DETECTION OF DELETION MUTATION CAUSATIVE FOR DUCHENNE MUSCULAR DYSTROPHY (DMD) IN BANGLADESHI COHORT

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

Department of Mathematics and Natural Sciences
BRAC University
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Declaration

It is hereby declared that,

1. The thesis submitted is my own original work while completing degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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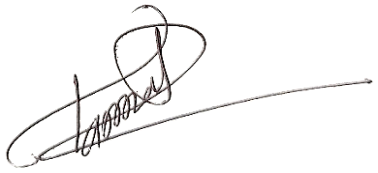

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Ethics approval and consent to participate:

The study was conducted after obtaining informed written consent including the use of peripheral blood and clinical data for research use and publication from the parents of every individual participant included in the study.

This article does not contain any studies with animal participants performed by any of the authors.

Abstract

Background: Duchenne muscular dystrophy (DMD) is an inherited genetic disorder resulting progressive skeletal, respiratory and cardiac muscle weakness. DMD is caused by a variety of mutations in DMD gene located on the X chromosome, which lead to a lack of functional dystrophin protein expression in males. Approximately 65-70% of individuals with DMD have intragenic deletions in the dystrophin gene. The aim of this study is to identify the frequency of deletion mutations in clinically DMD suspected Bangladeshi cohort.

Method: We have conducted multiplex PCR test for the deletion analysis of 35 DMD suspected male patients. A panel comprised of 26 hotspot exons of DMD gene have been used for the analysis. Variant classification analysis was conducted based on the American College of Medical Genetics (ACMG) guidelines.

Results: The cohort comprises 35 male patients. Multiplex PCR analysis revealed pathogenic deletion in 60% (21) patients. No clinically relevant variants were found in 40% (14) patients. The diagnostic yield for this test is 60%. The frequency of deletions in exon 48 (38%) was the most common deletion associated with our cohort. Most common symptoms seen among them were positive Gower sign (86%), poor walking and running ability (83%), high CPK level (71%), calf hypertrophy (57%).

Conclusions: Deletion mutations are present in 60% of cases. The aim of this study is using the first-tier test for DMD diagnosis to generate the frequency of deletion mutations in Bangladeshi DMD cases. The result shows the utility of using multiplex PCR test as genetic diagnosis that will help clinicians in monitoring and organizing future therapeutic management of DMD suspected cases. To our knowledge, this study is the first report of DMD gene deletion analysis in Bangladesh.

Keywords: Duchenne muscular dystrophy, Multiplex PCR, Exon, CPK, X-linked recessive disorder.

Dedicated to my lovely parents

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Tamannyat Binte Eshaque

October 27th, 2022

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List of Acronyms

Duchenne Muscular Mystrophy (DMD)

Kilo Dalton (KDa)

Dystrophin Proteins (Dp)

Creatine Phosphokinase (CPK)

Manifesting Carriers (MCs)

Dilated Cardiomyopathy (DC)

Intellectual Disability (ID)

Becker Muscular Dystrophy BMD

Fragile X Syndrome (FXS)

Klinefelter Syndrome (KS)

Spinal Muscular Atrophy (SMA)

Rare Diseases (RDs)

Multiplex Polymerase Chain Reaction (mPCR)

Genomic DNA (gDNA)

Ethylenediamine tetraacetic acid (EDTA)

Integrated DNA Technologies (IDT)

University of California, Santa Cruz (UCSC)

Food and Drug Administration (FDA)

Antisense Oligonucleotides (AOs)

Clustered regularly interspaced short palindromic repeats (CRISPR)

Pre-messenger RNA (pre-mRNA)

American College of Medical Genetics (ACMG)

Multiplex Ligation-dependent Probe Amplification (MLPA)

Thesis Title:

Detection of deletion mutation causative for Duchenne Muscular Dystrophy (DMD) in Bangladeshi cohort

Chapter 1: Introduction

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1.1 Background:

Duchenne muscular dystrophy (DMD) is an inherited genetic disorder that results in progressive skeletal, respiratory and cardiac muscle weakness that ultimately leads to loss of ambulation as well as respiratory and heart failure. A review of newborn screening studies indicates DMD affects 1:3802 to 1:6002 male births. DMD is a severe, progressive condition affecting 1 in 3600–6000 live male births. (Koeks et al., 2017). Approximately two-thirds of DMD-associated mutations are inherited from unaffected carrier mothers. Since the remaining third of DMD mutations arise from de novo mutations of the germ line, DMD mutations will likely always be with us. DMD is caused by a variety of mutations in DMD gene located on the X chromosome, which lead to a lack of functional dystrophin expression in males. (Magrath et al., 2018). The absence of dystrophin destabilizes the muscle membrane, leading to the clinical features of motor developmental delay, calf hypertrophy, joint contractures, and progressive muscle weakness in affected boys, with markedly elevated serum CK that reflects ongoing muscle damage. In addition, boys with DMD may have a variable degree of speech delay, learning disability, and/or cognitive impairment. Progressive muscle degeneration eventually leads to loss of independent ambulation by early adolescence, scoliosis, cardiomyopathy, respiratory insufficiency, and reduced life expectancy, with death occurring before the third or fourth decade of life due to cardiorespiratory complications, according to recent DMD natural history studies. (Mah., 2016).

1.2. Significance of Dystrophin protein:

The Dystrophin gene is the largest human gene, containing 79 exons that encode a 14-Kb mRNA and produce a 427-Kd membrane protein called Dystrophin. (Mohammed et al., 2018). DMD is caused by mutations of the DMD gene, located on chromosome Xp21, which encodes for dystrophin, a 427 kDa protein that is expressed at the muscle sarcolemma. Interestingly, in healthy skeletal muscle, dystrophin accounts for only 0.002% of total muscle protein, but its absence leads to tremendous detrimental effects on muscle functionality.(Łoboda et al., 2020). Dystrophin is a critical protein linking actin to the sarcolemma in skeletal, respiratory and cardiac muscle. Deficiency of functional dystrophin results in contraction induced injury of the sarcolemma, detected as leakage of muscle proteins, including creatine kinase, and subsequent

development of skeletal and cardiac muscle damage and susceptibility to necrosis and fibro fatty replacement. (Magrath et al., 2018). Gene mutations in the 2.24 million base pair DMD gene on the X chromosome result in biochemical loss or abnormalities of the dystrophin protein. (Takeda et al., 2021).

Because this muscular disease is caused by an absence of dystrophin, a 427-kDa protein associated with sarcolemma in skeletal and smooth muscle and two alternative 427-kDa isoforms are also expressed in the cerebral neocortex. In the cerebellum, dystrophin appears to play a role in normal neuronal function or development. Two carboxy-terminal dystrophin proteins (Dp), Dp71 and Dp140, are both expressed in the brain, in addition to full-length central nervous system dystrophins, and are initiated between exons 62 and 63, and upstream from exon 44, respectively. (D'Angelo et al., 2011). These mutations lead to a loss of dystrophin protein expression resulting in a severe muscle wasting, respiratory and cardiac failure and death before the age of 30. The reason is that the loss of dystrophin disrupts the DGC complex, causes membrane instability with increased susceptibility to injury, and fiber necrosis. (Falzarano et al., 2015). Contractile tissues in general and skeletal muscle fibres in particular, occupy a special position in the physiological systems of the human body, making up approximately 40% of body weight. Voluntary contractile fibres and their associated cell types display a remarkable array of special features on various levels of biological organization ranging from genotype to phenotype. Many of the functional and structural specializations of the muscular system play body-wide roles in health and disease, affecting especially locomotion, posture, heat homeostasis and metabolic networks and their integration. Given this context, the complexity and multifunctionality of the constituents of the skeletal muscle proteome is reflected by the diversity of muscular disorders. In addition to neurological, metabolic and autoimmune diseases that indirectly affect the motor system, intrinsic disorders of skeletal muscles manifest as inflammatory myopathies, myotonias, congenital myopathies, pharmacogenetic myopathies and muscular dystrophies. (Ohlendieck et al., 2021).

In skeletal muscle fibres, one of the largest genes in the human genome, the X-chromosomal 79-exon spanning DMD gene, exhibits the highest expression levels in form of the full-length Dp427-M isoform of the membrane cytoskeletal protein dystrophin. The DMD gene is positioned on the short arm of the X-chromosome at the Xp21.2 band. As outlined in Fig. 1, the 79 exons encode anamino-terminal region with an actin-binding site, 4 prolinerich hinge regions,

large central spectrin-like rod domains, a cysteine-rich domain and a carboxy-terminal domain with binding sites for various dystrophin-associated proteins. The large number of distinct binding sites displayed by the protein product of the DMD gene provides the structural basis for a supramolecular dystrophin node at the sarcolemma. The tissue-specific expression of dystrophin isoforms is driven by seven different promoters. The protein products include three full length dystrophins in brain, muscle and Purkinje cells, i.e. isoforms Dp427-B, Dp427-M and Dp427-P and shorter isoforms in the retina, brain/kidney, Schwann cells, the brain and a variety of other tissues, i.e. Dp260-R, Dp140-B/K, Dp116-S, Dp71-G and Dp45. (Ohlendieck et al., 2021).

