sitting or standing without help; toe walking; an unusual, waddling manner of walking (gait); difficulty in climbing stairs or getting up from sitting position (Gower's sign); and repeated falling. Toddlers and

young children may seem clumsy and may have abnormal enlargement of the calves due to scarring of muscles (pseudo hypertrophy). As the disease progresses, additional abnormalities may develop such as progressive curvature of the spine (scoliosis or lordosis), wasting of thigh and pectoral muscles, and abnormal fixation of certain joints (contractures). Without any physical therapy treatment, leg braces might be needed by the age of 8-9 to assist affected individuals to walk. By approximately ages 10 to 12, most affected individuals require a wheelchair [6].

1.1.3 Dystrophin Gene and Mutations

Dystrophin is the largest gene in human genome having 79 exons and 2.6 million base pairs of DNA. DMD is caused by mutations in dystrophin gene which is located on the short arm (p) of the **X chromosome (Xp21.2)** which has the gene for the large protein Dp427, also known as dystrophin. Dystrophin is not only distributed in skeletal, smooth muscles and cardiac muscle but also in the brain. [7]

The most common form of mutation found in the dystrophin is a **deletion** within the gene (65%-70% cases). **Duplication or partial duplications** are found in 5-10% of patients. Both deletion and duplication can occur almost anywhere in the dystrophin gene; however, **two hot-spots (exon3-7) and exons (44-53)** are known. Approximately 90% of the mutations occur in these two hot spots. The mutations for DMD are mainly **Out-of-frame** mutations. [8]

1.2 Becker's Muscular Dystrophy

Becker's Muscular Dystrophy (BMD) is a **milder form of DMD** which is caused by reduction in amount or alteration in the size of dystrophin protein.[9] It is caused by an **in frame deletion** in the dystrophin gene. The in-frame deletion results in truncated gene rather than complete loss of gene as found in DMD. The reading frame of the gene is preserved.[10] As a result, the symptoms observed are less severe and life expectancy of the patient is high as compared to DMD. [11]

1.3 Limb Girdle Muscular Dystrophy (LGMD)

LGMD are a group of diseases which cause weakness and wasting of the muscles in arms and legs. The muscles closest to the body are mostly affected; specifically the muscles of shoulders, upper arms, thighs and pelvic area. They are heterogeneous group of genetically determined myopathies.[12]

With the help of molecular studies, around 31 different types of LGMDs have been identified so far. The type of disorder can be autosomal dominant form or recessive form. The dominant forms are written as LGMD1 and recessive forms are written as LGMD2. A total of 23 autosomal recessive forms (LGMD2A-W) and 8 autosomal dominant forms (LGMD1A-H) have been identified. The recessive forms account for 75-90% of LGMD cases and dominant forms account for 10-25% of the cases [13]. The LGMD2 cases are more frequent with a cumulative incidence of 1:15000. The difference in the prevalance among different countries depends on the carrier distribution. [14] LGMD2A (Calpainopathy) is the most frequent type of LGMD accounting for 20-40% of cases followed by LGMD2B (Dysferlinopathy) with 10-15% of cases. Most of the autosomal recessive disorders are caused due to mutations in the genes which encode for the structural proteins that make up cytoskeletal scaffold of that particular muscle.[15]

1.3.1 Clinical Symptoms and Diagnosis

The general symptoms of patients suffering from LGMD are weak thigh muscles (need to use arms to get up from squatting position), walking or waddling on balls of feet, difficulty in running, lordosis (abnormally curved lower back), scoliosis (sideways curvature of back), overgrowth of calf muscles[16]. The initial diagnosis of LGMD includes the clinical presentation; serum creatinine kinase (CK) levels and muscle biopsy. However, the exact diagnosis of the subtype can only be achieved through molecular characterization of subtype.[17]

1.3.2 Types Of Limb Girdle Muscular Dystrophy

1. LGMD2A (Calpainopathy): Calpainopathy is caused by mutations in the CAPN3 gene which is present at chromosome 15q15.2 covering 53kb of genomic sequence. The mutations which cause calpainopathy can be missense or nonsense substitutions, small insertions and

deletions widely spread over the gene. The onset of symptoms ranges from 2 to 40 years in LGMD2A. The disorder is characterized by progressive and symmetric weakness of proximal muscles (limb and girdle), joint contractures and scapular winging. [18]

2. LGMD2B (Dysferlinopathy): Dysferlinopathies are caused by mutations in the **DYSF gene**. It is the most common adult onset forms of LGMD. The onset of symptoms occurs in late teenage or early adulthood. There is elevation of CK levels in LGMD2B patients; even presymptomatically. Mutations in DYSF gene cause different phenotypes like Miyoshi myopathy, LGMD2B, rare axial myopathies, distal myopathies with anterior tibial weakness. Same mutation in different individuals leads to different phenotypes in the same family. It is characterised by high serum CK levels, disease progration and frequent inflammatory reactions.[19]

3. Sarcoglycanopathies: It is a group of LGMD which are caused by mutations in the sarcoglycan proteins present in the sarcolemma of the muscle cell. (Fig 2.)[21] Different genes of the sarcoglycans are responsible for causing sarcoglycanopathies. (Table 1). The common characteristics of these group disorders include progressive weakness and degeneration of skeletal muscles, which leads to loss of ambulation during adolescence in most of the patients. The first symptom observed is positive Gower's maneuver (difficulty in getting up from sitting position). Scoliosis and contractures often develop in the course of the disease. There is a difficulty in breathing caused in some patients which leads to premature death sometimes. [20]