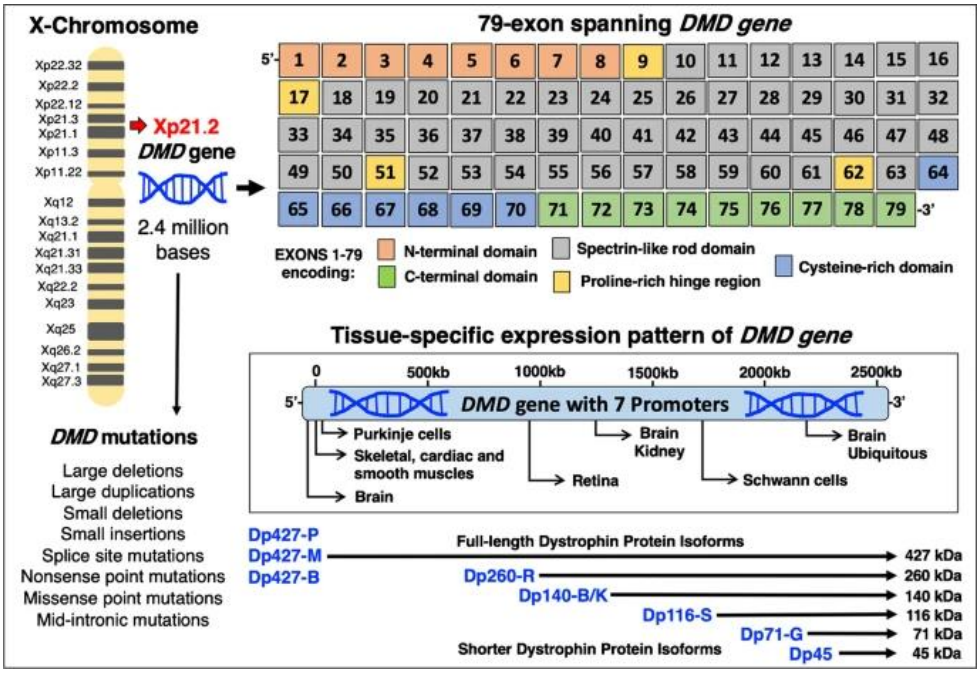

Fig.1: Overview of the *DMD* gene, its promoter structure and tissue-specific expression pattern of dystrophin isoforms. Abbreviations used: B, brain; B/K, brain/kidney; Dp, dystrophin protein; G, general; M, muscle; P, Purkinje cell; R, retina; S, Schwann cell.
(Ohlendieck et al., 2021)

1.3. DMD mutation type:

Although no true complete population-based systematic assessments have been performed, most mutation surveys indicate that approximately 70% of all DMD-causing dystrophin mutations are due to single or multiexon deletions with a higher mutational frequency observed within exons

44–55, which corresponds to the rod domain of dystrophin. Such deletions alter the reading frame of dystrophin and result in a prematurely truncated protein. (Nelson et al., 2009). The phenotypic severity depends mainly on the reading frame rule. This rule postulates that mutations destroying the reading frame cause absence of dystrophin in skeletal muscle and the DMD phenotype, whereas mutations preserving the reading frame permit the expression of semi functional dystrophin and BMD phenotype. (Juan-Mateu et al., 2015)

Because approximately 70% of individuals with DMD have a single-exon or multi-exon deletion or duplication in the dystrophin gene, dystrophin gene deletion and duplication testing is usually the first confirmatory test. If deletion or duplication testing is negative, genetic sequencing should be done to screen for the remaining types of mutations that are attributed to DMD (approximately 25–30%). These mutations include point mutations (nonsense or missense), small deletions, and small duplications or insertions, which can be identified using next-generation sequencing. (Birnkrant et al., 2018). The most common mutational event is represented by intragenic deletions accounting for 65-70% of all mutations; duplications account for 10% of all mutations. Both might occur almost anywhere in the gene. However two deletion hot-spots are known – one located towards the central part of the gene, encoding for exons 45-55, and the other towards the 5', including exons 2-19. (Taglia et al., 2015). The majority of identified mutations are deletions, accounting for approximately 60%-65% of DMD and 85% of BMD mutations, and duplications have been observed in 5%-15%. (Lee et al., 2012). Deletions can vary in size from a few kb to several thousand kb, and cluster around two regions of the gene, between exons 3 and 20 and 44 and 53. (Davies et al., 2005).

1.4. Disease progression in DMD individual:

Duchenne muscular dystrophy patients suffer progressive and irreversible muscular damage, as is evident from them having dramatically elevated levels of serum creatine kinase (CK), followed by the development of proximal muscle weakness before the fifth year of age and then loss of ambulation by 12–15 years of age. The range of CK value in normal persons remains between 50-150 IU/l. The CK Value of DMD and BMD patients is abnormally elevated (five-ten times to 100 times or even more). (Basak et al., 2009). Cardiac and/or respiratory impairment frequently develop as a result of increased muscle weakness with age, leading to death by the 20–30 s. (Echigoya et al., 2018). The presence of motor developmental delay with or without

speech delay and muscle hypertrophy in a young boy should trigger the order of serum CK as an initial diagnostic screen for DMD, especially if the child also has signs of proximal muscle weakness, manifesting as an abnormal waddling gait, or a positive Gowers' sign.(Mah et al., 2016). The presence of nonprogressive cognitive impairment is widely recognized as a common feature in a substantial proportion of patients. Interestingly, delay in global developmental and language disorders can constitute the signs of onset in this disease. (Ciafaloni et al., 2009). The presence of Gower's sign in Duchenne patients relates to proximal muscular weakness in hip and thigh muscles, which requires the help of both hands and arms for rightening the body to reach a standing position. Muscular dystrophy-associated temporal and spatial variations in gait were shown to include changes in cadence, anterior pelvic tilt and dorsifexion during swing. The proper assessment of pathological gait patterns and functional ambulation are crucial for prediction of disease progression, as well as monitoring of drug treatment and physiotherapeutic interventions. Detailed studies of gait abnormalities have established a drastic decrease in walking speed, stride length, step length, maximal power generation at the hip, maximal knee extension torque, maximal dorsifexion torque and maximal power generation at the ankle in Duchenne children. (Ohlendieck et al., 2021).

Disease progression in Duchenne muscular dystrophy due to DMD gene mutation

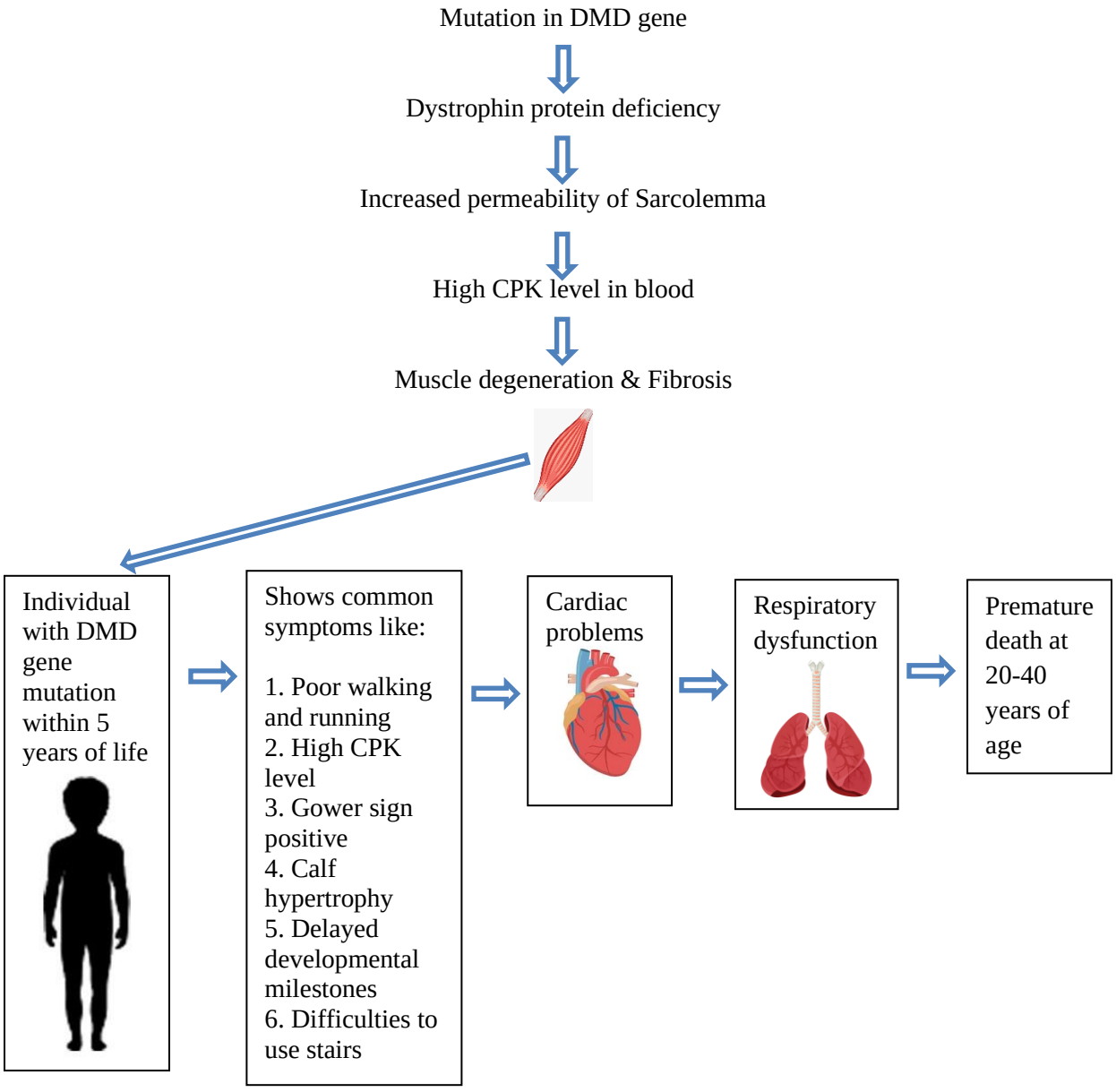

Fig.2: Disease progression in DMD affected individual with disrupted Dystrophin protein. (Łoboda et al., 2020)(Ohlendieck et al., 2021)

1.5. Diagnostic approaches:

DMD is X-linked and ~65% of the mutations are deletions, rapid polymerase chain reaction (PCR) assays could be developed to allow deletion detection in male patients in as little as 1 day. Multiplex PCR methods allow detection of approximately 98% of deletions, which accounts for 65% of all mutations. Locations of deletions in the dystrophin gene are apparently nonrandom with a preponderance found in two "'hot spot" regions at the 5' terminus and in the distal half of the central rod domain around exons 44-53. (Basumatary et al., 2013). The diagnosis of DMD can usually be made after a careful review of the clinical history, physical examination, and confirmation by additional investigations, including muscle biopsy and/or molecular genetic testing. (Mah, 2016). Correct DNA diagnostic analysis is crucial for DMD patients since it is important for optimal care and family planning, but also provides information on eligibility for mutation-specific treatments. (Aartsma-Rus et al., 2016). A positive family history of DMD is not required, as approximately one-third of cases may occur as a result of spontaneous mutation. (Mah et al., 2016).