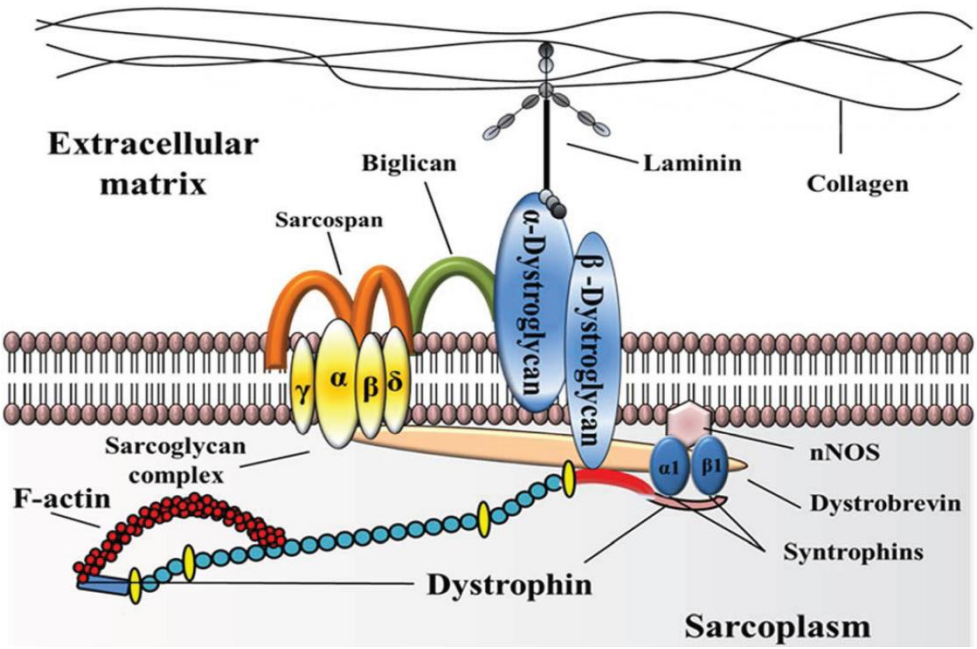


Fig 2. Molecular organization of the dystrophin–glycoprotein complex (DGC) in sarcolemma. α-Dystroglycan links the extracellular components (laminin, neurexin, and agrin) with β-dystroglycan, which is a transmembrane glycoprotein and binds not only to dystrophin, but also to sarcoglycans. [21]

Sarcoglycanopathy	Gene Responsible
LGMD2C	SGC γ (SGCG)
LGMD2D	SGCα (SGCA)
LGMD2E	SGCβ (SGCB)
LGMD2F	SGCδ (SGCD)

Table 1. Different sarcoglycanopathies with the corresponding genes.

1.4 Spinal Muscular Atrophy (SMA)

SMA is a neurodegenerative disorder that affects the peripheral nervous system, central nervous system, and voluntary muscles system (skeletal muscles). It is caused by a deficiency of SMN (Survival of Motor Neuron) protein which present on chromosome 7. It results in the loss of motor neurons in the Spinal cord. These groups of disorders are characterized by degeneration of anterior horn cells and resultant muscle atrophy and weakness.[22]

In normal conditions, a normal SMN1 gene translates to a fully functional SMN protein. In case of a mutation in the SMN1 gene, little or no full-length functional SMN protein is transcribed. In absence of SMN protein, there is a loss of motor neurons. This loss is generally compensated by protein from the SMN2 gene which present on the same chromosome. SMN2 genes are 99% similar to SMN1. Most of the proteins translated from the SMN2 gene are short and nonfunctional but some are functional. Hence, it is less effective. The number of SMN2 proteins present depicts the severity of the disorder. If more SMN2 genes are present, it means more SMN protein would be available and hence the milder symptoms of the disease are present and later the onset of the symptoms [23].

1.4.2 Genetics

SMN1 and SMN2 genes both are present on the same chromosome and have a similar DNA sequence except for a T at +6 position of exon 7. SMN2 gene is inverted duplication copy of SMN1 gene.(Fig 3)[24] (Fig 4)[23]