1.6. DMD mutation carriers:

The majority of DMD carriers remain asymptomatic, while only 2.5-10% of carriers are symptomatic and classified as manifesting carriers (MCs). The symptoms of MCs range from mild muscle weakness to severe abnormal gait with frequent falls, and difficulty in rising from the floor or walking on tiptoes. (Zhong et al., 2019).

1.7. Treatment:

Currently there is no cure for DMD. The implementation of care recommendations (including corticosteroids, cardiac medications and assisted ventilation) improve outcomes and quality of life, but their effects on the underlying disease mechanisms are as yet unknown. (Koeks et al., 2017). The most encouraging therapeutic options available to date thus far involve an exon-skipping approach and a stop codon read through approach. The former enables DMD patients to produce dystrophins such as found in Becker patients, albeit at significantly lower levels, leading to a slowdown in disease progression, while the latter attempts to reestablish the functional

integrity of the protein. Both these approaches require very precise identification of the mutational status of the DMD gene in DMD/BMD patients. (Kumar et al., 2020).

1.8. Aims and objective of this study:

In this study, we have selected 35 Duchenne muscular dystrophy (DMD) suspected male cases of Bangladesh. Aim of this study is to perform multiplex PCR test to find the frequency of deletion mutation within the hotspot region of DMD gene found in Bangladeshi population and also identifying the frequently deleted exons among them.

Clinical significance of this study gives the possibility of accurate diagnosis of DMD and help clinicians for early intervention and management. Proper diagnosis of DMD can remove the confusion that arises with other muscle related disorders due to similar phenotypes. Besides, we have also compared our findings of deletion frequency with other Asian and distant country's deletion frequency to analyze the similarities between them. To our knowledge, it is the first genomic deletion analysis work that analyzed the maximum number of hotspot regions of DMD gene in DMD suspected cohort in Bangladesh.

Chapter 2:

Materials and Methodology

Chapter 2: Materials and Methodology

2.1. Subjects:

A total number of 35 unrelated DMD suspected patients from different regions of Bangladesh were studied for this work. Patient's sample and data were collected from NeuroGen Healthcare Limited, West Panthapath, Dhaka, Bangladesh. These 35 males were suspected to have DMD gene mutation. They were referred to do the Multiplex PCR DMD deletion test for confirmed diagnosis by child neurologists and child specialists from different region of Bangladesh.

2.2. Clinical characteristics of patients:

The patients were presented for genetic test to identify the deletion mutation in their DMD gene. On physical examination, the most common presenting symptoms were Gowers' sign positive, poor running and walking ability, hypertrophy of calf muscles present in many patients. From biochemical analysis high serum creatine phosphokinase was found in most of the patients. The remaining symptoms among all the 35 cases presented were developmental delay, poor ability of using stairs, muscle weakness, skinny legs and arms. A few had waddling feet and abnormal gait. Some of them had a family history of muscular dystrophy. Phenotypes suggesting neurological problems were present among the cases as well. Speech delay, intellectual disability and hyper-activeness were found in some patients at the time of diagnosis. Although, seizure is not a symptom of DMD. We found patients to have seizure once in their life so far. A table showing the overview of all the clinical characteristics of 35 DMD suspected male patients of this study is given below **(Table 1).**

Table 1: Overview of the clinical characteristics of 35 DMD suspected male patients of this study

Sl No.	Age of onset (years)	Calf hypertrophy	Gower sign positive	High CPK level	CPK Level (U/L)	Feeding difficulties	Toe walking	Poor walking/running ability	Poor ability for using stairs	Waddling feet/gait	Seizure	Muscle weakness	Skinny legs/arms	Developmental milestones	Intellectual Disability	Speech delay	Hyper activeness	Positive Family history
1			yes	yes	7840													
2	1.5		yes		8800	yes		yes				yes		on time				
3	11		yes	yes	13709	yes		yes	yes	yes				on time				
4		yes		yes				yes		yes		yes		Delayed		yes		yes
5			yes									yes		Delayed			yes	
6	6	yes	yes	yes	15,057	yes		yes				yes		on time				
7	6	yes	yes	yes	22,142			yes	yes	yes				on time				
8		yes		yes				yes			Convulsion (once)	yes	yes	Delayed		yes		
9				yes	24,420			yes						Delayed				
10	2.5		yes					yes						on time				yes
11	4	yes	yes					yes	yes			yes		on time	yes			
12		yes	yes	yes				yes			after birth (1st), at 9 months	yes		Delayed				
13		yes		yes	18160	yes				yes				Delayed	yes	yes	yes	yes
14	2		yes	yes	6190			yes	yes			yes		Delayed				
15			yes					yes						on time				
16	8.5		yes				yes	yes										
17			yes	yes	7377			yes						Delayed	yes	yes		
18	2.5	yes	yes	yes	15,077			yes	yes			yes		Delayed	yes			
19		yes	yes					yes	yes					Delayed	yes	yes		yes
20		yes	yes	yes	7800		yes	yes	yes					on time		yes		
21	4			yes	3100			yes	yes					Delayed				
22		yes	yes	yes	12,073			yes						Delayed	yes	yes	yes	
23	4.6	yes	yes										yes	on time				yes
24	7	yes	yes						yes			yes		on time				
25		yes	yes	yes	24,975			yes	yes			yes		Delayed				yes
26			yes		36,210		yes		yes					Delayed	yes	yes	yes	
27	5		yes	yes	12430	yes	yes	yes				yes		on time			yes	
28	6	yes	yes	yes	7958			yes				yes		on time				
29	4		yes	yes	14,257			yes						on time				yes
30	3	yes	yes	yes	26568			yes	yes			yes		Delayed		yes		yes
31	3		yes	yes	17,333			yes	yes					On time				
32	5	yes	yes	yes	8064			yes	yes			yes		Delayed				
33		yes	yes	yes	10060	yes		yes	yes		once	yes		Delayed		yes		
34	4y	yes (calf muscle and deltoid	yes	yes	3198			yes	yes					Delayed				
35	7y	yes (calf muscle and deltoid hypertrophy)	yes	yes	14438			yes	yes					On time				Yes

2.3. Genomic DNA extraction and quality analysis:

For genetic analysis of patient, three milliliters of peripheral blood samples were collected from 35 clinically suspected DMD patients in an EDTA vacutainer tube. Genomic DNA was isolated using ReliaPrep™ Blood gDNA isolation kit (Promega, USA) according to manufacturer instructions. The concentration and quality of DNA was determined using NanoPhotometer C40 (Implan, Germany). The quality of DNA was also checked by 0.8% agarose gel electrophoresis. The volume, concentration and purity of all the 35 DNA samples are given in **Table 2**.

Table 2: Biospecimen description of the 35 DMD suspected samples

Patient Sl No.	Eluted Volume (µl)	DNA Concentration (ng/µl)	DNA Purity	
			260/230	260/280
1	40	170.1	2.22	1.86
2	40	170.1	2.22	1.86
3	40	198.7	1.91	1.91
4	40	190.85	2.29	1.88
5	40	111.35	2.05	1.90
6	40	140.65	2.31	1.88
7	40	166.15	2.01	1.86
8	40	135.0	1.86	1.84
9	40	225.6	2.47	1.87
10	40	183.65	2.31	1.92
11	40	187.60	2.24	1.86
12	40	162.1	2.2	1.87
13	40	95.05	1.9	1.89
14	60	85.2	1.7	1.9
15	60	61.1	2.1	1.88
16	40	145	2.2	1.87
17	40	91.5	1.95	1.91
18	40	145.1	2.2	1.91
19	40	72.1	2.1	1.88
20	60	67.8	1.9	1.87
21	40	100.7	2.2	1.9
22	60	54.1	1.9	1.87
23	40	89.2	2.2	1.91
24	60	36.80	2.1	1.9
25	60	61.1	1.9	1.88
26	60	47.5	2.3	1.85
27	60	50.2	2.3	1.92
28	60	35.1	2.0	1.88
29	60	180.8	1.87	1.87
30	60	155.2	2.37	1.92
31	60	59.5	2.2	1.85
32	60	87.8	2.4	1.9
33	60	54.2	2.4	1.89
34	60	83.2	2.4	1.91
35	60	60.1	2.4	1.89

2.4. Diagnostic Multiplex PCR for amplification:

Multiplex PCR was used to examine the exons for a large (single or multiple) deletion in the hotspot regions of the dystrophin gene to clinically diagnose the DMD suspected cases. A panel comprised of 26 hotspot exons of DMD gene have been developed. Using Primer 3 plus software, IDT and UCSC Genome Browser 17 sets of primers were designed targeting 17 exons. Additionally, 9 primer set have been taken that was validated by den et al. 2006 (Dunnen & Beggs, 2006) for DMD gene. In total, 5 sets of multiplex PCR was carried out to amplify all the 26 amplicons using GoTaq® Hot Start Colorless Master Mix (Promega, USA). To run the amplification, the Applied Biosystems® 2720 Thermal Cycler has been used with 96-well reaction plates. In total, 32-35 cycles were run in this test method. The amplicons were visually confirmed by 2.0% agarose gel electrophoresis. The exons tested were: 1, 3, 4, 6, 8, 12, 13, 16, 17, 19, 32, 34, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53 and 60. (**Table 3).**

Table 3: Set arrangements for the amplification of tested exons with PCR steps

Set	Exon	Size (bp)
Set 1	Exon 47	578
	Exon 44	473
	Exon 6	420
	Exon 16	314
	Exon 4	250
	Exon 1	146

95°C- 2 mins → Initial denaturation
95°C- 30s → Denaturation
55°C- 30s → Annealing
72°C- 40s → Elongation
72°C- 5 mins → Final elongation
4°C- 5 mins → Short time incubation