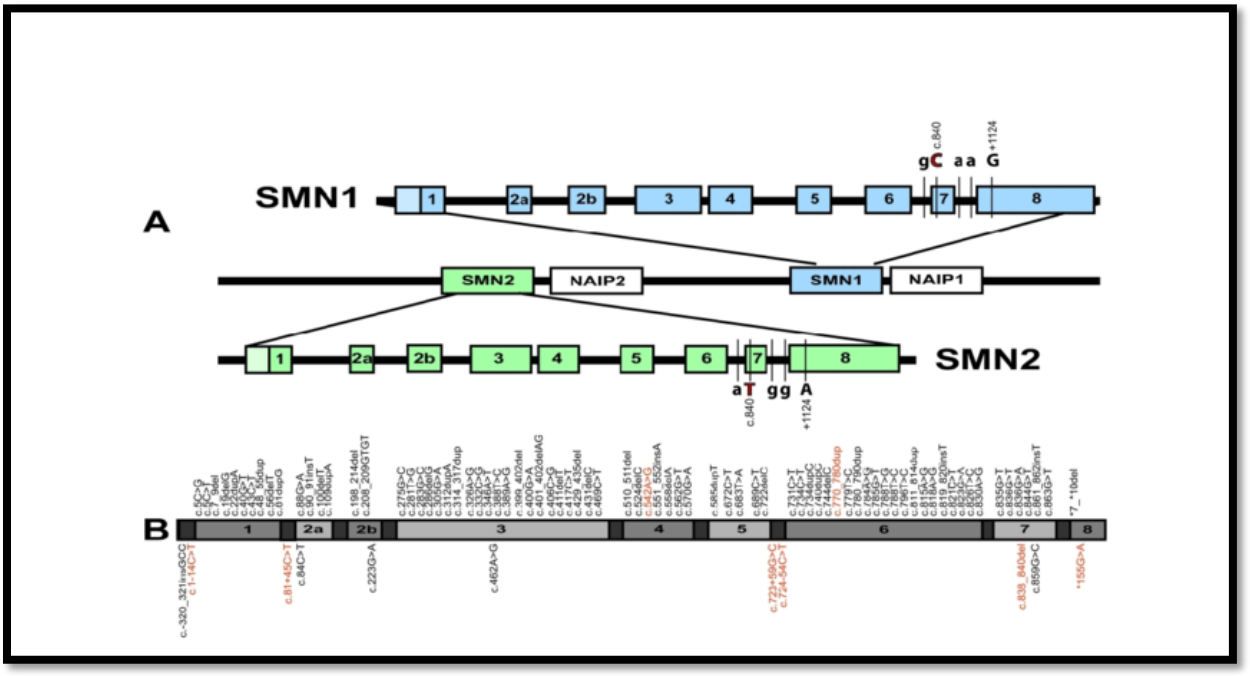


Fig 3. SMN1 and SMN2 have similar DNA sequence except C at +6 in SMN1 gene and T at +6 in SMN2 gene.

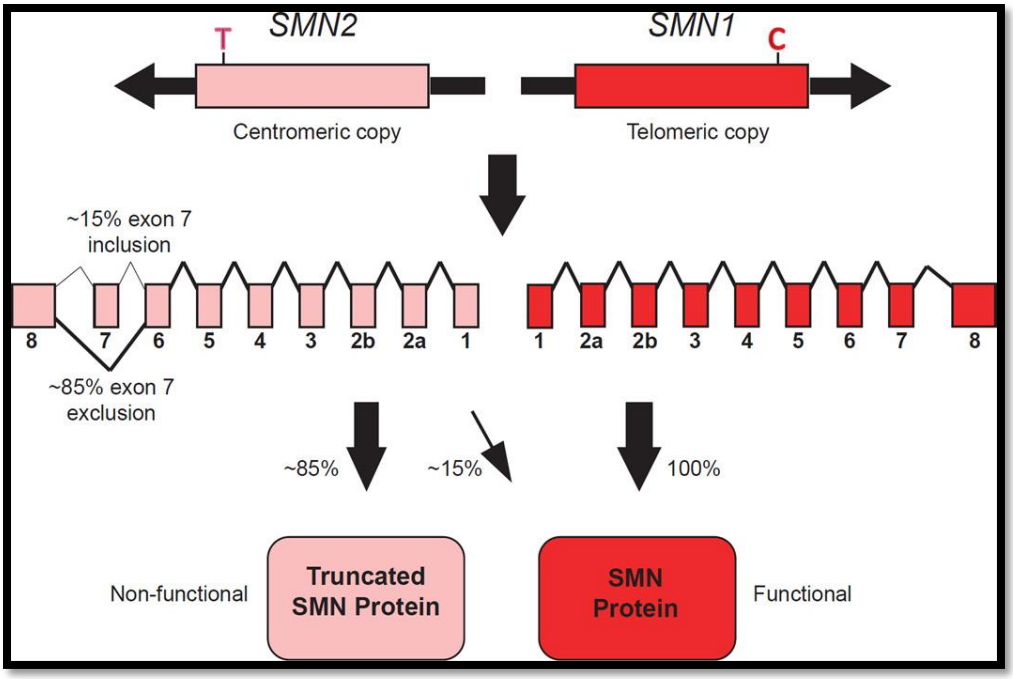


Fig 4. Both of the copies of SMN1 exhibit deletions or mutations in SMA patients. The SMN2 gene is expressed, but because of a C-to-T substitution at exon 7's position 6, the bulk of the resulting SMN2 pre-mRNA lacks exon 7. The SMN protein that has been shortened is unstable and useless. From the SMN2 pre-mRNA, only a small fraction of full-length mRNA containing exon 7 is generated, but this results in functioning full-length SMN protein.

SMA Type 1: SMA Type 1 is also known as **Werdnig-Hoffman disease**. It has infantile onset and the symptoms are visible by birth **or by the age of 6 months**. The children having SMA type 1 have **2 copies of SMN2 gene**. The symptoms include generalized muscle weakness, a weak cry and breathing distress. There is difficulty in swallowing and sucking. In case of SMA type 1, there is an increased risk of aspiration (food/liquid entering the food pipe) and failure to thrive. The major characteristic symptom is that **the child is not able to sit.**

SMA Type 2: SMA Type 2 is also known as **Dubowitz Disease or intermediate SMA**. The symptoms are visible between the age of **3 to 15 months**. Patients suffering from Dubowitz disease generally have **3 copies of SMN2 gene**. The symptoms include proximal muscle weakness which involves upper limbs less than lower limbs. However the face and eye muscles remain unaffected. The characteristic symptom is that the **child cannot walk.**