Set	Exon	Size (bp)
Set 2	Exon 60	742
	Exon 50	680
	Exon 49	618
	Exon 42	509
	Exon 8	336
	Exon 12	257

95°C- 2 mins → Initial denaturation
95°C- 30s → Denaturation
53°C- 30s → Annealing
72°C- 50s → Elongation
72°C- 5 mins → Final elongation
4°C- 5 mins → Short time incubation

Set	Exon	Size (bp)
Set 3	Exon 52	648
	Exon 45	533
	Exon 41	470
	Exon 34	415
	Exon 13	257
	Exon 3	198

95°C- 2 mins → Initial denaturation
95°C- 30s → Denaturation
55°C- 30s → Annealing
72°C- 40s → Elongation
72°C- 5 mins → Final elongation
4°C- 5 mins → Short time incubation

Set	Exon	Size (bp)
Set 4	Exon 53	661
	Exon 51	610
	Exon 19	459
	Exon 17	416
	Exon 46	148

95°C- 2 mins → Initial denaturation
95°C- 30s → Denaturation
54°C- 30s → Annealing
72°C- 40s → Elongation
72°C- 5 mins → Final elongation
4°C- 5 mins → Short time incubation

Set	Exon	Size (bp)
Set 5	Exon 48	707
	Exon 43	559
	Exon 32	424

95°C- 2 mins → Initial denaturation
95°C- 30s → Denaturation
61°C- 30s → Annealing
72°C- 25s → Elongation
72°C- 5 mins → Final elongation
4°C- 5 mins → Short time incubation

2.5. Analyzing the PCR products by agarose gel electrophoresis to detect DMD gene deletions:

After multiplex PCR, the reaction products were separated on a 2% agarose gel and analyzed for the presence of deletions. All reactions are run from each primer set together to allow for direct comparison between positive and negative controls and patient sample. The gels are photographed and results are recorded. All the gel images are visually inspected with care as it is photographed to ensure the presence of all the reproduced bands. The images are used for interpretation of our results. The clinical interpretation of this result closely follows American College of Medical Genetics (ACMG) guideline. (Richards et al., 2015).

Chapter 3: Results

Chapter 3: Results

3.1: Findings from clinical phenotype analysis:

The mean age of 34 DMD patients at the time of genetic testing was 7.2 years (1.5 years-14.9 years). 1st patient's data of age was not available. Data for the age of disease onset were available from 20 patients. The mean age of initial symptoms was noticed was 4.8 years (1.5 years-11 years). On physical examination, the most common presenting symptoms was Gowers' sign positive, which was seen in 30 patients (86%), followed by poor running and walking ability in 29 patients (83%). Hypertrophy of calf muscles was present in 20 patients (57%). From biochemical analysis high serum creatine phosphokinase was found in 25 patients. The mean level of serum creatine phosphokinase was measured 14,051.5 U/L (3100-36210 U/L). The remaining symptoms among all the 35 cases presented were developmental delay (n = 18), poor ability of using stairs (n=17), muscle weakness (n=16), skinny legs and arms (n=2). 11% had waddling feet and abnormal gait (n=4). Nine of 35 patients (25%) had a family history of muscular dystrophy. Ten patients (29%) had speech delay at the time of diagnosis, seven patients (20%) had intellectual disability and five patients had hyper-activeness (14%). We found 3 patients (9%) to have seizure once in their life so far. Figure 3 shows all the clinical characteristics of 35 DMD suspected male patients of this study.

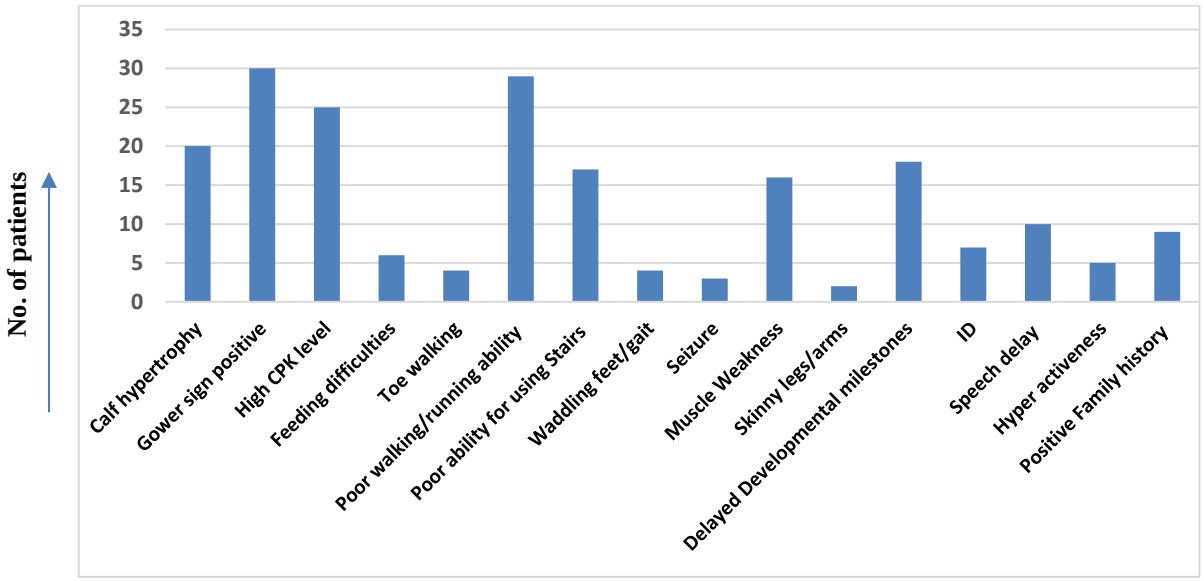

Fig. 3: Most common phenotypes identified among 35 DMD suspected male patients of this study.

3.2: Molecular findings interpreting from Gel electrophoresis:

The present study was aimed to diagnose the deletion mutation pattern of 35 DMD suspected patients. The average age at the time of genetic testing was 7.2 years (1.5 years-14.9 years). The study revealed that, out of 35 unrelated male DMD suspected patients 21 patients (60%) were having positive results and 14 patients (40%) were having negative results. This means that among 35 DMD suspected individuals, single or multiple gene deletion was found in 21 patients. A total of 81 exons were deleted in 21 patients. The average number of exons deleted per patients was 3.8. The diagnostic yield in this case from multiplex PCR method for the identification of deletion mutation in DMD gene is 60%. It was found that out of 21 patients with deletion mutation causative for Duchenne muscular dystrophy, only 5 patients (23.8%) carried single exon deletion. Rest of the 16 patients (76.1%) had multiple deleted exons. Multiple deletions were seen covering the major hot spot regions in the DMD gene. Multiplex PCR corresponding gel electrophoresis images of 21 DMD positive patients are attached in the **appendix section** (Page No. 38-58). No clinically relevant deletion mutation was found in 40% (14) patients with negative test results. This means that no deletion mutation was found in our selected hotspot region of these 14 patients. **Figure 4** shows simple representations of the positive and negative findings of our study. **Table 4** demonstrates exon deletion status of all the 35 cases of our cohort.

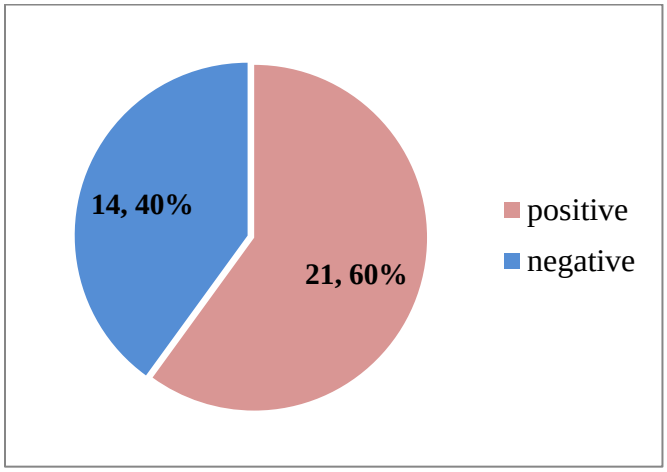

Fig.4: Pie-chart illustrates the frequency of deletion mutation in DMD patients in Bangladeshi cohort.

Table 4: Presence of the deletion mutation status for both positive and negative DMD suspected cases found in this present study along with their age of diagnosis.

Sl. No.	Age of diagnosis (years)	Result output	Deleted Exons
1		**Positive**	**8, 12, 13, 17 & 19**
2	5.5	**Positive**	**50**
3	22	Negative	
4	7.9	Negative	
5	7	**Positive**	**49 & 50**
6	7	Negative	
7	9	**Positive**	**45**
8	1.5	**Positive**	**45, 46, 47, 48, 49, 50, 51, 52 & 53**
9	4	**Positive**	**46, 47, 48, 49, 50 & 51**
10	5.4	Negative	
11	14.5	**Positive**	**3, 4, 6, 16, 8 & 12**
12	2.11	Negative	
13	4.3	Negative	
14	6.1	**Positive**	**46, 47, 48, 50, 52 & 51**
15	5.1	Negative	
16	9.7	Negative	
17	8.1	Negative	
18	4.1	Negative	
19	3.8	Negative	
20	7.3	**Positive**	**48, 49 & 50**
21	14.9	**Positive**	**1, 3, 4, 6, 8, 12, 16 & 17**
22	3.5	**Positive**	**51**
23	5.2	Negative	
24	10.1	**Positive**	**12,13, 16, 17, 19, 32, 34, 41,42, 43 & 44**
25	8.1	Negative	
26	4.3	**Positive**	**52**
27	11	**Positive**	**50 & 52**
28	11.2	**Positive**	**45, 46 & 47**
29	3.3	**Positive**	**46, 47 & 48**
30	7	**Positive**	**52 & 53**
31	7.2	**Positive**	**46, 47, 48, 51, 52 & 53**
32	8.3	**Positive**	**48**
33	4.5	**Positive**	**47 & 48**
34	6.9	**Positive**	**53 & 60**
35	8	Negative	

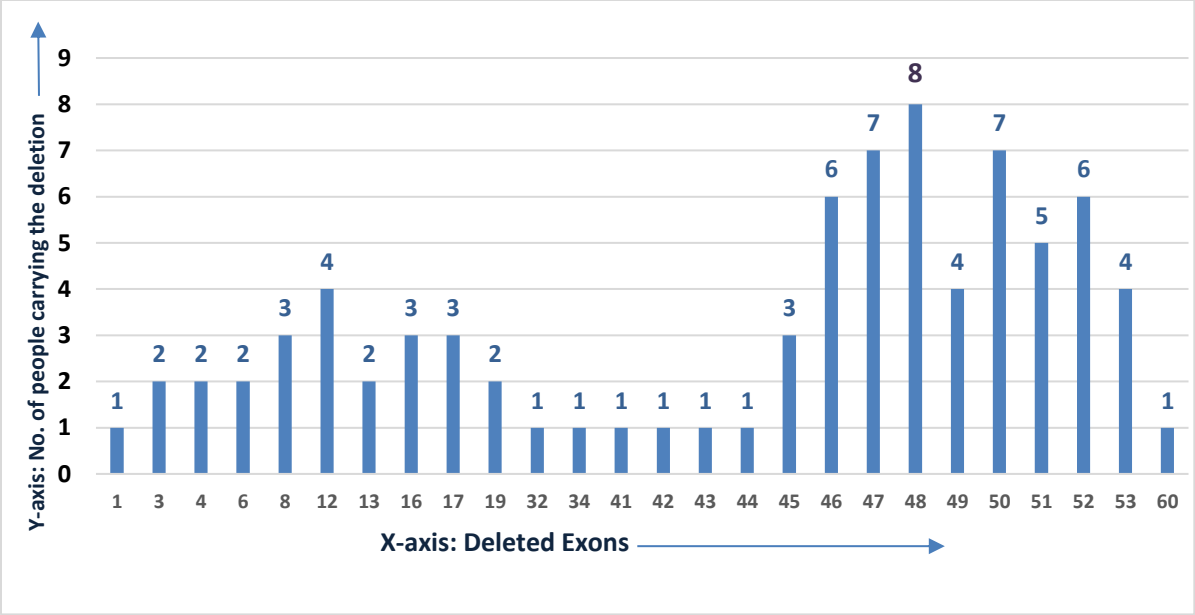

Fig.5: Bar-diagram representing the most commonly deleted exons in DMD gene and the frequency of deletions in 21 patients. The data showed that deletion of exon 48 is frequently seen in the DMD affected individuals. It was found to be deleted in 8 out of 21 positive test results.

The above data presented in **figure 5** illustrates that the 21 patients with deletion mutations are mostly carrying their mutation near two important areas of the gene, one in the 3' end (exons 3-19) and one in the 5' end (exons 45-53) of the DMD gene. For this study, 26 hot spot exons were selected where most of the deletion mutation occurs. Within this region, deletion of exon number 48 near the central part of DMD gene was found in maximum number. Exon 48 was identified to be missing in 8 out of 21 patients. This may describe that our population carries most of the deletion mutation of DMD near this location of Dystrophin gene.

Additionally, exon 47 was found to be missing in 7 patients and also exon 50 was found to be missing in 7 patients. Besides, exon 46 and 52 were also found to be frequently deleted in these patients. Deletions near the 3’ end (exons 3-19) were comparatively less frequent. However, within this region exon 12 was found to be missing in total 4 patients. Deletion in exons 32, 34, 41, 42, 43, 44 were missing in lowest number of patients.

The overall results of our study suggest that, 60% of the patients in our cohort carries deletion mutation causative for Duchenne muscular dystrophy. That is the frequency of deletion mutation in Bangladeshi population. We have also identified the most frequently deleted exon among this population which is found to be exon 48. We have used multiplex PCR method and gel electrophoresis to diagnose these deleted regions from our 35 samples.

Chapter 4: Discussions

Chapter 4: Discussions

Rare Diseases (RDs) represent a major challenge worldwide, with many initiatives devoted to achieving an appropriate genetic and clinical diagnosis launched in many countries (http://www. udninternational.org). Duchenne muscular dystrophy (DMD), the most common muscular dystrophy of childhood, is degenerative, life-limiting and incurable. Affected boys have increasing muscle weakness, cardiorespiratory and orthopedic complications, and are at a risk of cognitive, behavioral and language difficulties. DMD can initially present with global developmental delay. (Wong et al., 2015). Early and accurate genetic diagnosis is recommended by all guidelines and is now considered compulsory for mutation identification, allowing for prevention and family planning, and applies to all dystrophinopathy mutation groups (Koeks et al., 2017). In addition, the emergence of new therapeutic and often personalized approaches in DMD has further highlighted the need for an early genetic definition in order to identify eligible patients. (Neri et al., 2020)

Our target in this study was to find out the frequency of deletion mutation that causes DMD in our Bangladeshi cohort. Before this no study has been developed targeting this factor of diagnosis in our country. According to our analysis, we used multiplex PCR method to identify the deletion mutation status in 35 DMD suspected cases. In our results, it was seen that 21 out of 35 are carriers of deletion mutation which was the reason behind their symptoms confirming DMD. Among the 21 patients, we found 5 of them to have only single exon deleted. Patient serial no. 2, 7, 22, 26 and 32 were carrying only single exon deletion those are 50, 45, 51, 52 and 48 respectively. The five most frequent single exon deletions recorded in the database (all reported more than 100 times) were deletion of exon 45 (4%), 51 (3%), 44 (3%), 52 (3%), and 50 (2%). (Bladen et al., 2015). Similarly, in an Egyptian study with DMD cases most single deletions were variable within the major hot spot and included exons 43, 44, 45, 47, 48, 50 and 51.(Effat et al., 2000).

The present study also focused on finding the most frequently deleted exon in our cohort. Exon 48 is the most commonly seen exon to be deleted and was found in 8 patients. However, we found only one patient who had single exon 48 deletion in DMD gene. In 2011, three Italian patients with the deletion of exon 48 were described who were diagnosed at the ages of 25, 38 and 39 years respectively with severe cardiologic disorders. The two older ones died of

congestive heart failure and the youngest one had heart transplantation. (Zimowski et al., 2017). In a study performed among 227 families with Becker muscular dystrophy (BMD), it was found that nine non-consanguineous families with 17 male individuals carrying a rare single exon 48 deletion of the dystrophin gene. They were affected with a very mild or subclinical form of BMD. (Zimowski et al., 2017). In particular, in frame deletions removing single or few exons, but associated with more severe than expected phenotypes, might indicate that a specific portion of dystrophin is crucial for its function. (Muntoni et al., 1994).

Deletions in the N-terminal region and in the rod-domain proximal to exon 45 have been associated with earlier onset of symptoms than mutations in the distal region. In particular patients sharing deletion of exons 45-55 seem to have a less severe muscular involvement, with only a few cases of dilated cardiomyopathy so far described. (Taglia et al., 2015). Here we can establish a genotype-phenotype correlation with our present study by demonstrating that we found patients within our deletion findings those who are carrying mutations within the region 45-55 exons. Patient no. 8 was found to have deletions in exons 45, 46, 47, 48, 49, 50, 51, 52 and 53. Patient no. 9 had deletions in exons 46, 47, 48, 49, 50 and 51. Again, exons 46, 47, 48, 50, 52 and 51 were not found in patient no. 14. Finally, patient no. 31 was found with missing exon numbers 46, 47, 48, 51, 52 and 53. Within these four patients, except patient no. 31, all had delayed developmental milestones (Table. 1). From the available supporting data regarding the age of first symptom onset and the age of diagnosis given in Table. 1 and Table. 4, it can be understood that all the 4 patients were having the signs of the disease at an early age. Our cohort patient's maximum age has been seen up to 14 years. So far cases of dilated cardiomyopathy have not been reported yet. Deletions around exons 45-53 were most common and generally caused typical BMD. However, phenotypic variability among patients with similar mutations suggests that epigenetic and/or environmental factors play an important role in determining the clinical progression. (Beggs et al., 1991).

The clinical distinction between DMD and BMD is conventionally based on the age of wheelchair dependency: before age 13 years in DMD and after age 16 years in BMD. However BMD patients may remain ambulant until the late 40s and over. (Taglia et al., 2015). The central and the distal rod-domains are likely to be functionally dispensable, as deletions in these domains have been associated with isolated hyperCKemia, myalgia and cramps, but not with weakness. This is the case of deletions in exons 32-44, 48-51 and 48-53, who had normal or near

normal dystrophin concentrations. (Taglia et al., 2015). In our cohort, we find out 5 such patients having deletions within this region. Our cases no. 5, 20, 28,29 and 33 had deletions from exons 45-50. In two cases of dilated cardiomyopathy (DC) caused by dystrophinopathy, one patient a 24-year-old man, had a family history of X linked DC, while the other, a 52-year-old man, had sporadic disease. Analysis of dystrophin gene mutations showed a deletion of exons 48–49 in the patient with familial DC and of exons 49–51 in the other. (Muntoni et al., 1997).

The multi-system pathophysiology of Duchenne muscular dystrophy is characterized by a variety of neurological complication. Neurological issues are characterized by social, behavioral and emotional problems, delayed milestones of language development, adaptive deficiencies, impaired working memory and a variety of neuropsychiatric diseases such as hyperactivity, obsessive–compulsive behavior, attention deficit and autism spectrum disorders. (Ohlendieck et al., 2021). Our Bangladeshi studying cohort of 35 DMD suspected cases showed variable range of neurological difficulties including speech delay, intellectual disabilities, hyperactivity along with overall delay in developmental milestones. Besides, seizure was noted in 3 of our patients where one patient (patient no. 12) showed no deletion mutation after diagnosis.