SMA Type 3: It is also known as **Kugelberg Welander disease or adult onset SMA**. The symptoms are visible **after 18 months** with patients having **3-4 copies of SMN2 gene**. Type 3 accounts for around 30% of SMA cases. The patients suffering from SMA Type 3 can walk but have difficulty in climbing stairs and they tend to fall more because of proximal weakness. Overtime, the patients tend to lose the ability to walk and stand and might need a wheelchair.[25]

TYPE	ONSET	FUNCTION	MEDIAN SURVIVAL*
0	Prenatal	Respiratory failure at birth	Weeks
1	0–6 months	Never sit	< 1 years
2	< 18 months	Never Walk	>25 years
3	> 18 months	Stand or ambulatory	Adult
4	30 years	Ambulatory	Adult

Table 2. Types of SMA with the onset and function

2. Work Done and Experience

The diagnosis of the neuromuscular disorders is done with the help of molecular techniques such as RFLP for SMA and mPCR for DMD and BMD. For better results of DMD, the samples are outsourced for MLPA testing. There are more than 6000 genes responsible for LGMD and hence the NGS (Next Generation Sequencing) techniques are required for the diagnosis of LGMD. The samples are outsourced for the NGS reports. The patients mostly are come from Bombay Hospital and recommended by neurologists. The processing of 1 sample takes around 3-4 days in which the following steps are performed; DNA extraction, PCR cycle, restriction digestion and Gel electrophoresis. After which the reports are generated on the basis of the gel images which help to find if there is any mutation present or not. All the techniques are performed based on the kits available in the market. The cost of each RFLP test is around Rs.7,000 and for mPCR it goes around Rs. 8,000.

All the GLPs (Good Laboratory Practices) are followed including wearing of gloves and lab coats during performing the experiment and dealing with blood of the patients. The tips and all other disposables are thrown after using once. Discarding of the gel having EtBr was carried out in Potassium Permanganate solution.

The patient samples are either collected at the laboratory and the clinical history is noted down. It helps to correlate the physical symptoms with the results obtained from the tests.

I also got a chance to observe and study the NGS reports and it helped in identifying the various genes that are responsible causing LGMD. Some of the patients have both autosomal dominant and recessive genes causing pathogenecity. It could be concluded that more than one gene might be responsible for causing the disease. There were also some patients who had symptoms but the results showed variants of unknown significance which implies that there is scope of studying that variant and gene causing the symptoms.

3. Materials and Methodology

The first and foremost step in performing any molecular test is the isolation of DNA from the sample. This step is common for all the tests and takes approximately 2-3 hours.

RFLP is performed for SMA samples. The protocol for RFLP includes PCR (amplification of the desired gene) which is followed by a restriction digestion step. The restriction digestion product and the PCR product are loaded in Agarose gel and run for electrophoresis until a clear band is observed. The gel is then observed under UV light in a Gel Documentation system.

mPCR is performed for DMD and BMD samples. For running mPCR, the DNA isolate is run for multiplex PCR with different primer sets and then is loaded in the gel for Agarose gel electrophoresis. The PCR product obtained is very small in size and hence a gel with better band separation has to be used. Hence, Nusieve gel is used for better band formation in mPCR.

3.1 DNA Isolation

A DNA isolation kit is used for the isolation of DNA from the patient sample.

1. 20µL of Proteinase K solution is added to 200µL blood sample in a 1.7mL microcentrifuge tube. Spin and vortex the sample for 15 seconds each.
2. 200µL of lysis solution is added to the tube.
3. The tube is kept at 56^0C for 10 minutes.
4. 200µL of 95-100% ethanol is added. Spin and vortex for 15 seconds each.
5. All the contents are transferred in binding column in a 2mL collection tube.
6. Centrifuge at 8000 rpm for 1 minute.
7. Collection tube containing flow through is discarded and binding column is placed in a new 2mL collection tube.
8. 500µL pre wash solution is added to the binding column.
9. Centrifuge at 8000 rpm for 1 minute.
10. Collection tube containing flow through is discarded and the binding column is transferred to 2mL collection tube.
11. 500µL of was solution is added to the binding column.
12. Centrifuge at 12,000 rpm for 4 minutes.
13. Collection tube is discarded and column is placed in a 1.7mL centrifuge tube. (The cap of the column is kept open for 5 minutes).
14. 200µL of elution buffer is added to the binding column.
15. The column is kept at room temperature for 30-40 minutes.
16. Centrifuge at 8,000 rpm for 1 minute.
17. The binding column is discarded and collection tube containing the pure genomic DNA is kept at 2-8^0C for short term and incubated at -20^0C for long term storage.

3.2 Restriction Fragment Length Polymorphism (RFLP)

3.2.1 Polymerase Chain Reaction (PCR)

Master mix for PCR is available in the kit to which the primers and DNA isolate is added in a small PCR tube and the tube is kept in the Thermal cycler where the step of hotstart PCR is carried out.

The PCR cycle is carried out at the temperatures shown in the table. (Table 3).

Temperature	Time
94^0C	3 minutes
95^0C	15 minutes
94^0C	1 minute
55^0C	1 minute
72^0C	1 minute
72^0C	10 minutes

Table 3. Program run in the Thermal Cycler.

In the initial 18 minutes, the Taq polymerase enzyme gets activated after which the denaturation step is carried for 1 minute followed by annealing and extension each for 1 minute. The steps of denaturation of DNA strand, primer annealing and extension continues for 35 cycles. After this, the final extension takes place at 72^0C for 10 minutes. In the final extension step, any DNA strand which is left incomplete in the step of extension is completed.