We have studied the deletion frequency found on other countries as well to compare with our own data from Bangladeshi cohort. In Asian Indians the common “hot spot” deletions are located mainly in exons 45-51. (Singh et al., 2006). In Northeast India, 71% of patients showed deletions. (Basumatary et al., 2013). In an Egyptian study, the rate of deletion within the dystrophin gene was found 61.1%. (Therapy & Dystrophy, 2007).

In a Korean study, among all 29 DMD/BMD patients, 17 (58.6%) were found to have exon deletions in Korea. (Lee et al., 2012). In France, current release includes 2,411 entries that consist of 1,404 large deletions and 215 large duplications of one or more exons representing 67.4% and 10.3% of all mutations in the database, respectively. (Tuffery-Giraud et al., 2009). Canadian Neuromuscular Disease Registry (CNDR), Genetic testing data was available for 350 of 414 DMD patients (85%) and 61 of 78 BMD patients (78%) (Figure 1). The majority of mutations were deletions of at least one exon in the DMD gene in 69% (241/350) of DMD patients and 80% (49/61) of BMD patients, or 71% (290/411) of patients in total. (Lim et al., 2020).

A study from Kuwait shows data from DMD/ BMD patients who attended the Kuwait Medical Genetic Center during the last 20 years was retrieved from a Kuwait neuromuscular registry and

analyzed. The deletion and duplication rates were 66.2% and 4.4%, respectively. (Mohammed et al., 2018). Deletions were detected in 63.3% of patients in Greece and were mainly clustered in two areas of the gene, one in the 3' and one in the 5' end of the gene (exons 3-19 and 44-53). (Florentin et al., 1995). In the North countries of Africa, Egypt, for example, represented a relatively high frequency of DMD gene deletion frequency (51%; 78/152)(Elhawary Nasser et al., 2004). Some Asian populations, for example, Pakistani (40.75%) (Hassan et al., 2008), Malaysian (42%) (Marini et al., 2008), have nearly the same magnitudes and patterns of deletion frequencies to this Saudi study. (Tayeb et al., 2010). Relatively higher frequencies were recorded such as: 63% in an Italian study (Haven, 1992), 62.5% in a German study (Niemann-Seyde et al., 1992), and 62.3% in a Brazilian study (Zatz et al., 1998). Deletion accounts for 60% of the mutations within the 79 exons of the dystrophin gene. Seven exons (43, 44, 45, 46, 49, 50, and 51) were found to be most commonly deleted among the Asian patients. (Marini et al., 2008)

Table 5: Similarities of deletion mutation frequency in different countries.

Country	% Of Deletion	Authors
Northeast India (69)	71%	(Basumatary et al., 2013)
Pakistan (211)	40.75%	(Hassan et al., 2008)
Malaysia (20)	42%	(Marini et al., 2008)
Egypt (100)	61.1%	(Therapy & Dystrophy, 2007)
Korea (29)	58.6%	(Lee et al., 2012)
France (1404)	67.4%	(Tuffery-Giraud et al., 2009)
Canada (508)	71%	(Lim et al., 2020)
Kuwait (111)	66.2%	(Mohammed et al., 2018)
Greece (90)	63.3%	(Florentin et al., 1995)
Italy (152)	63%	(Haven, 1992)
Brazil (100)	62.3%	(Zatz et al., 1998)
Germany (56)	62.5%	(Niemann-Seyde et al., 1992)
Present study (35)	**60%**	

The frequency of deletion mutation in DMD gene among Bangladeshi population is 60%. This is comparatively similar to many of the countries we have mentioned above. The reported deletion rates were also identified using multiplex PCR in the respective population.

DMD is characterized by an X-linked pattern of inheritance, thus affecting mainly males. However, they have also been occasionally reported in females, in cases of skewed X-inactivation or X chromosome abnormalities. (Sbiti, El Kerch, et al., 2002). Serum CK levels are useful for diagnosis of DMD patients, as it is widely known that specificity of CPK is

approximately 94.1% with a sensitivity of 100% in DMD. However, CK levels are also increased in other muscular dystrophies. Hence CK levels are not that useful for carrier detection. (Anaya-Segura et al., 2015). However, detection of deletions provides accurate information for genetic counselling, and prenatal diagnosis can be proposed to female carriers. (Sbiti, Kerch, et al., 2002) Establishing the carrier status in X-linked recessive disorders is one of the basic dilemmas in genetic counselling because female carriers are usually asymptomatic. (M et al., 2013). The first essential step in genetic counselling 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. A genetically definite or obligate carrier is a woman with an affected son and an additional affected male relative. All other female relatives are possible carriers. (Tyrer et al., 1986). In this regard, genetic counseling for patients and their families must be emphasized. In providing a humane and compassionate platform through which disease ontology, disease progression and its inevitable outcome is disseminated to patients and their families, the importance of genetic counseling should not be underestimated. Genetic counseling also stresses the importance of carrier testing in female relatives of DMD patients, and serves to help families understand and manage the impact of having one or more DMD patients in the family. (Kumar et al., 2020). Despite advances in testing technologies and increasing awareness of DMD, the age at diagnosis has remained constant. This diagnostic delay continues to have a negative impact on parents' experiences, places families at risk of having a second affected child and may have a deleterious effect on affected children's treatment. Indeed, these experiences have been mirrored by parents of children with a range of other genetic conditions including fragile X syndrome (FXS), Klinefelter syndrome and childhood spinal muscular atrophy (SMA). (Wong et al., 2015). Parents experienced many emotions in their search for a diagnosis and consulted with a wide range of health professionals. An earlier diagnosis would mitigate the impact of the diagnostic odyssey on parents and facilitate access to a range of health interventions, including corticosteroid treatment and physiotherapy. There is increasing evidence that early treatment affects the outcome in DMD and early access to therapies will become increasingly important as novel, more effective treatments become available. (Wong et al., 2015).

Besides, several potential negative implications of carrier testing during adolescence have been highlighted in the literature. These include loss of autonomy, stigmatization, and anxiety following an unfavorable test result. In two studies, girls theorized that being found to be a

carrier of a condition could lead to fear of rejection by a future partner (Borry et al.2005; James et al.2003). It has also been suggested that a result that indicates that a daughter is a carrier of a genetic condition may distort the family's perception of her. (Fraser et al., 2018).

Health-care providers must have appropriate knowledge and skills related to genetic and genomic testing, interpretation of test results, communication of results to patients and families, and basic genetic counseling. In addition, the health-care system will require adequate numbers of trained medical geneticists and genetic counselors to assist in the role of specialty testing and interpretation of results. With the expected expansion of genetic and genomic testing, all health-care providers will need (1) educational programs that target relevant scientific, clinical, ethical, legal, and social topics and (2) support systems that address structural and systemic barriers to the integration of genetic medicine into clinical practice. (Botkin et al., 2015).

The most encouraging therapeutic options available to date thus far involve an exon-skipping approach and a stop codon read through approach. The former enables DMD patients to produce dystrophins such as found in Becker patients, albeit at significantly lower levels, leading to a slowdown in disease progression, while the latter attempts to reestablish the functional integrity of the protein. Both these approaches require very precise identification of the mutational status of the DMD gene in DMD/BMD patients. In the absence of curative treatment options, the early diagnosis of patients with suspected DMD is vital to implementing effective disease management strategies, keeping the quality of life of the patient in mind. (Kumar et al., 2020). Exon skipping therapies with antisense oligonucleotides (AOs) is a promising therapy for DMD, and is currently the focus of clinical trials. It uses antisense oligonucleotides to splice out selected exons from the pre-mRNA at or next to the mutation site, to generate a translatable transcript from the mutant DMD gene, which are partially functionally similar to milder dystrophinopathy Becker muscular dystrophy. It means that it is a treatment but not a cure. (Iskandar et al., 2019).

Exon skipping uses synthetic antisense oligonucleotide sequences to correct specific dystrophin gene mutations. It does so by inducing specific exons skipping during pre-messenger RNA (pre-mRNA) splicing of the dystrophin gene, resulting in restoration of the reading frame and partial production of an internally truncated protein, similar to the dystrophin protein expression seen in Becker muscular dystrophy. Antisense therapies that induce single or multiple exon-skipping could potentially be helpful for the majority of dystrophin mutations. (Mah et al., 2016).

Targeted skipping of DMD exons 8, 44, 45, 50, 51, 52, 53, and 55 is predicted to benefit 47% of affected individuals. (Wang et al., 2018). It was found in a study that, DMD patients with Δ45, skipping of either exon 44 or multi-exon skipping of exons 46 and 47 (or exons 46-48) are better potential therapies than skipping of exon 46 alone.(Findlay et al., 2015).

New treatment approaches to ameliorate the dystrophic phenotype include (i) pharmacological interventions using drugs that modulate the immune response and inflammation, abnormal ion homeostasis, impaired excitation–contraction coupling, cellular growth patterns, abnormal metabolic pathways, cholesterol metabolism, oxidative stress and cardio-respiratory complications ; (ii) myoblast transfer therapy; (iii) stem cell therapy; (iv) somatic genome editing using CRISPR/Cas9-mediated exon excision; (v) heat shock protein induction to enhance the natural cellular stress response provided by molecular chaperones ; (vi) stop codon read-through therapy; (vii) vector transfer therapy ; (viii) exon-skipping therapy ; (ix) electrical nerve stimulation to induce muscle transitions; and (x) utrophin substitution therapy. An interesting approach is the repurposing of established pharmacological substances and testing of multi-drug combinations in experimental trials using genetic animal models of Duchenne muscular dystrophy. Detailed discussions of the clinical advantages versus potential limitations of these new treatment options and their current validation status in preclinical or clinical studies have been published. Recently, several novel compounds have emerged which have been approved or await final approval by medicines agencies. This includes the FDA-approved novel corticosteroid Deflazacort that was shown to be associated with improved muscle strength and agents with conditional approval such as Casimersen for skipping exon 45 resulting in the elevated production of dystrophin in skeletal muscle and Eteplirsen for skipping of exon 51 which causes delayed loss of ambulation in some patients and Golodirsen and Viltolarsen for skipping exon 53 resulting in increased dystrophin levels, as well as the oxadiazole drug named Ataluren, approved by the European Medicines Agency, which is supposed to help restore dystrophin by suppressing nonsense mutations. (Ohlendieck et al., 2021).