3.2.2 Restriction Digestion

The PCR product, restriction enzyme (DraI) and buffer (1X Tango) is added to a PCR tube and kept in Thermal cycler for 35 cycles. The program followed is same as that for PCR. (Table 3)

3.3.3 Agarose Gel Electrophoresis

Things to keep ready before starting: 1. Pipettes

2. EtBr (from fridge)
3. Tray with comb
4. Buffer 1X TBE (should be at room temp)
5. Agarose (select the one for SMA)
6. H_2O – Warm water keep aside

Protocol:

1. Weigh 20ml TBE Buffer(1X) + 0.5g Agarose (for small 4 well gel) or 50ml TBE Buffer(1X) + 1.25g Agarose (for big 10 well gel).
2. Soak for 5 minutes.
3. Weigh on weighing balance (Beaker + 1X TBE Buffer + Agarose)
4. Heat for 1 min at high power
5. Weigh the mixture again (use tongs to hold and mix).
6. Add the pre-heated H 2 O around 8ml-10ml more than original weight (*check that everything is mixed nicely)
7. Heat for 2 min. at power 80 (press power thrice)
8. Weight again

(If weight decreases then add water accordingly to buffer it back to original weight)

(If weight increases then heat to mixture again by adding some water if there are clumps)

9. Let the mixture cool a little, mix with tongs
10. Add 0.5 µl EtBr (for both 20 ml & 40 ml gel)
11. Pour the Agarose
12. If there are any bubbles in the gel then use a micropipette tip end to remove them
13. Let the gel set for 45 min. (Minimum)

For Loading the Sample:

1. Gel dye (Fast Cyclin) (2µl) + EtBr (0.5µl)

[To make a working stock for 10 samples: Dye: 10 x 2µl = 20µl and EtBr: 10 X 0.5µl = 5µl]

2. Then take a Para film and place the inner (cleaner) side up
3. Add 2.5µl of Gel dye + EtBr solution on the Parafilm, equally spaced from each other (make sure it does not mix)
4. Add 10µl of sample to it. Mix it nicely by increasing the count to 13 or so.
5. Load in wells

6. Switch on the Power pack (keep the wires connected before). Voltage should be 120-125V

3.3 Multiplex PCR

3.3.1 Polymerase Chain Reaction

Master mix for PCR is available in the kit to which the primers sets and DNA isolate is added in a small PCR tube and the tube is kept in the Thermal cycler where the step of hot start PCR is carried out.

The 3 primer sets have primers for different exons as shown in table 4.

Exon	Size (in bp)
Pm	535
49	439
3	410
43	357
50	271
13	238
53	212
6	202
47	181
46	148
60	139
7	119
52	113

A

Exon	Size (in bp)
45	547
48	506
19	459
17	416
51	388
8	360
12	331
44	268
4	196

B

Exon	Size (in bp)
2	294
20	242
1	220
55	190
10	175
11	165
21	155
54	140
9	129
5	93

C

Table 4. (A) Primer set of 13 exons (B) Primer set of 9 exons (C) Primer set of 10 exons.

The PCR cycle is carried out at the temperatures shown in the table (Table 5)

Temperature	Time
94.0 ^{0}C	6 minutes
95.0 ^{0}C	15 minutes
94.0 ^{0}C	30 seconds
52.0 ^{0}C	30 seconds
65.0 ^{0}C	4 minutes
65.0 ^{0}C	7 minutes

Table 5. Standardized mPCR reaction

3.3.2 Agarose Gel Electrophoresis

Things to keep ready before starting: 1. Pipettes

2. EtBr (from fridge)
3. Tray with comb
4. Buffer 1X TBE (should be at room temp)
5. Nusieve 3:1 Agarose (select the one for DMD)
6. H_2O – Warm water keep aside

Protocol:

1. Weigh 50ml TBE Buffer(1X) + 1.5g Nusieve Agarose.
2. Soak for around 30-45 minutes.
3. Weigh on weighing balance (Beaker + 1X TBE Buffer + Agarose)
4. Heat for 1 min at high power
5. Weigh the mixture again (use tongs to hold and mix).
6. Add the pre-heated H 2 O around 8ml-10ml more than original weight (*check that everything is mixed nicely)
7. Heat for 2 min. at power 80 (press power thrice)
8. Weigh again

(If weight decreases then add water accordingly to buffer it back to original weight)

(If weight increases then heat to mixture again by adding some water if there are clumps)

9. Let the mixture cool a little, mix with tongs
10. Add 0.5 µl EtBr.
11. Pour the Agarose
12. If there are any bubbles in the gel then use a micropipette tip end to remove them
13. Let the gel set for 90 minutes. (Minimum)

For Loading the Sample:

1. To the first well, add DNA ladder
2. Gel dye (Fast Cyclin) (2µl) + EtBr (0.5µl)

[To make a working stock for 10 samples: Dye: 10 x 2µl = 20µl and EtBr: 10 X 0.5µl = 5µl]

3. Then take a Para film and place the inner (cleaner) side up
4. Add 2.5µl of Gel dye + EtBr solution on the Parafilm, equally spaced from each other (make sure it does not mix)
5. Add 10µl of sample to it. Mix it nicely by increasing the count to 13 or so.
6. Load in wells
7. Switch on the Power pack (keep the wires connected before). Voltage should be 120-125V

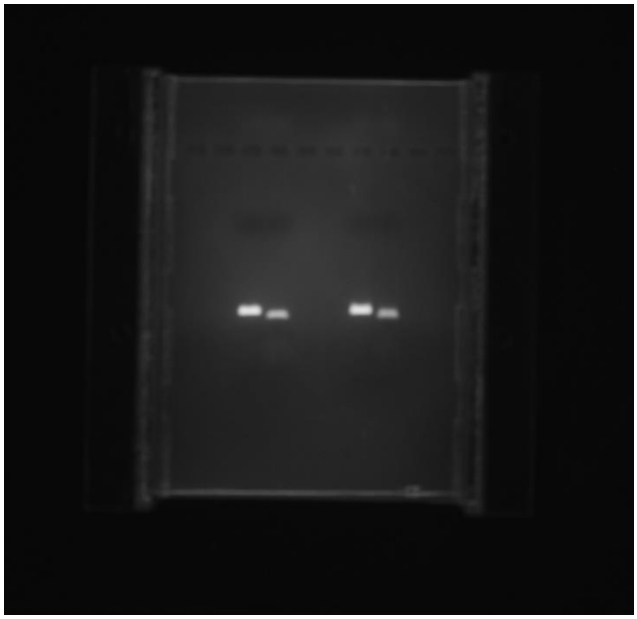
4. Results

The gel is observed under UV light in Gel Documentation and images are taken for results.

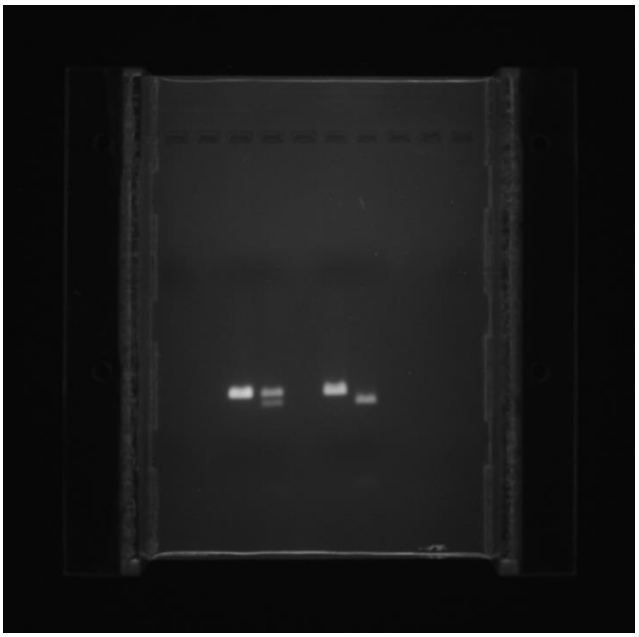
SMA Gels: (Result images 1-10): The 2 bands visible in the restriction digestion product lane shows that there is no deletion as there is amplification of both the restriction digestion products. This implies that the test is negative and the patient is not suffering from the disease.

The singular lower band in the same lane shows that only one of the restriction digestion products was able to amplify and there is deletion in the gene. This implies that the test is positive and the patient is suffering from the disease.

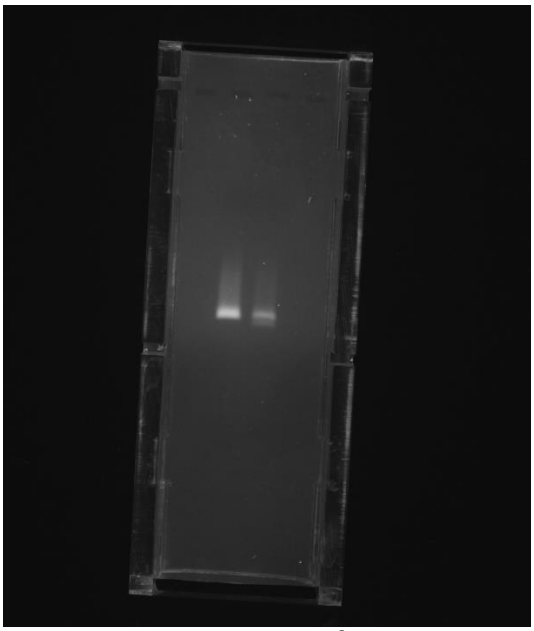
DMD Gels: (Result image 11): The missing bands in the gel shows the deletion of the corresponding exon.



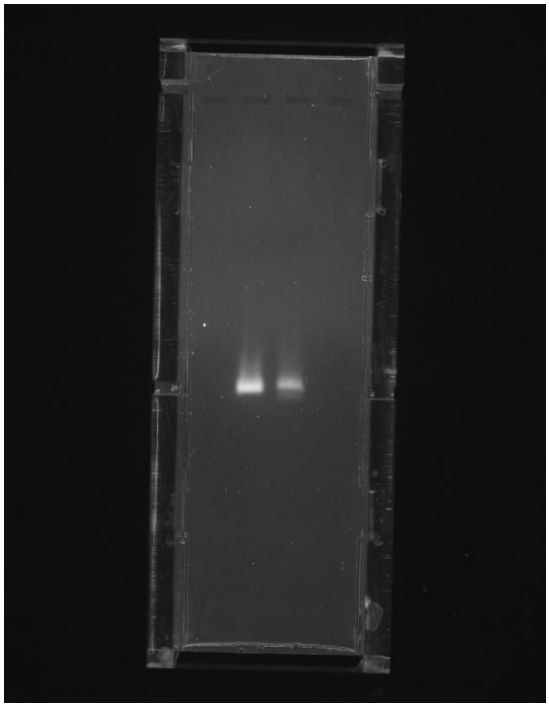
Result 1. The 1st and 3rd lane have the PCR product; 2nd and 4th have the restriction digestion product of patient 1 and 2 respectively. (SMA)