The pathogenesis affecting DMD is complex and multiple interventions targeting different disease processes are needed. Early recognition and precise genetic diagnosis will allow for individualized therapeutic options for DMD. Even though there is presently no cure, respiratory intervention and other supportive strategies as outlined in the current standard of care for DMD have led to improved survival and better health-related quality of life for many affected

individuals. (Mah et al., 2016). Multidisciplinary management of symptoms is currently the standard of treatment for DMD, with interventions primarily focused on delaying disease progression. (Lim et al., 2017).

Multiplex PCR test following gel electrophoresis allows rapid diagnosis of deletion mutation causative for Duchenne muscular dystrophy. This identification of deletion provides accurate information for genetic counselling and further prenatal diagnosis can be planned for female carriers in the family. Because of its simplicity, rapidity, reduced cost, and its nonradioactive approach, this technique is very practical for the molecular diagnosis of this disorder in our country. According to the present study, this technique can be considered as a first-tier diagnostic test for identifying DMD in our population as around 60% of the reason of this disease are deletion mutation of single or multiple exons. Studying the genotype-phenotype correlations in patients with these deletions, and generating relevant cell and animal models, can provide insight into the genetic complexity of dystrophinopathies and help guide the design of therapeutic strategies for dystrophin restoration. (Gibbs et al., 2020). Early and precise diagnosis of the disease is an essential part of an effective disease management strategy as care guidelines and prevention through counseling need to be initiated at the earliest particularly since therapies are now available for a subset of patients. (Kumar et al., 2020).

This present study of analyzing the deletion mutations of DMD gene within our population will surely help our clinicians getting a clear picture of the disease and its associated clinical phenotypes for diagnosis. It will lessen the confusion between overlapping phenotypes that might show up in many myopathies and complex muscle related disorders. Moreover, this study is the first report of DMD gene deletion analysis in Bangladesh.

Limitations of the study:

Multiplex PCR method will capture deletions impacting the targeted hotspot exons of the gene and will not cover any mutations that are outside of the targeted hotspot region. Patient samples negative for deletion mutations in the DMD gene by mPCR, or where the borders of the identified mutations were unclear, were subjected to MLPA analysis. (Kumar et al., 2020).

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Appendix

Multiplex PCR corresponding gel electrophoresis images of 21 DMD positive patients

Patient serial No. 1

Test Result: Positive
Interpretation: Dystrophin Gene deletion observed in 5 Exons (Exon 8, Exon 12, Exon 13, Exon 17 and Exon 19) indicative of DMD.

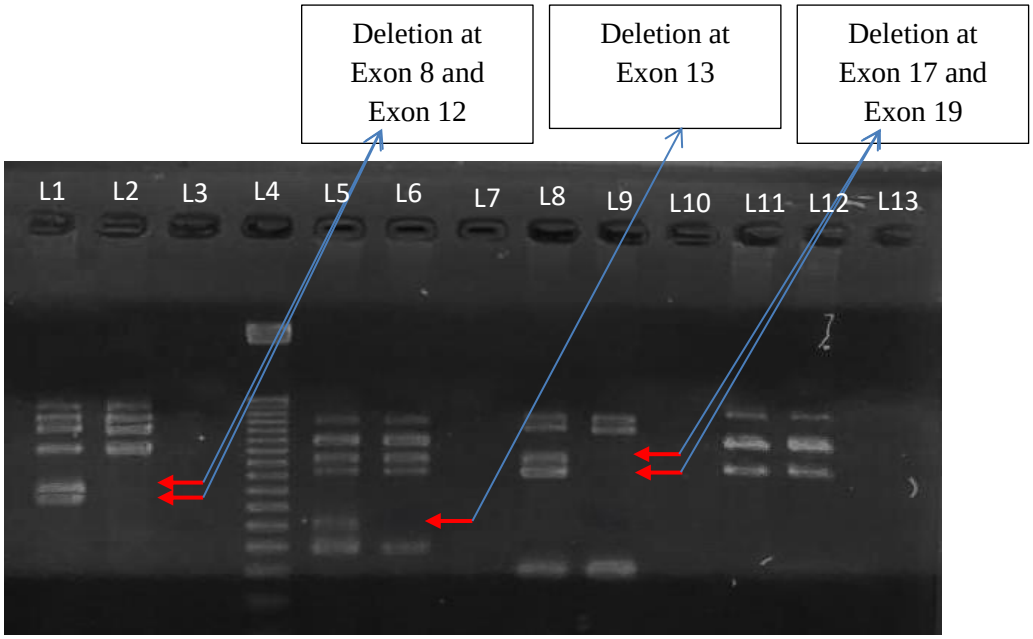

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L5, L8 and L11 = Control's PCR products
L2, L6, L9 and L12 = Patient's PCR products
L3, L7, L10 and L13 = Negative PCR products
L4 = Low molecular weight 50bp ladder

Patient serial No. 2

Test Result: Positive
Interpretation: Dystrophin Gene deletion observed in exon 50, indicative of DMD.

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L7, L11 and L14 = Control's PCR products
L2, L5, L8, L12 and L15= Patient's PCR products
L3, L6, L9, L13 and L16= Negative PCR products
L10 = Low molecular weight 50bp ladder

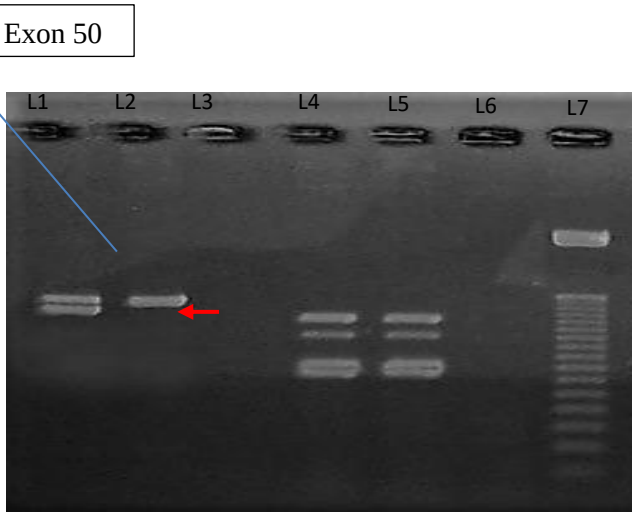

Figure B: Validation of PCR products of Set 2

L= Lane
L1 and L4 = Control's PCR products
L2 and L5 = Patient's PCR products
L3 = Negative PCR products
L7 = Low molecular weight 50bp ladder

Patient serial No. 5

Test Result: Positive
Interpretation: Dystrophin Gene deletion observed in exon 49 and 50, indicative of DMD.

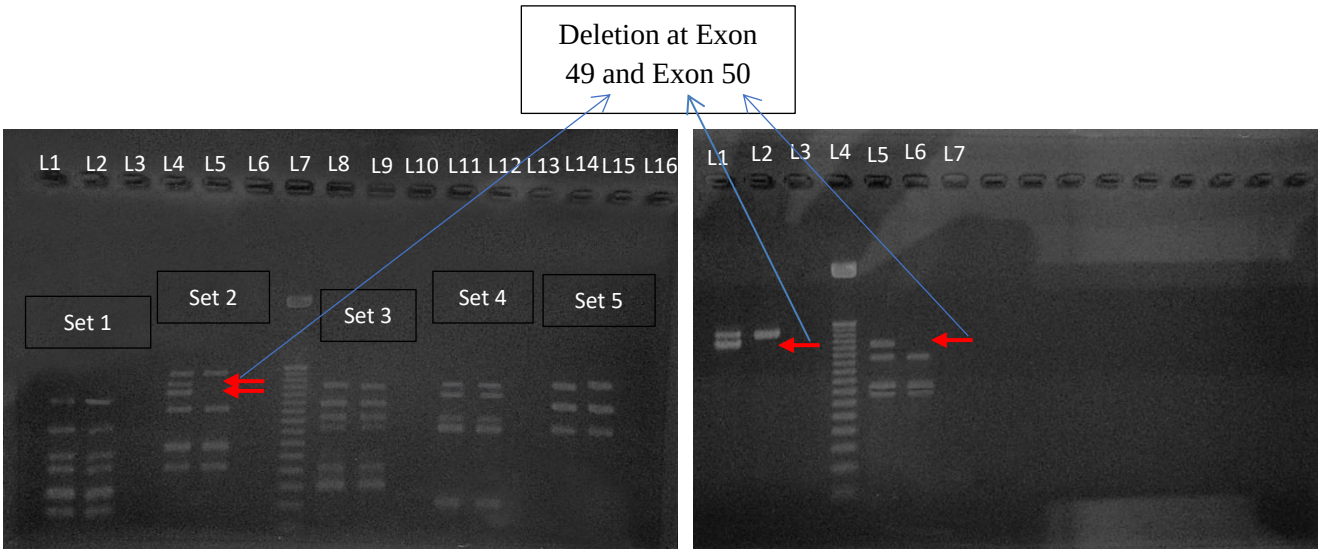

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L8, L11 and L14 = Control's PCR products
L2, L5, L9, L12 and L15= Patient's PCR products
L3, L6, L10, L13 and L16= Negative PCR products
L7 = Low molecular weight 50bp ladder

Figure B: Validation of PCR products of Set2

L= Lane
L1 and L5 = Control's PCR products
L2 and L6 = Patient's PCR products
L3 and L7 = Negative PCR products
L4 = Low molecular weight 50bp ladder

Patient serial No. 7

Test Result: Positive

Interpretation: Dystrophin Gene deletion observed in exon 45 indicative of DMD.