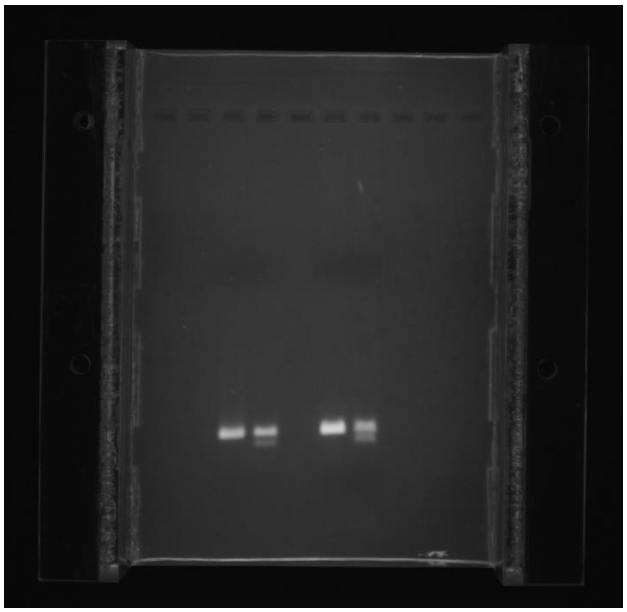
Result 2. The 1st and 3rd lane have the PCR product; 2nd and 4th have the restriction digestion product of patient 3 and 4 respectively. (SMA)



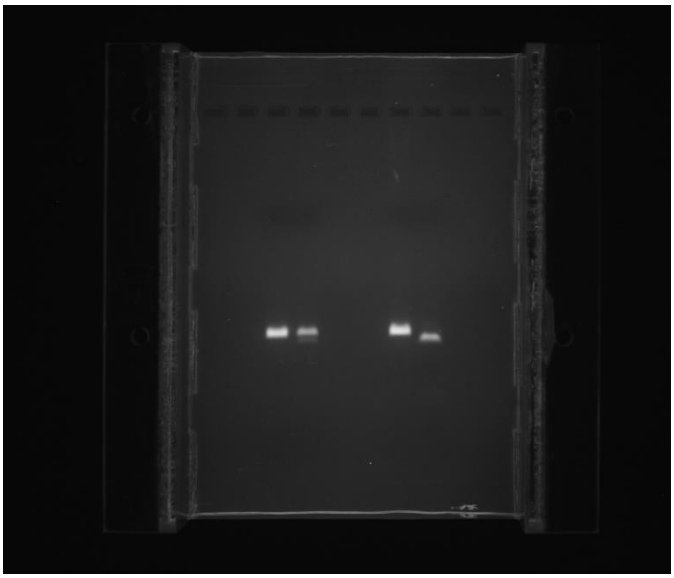
Result 3. The 1st lane has the PCR product; 2nd has the restriction digestion product of patient 5. (SMA)



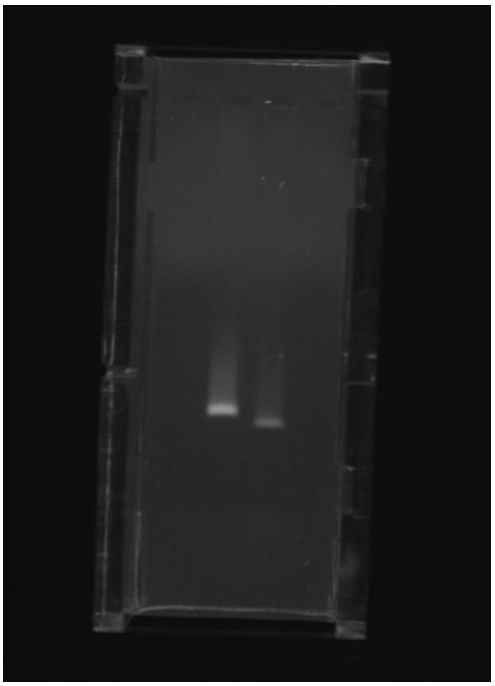
Result 4. The 1st lane has the PCR product; 2nd has the restriction digestion product of patient 6. (SMA)



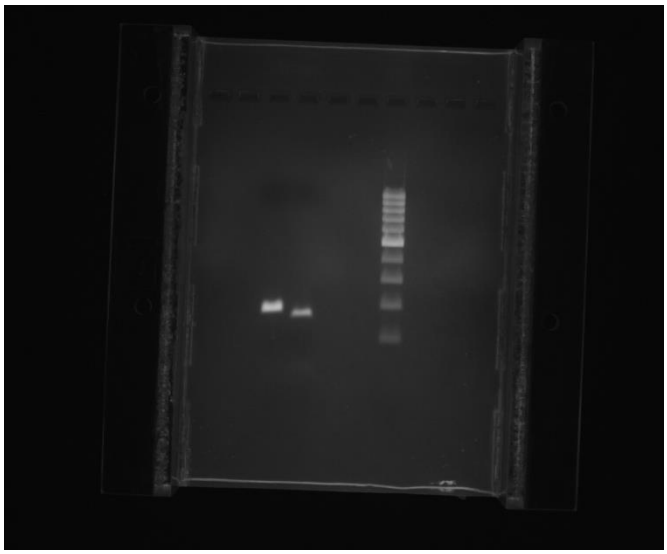
Result 5. The 1st and 3rd lane have the PCR product; 2nd and 4th have the restriction digestion product of patient 7 and 8 respectively. (SMA)



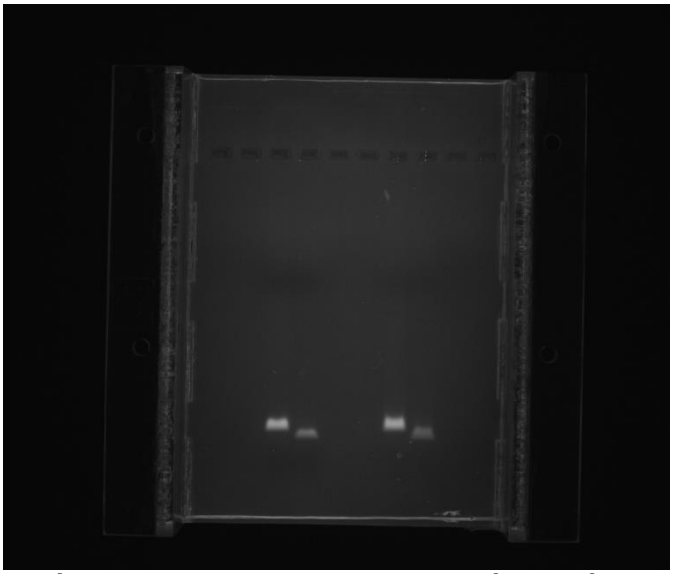
Result 6. The 1st and 3rd lane have the PCR product; 2nd and 4th have the restriction digestion product of patient 9 and 10 respectively. (SMA)



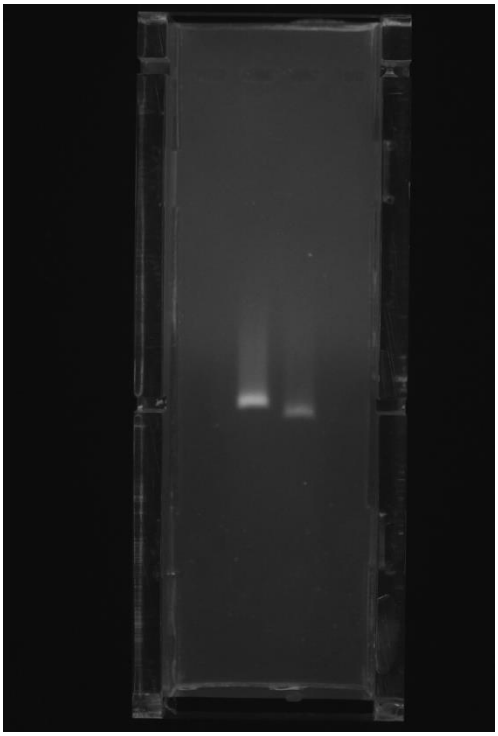
Result 7. The 1st lane has the PCR product; 2nd has the restriction digestion product of patient 11. (SMA)



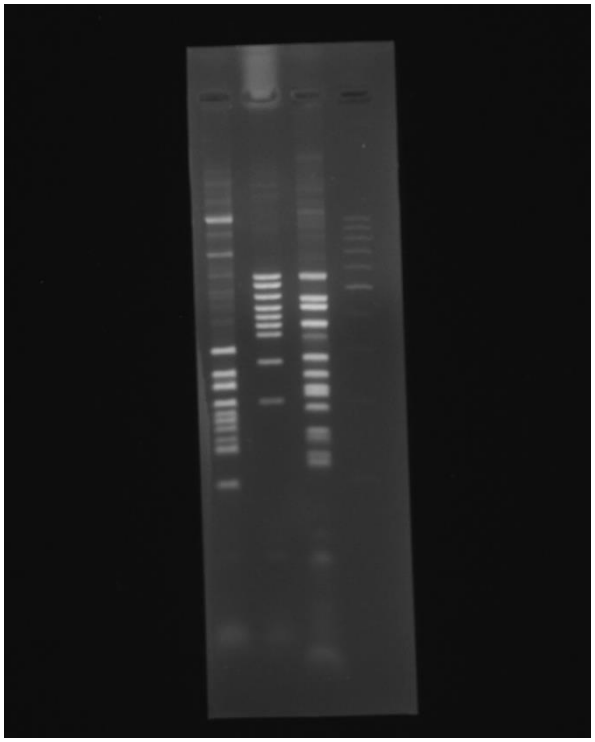
Result 8. The 1st lane has the PCR product and the 2nd lane has Restriction digestion product of patient 12. (SMA) The 3rd lane has DNA ladder which was loaded to check the viability of the ladder.



Result 9. The 1st and 3rd lane have the PCR product; 2nd and 4th have the restriction digestion product of patient 13 and 14 respectively



Result 10. The 1st lane has PCR product and the 2nd lane has the restriction digestion product of patient 15. (SMA)



Result 11. 1st lane has DNA ladder, 2nd lane has patient sample with Primer set I, 3rd lane with sample and Primer set II and the 4th lane has patient sample with Primer set III. (DMD Practice Gel)

5. Summary

SMA patient samples were mostly received during the internship period. No DMD patient samples were there for testing. So, a practice gel for DMD was made.

Patient	Deletion	No Deletion
Patient 1	✓	
Patient 2	✓	
Patient 3		✓
Patient 4	✓	
Patient 5		✓
Patient 6		✓
Patient 7		✓
Patient 8		✓
Patient 9		✓
Patient 10	✓	
Patient 11	✓	
Patient 12	✓	
Patient 13	✓	
Patient 14	✓	
Patient 15	✓	

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