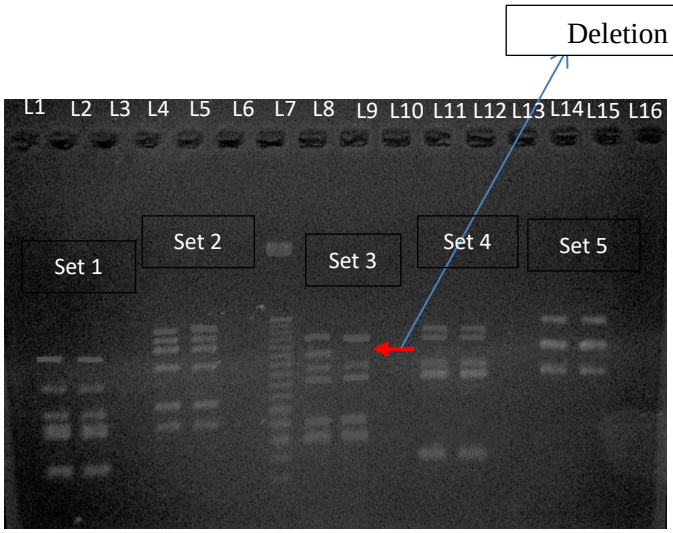

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L8, L11 and L14 = Control's PCR products
L2, L5, L9, L12 and L15= Patient's PCR products
L3, L6, L10, L13 and L16= Negative PCR products
L7 = Low molecular weight 50bp ladder

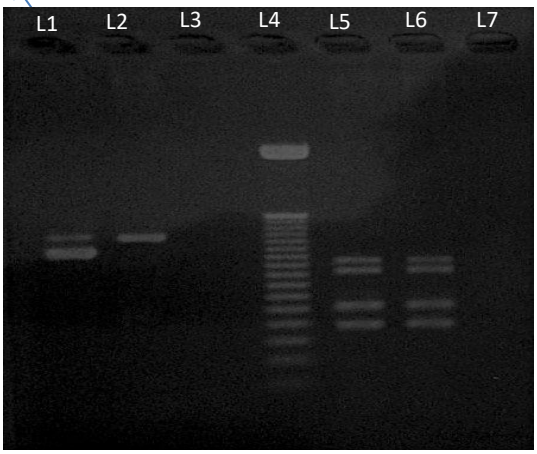

Figure B: Validation of PCR products of Set3

L= Lane
L1 and L5 = Control's PCR products
L2 and L6 = Patient's PCR products
L3 and L7 = Negative PCR products
L4 = Low molecular weight 50bp ladder

Patient serial No. 8

Test Result: Positive
Interpretation: Dystrophin Gene deletion observed in exon 45-53 indicative of DMD.

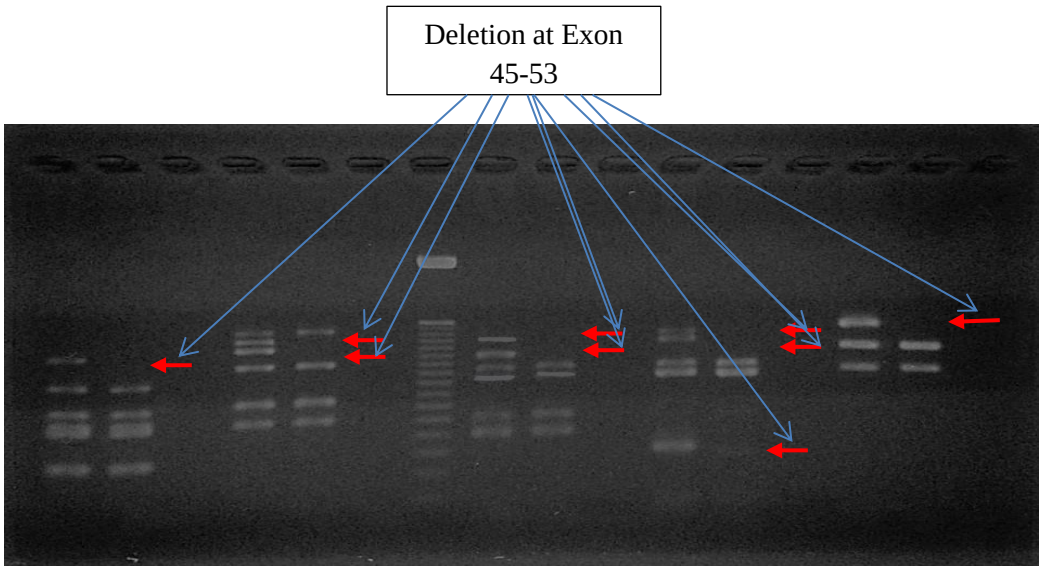

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L8, L11 and L14 = Control's PCR products
L2, L5, L9, L12 and L15= Patient's PCR products
L3, L6, L10, L13 and L16= Negative PCR products
L7 = Low molecular weight 50bp ladder

Patient serial No. 9

Test Result: Positive
Interpretation: Dystrophin Gene deletion observed in exon 46-51 indicative of DMD.

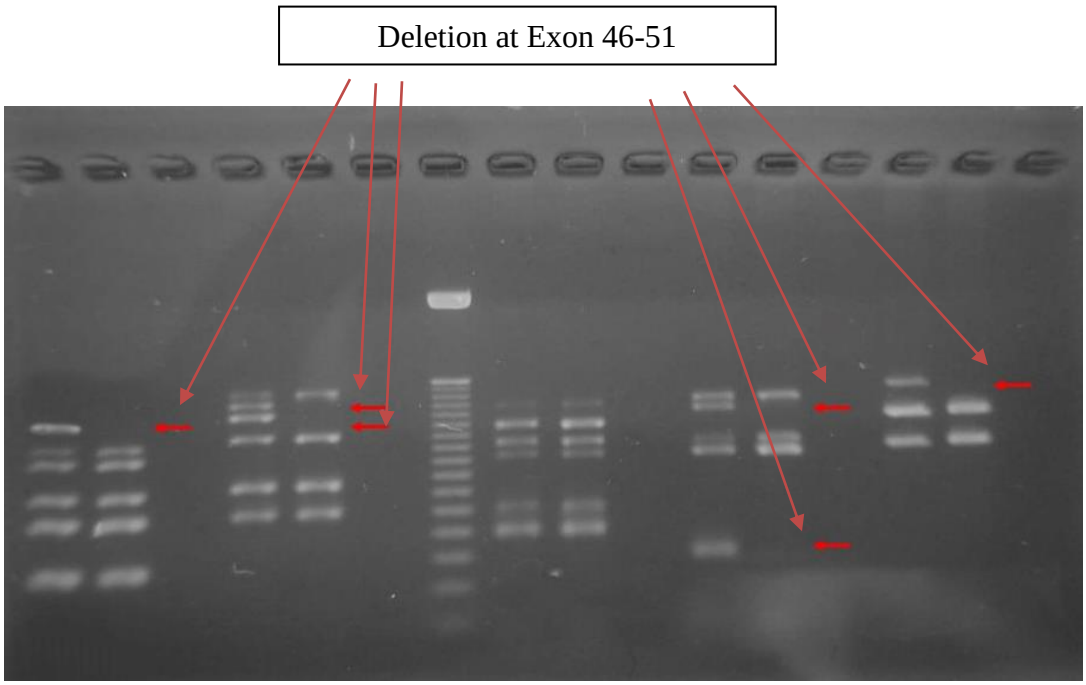

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L8, L11 and L14 = Control's PCR products
L2, L5, L9, L12 and L15= Patient's PCR products
L3, L6, L10, L13 and L16= Negative PCR products
L7 = Low molecular weight 50bp ladder

Patient serial No. 11

Test Result: Positive

Interpretation: Deletion of exon 3, 4, 6, 8, 12 and 16 in the DMD gene indicative of Duchenne Muscular Dystrophy**.**

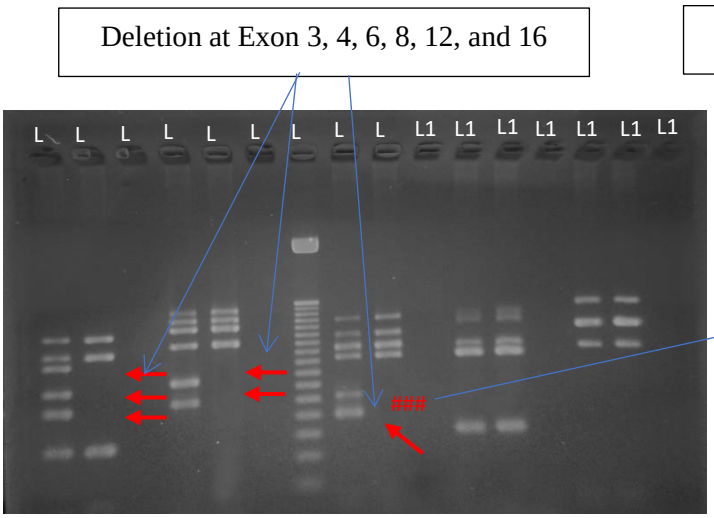

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image

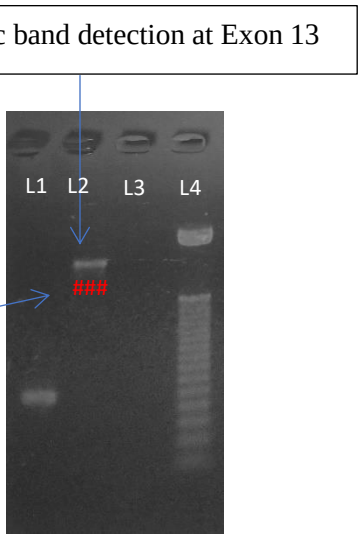

Figure B: Nonspecific band detected with Exon 13

L= Lane (Fig. 1)
L1, L4, L8, L11 and L14 = Control's PCR products
L2, L5, L9, L12 and L15= Patient's PCR products
L3, L6, L10, L13 and L16= Negative PCR products
L7 = Low molecular weight 50bp ladder

L= Lane (Fig. 2)
L1 = Control's PCR products
L2 = Patient's PCR products
L3 = Negative PCR products
L4 = Low molecular weight 50bp ladder

Patient serial No. 14

Test Result: Positive
Interpretation: Deletion of exon 46, 47, 48, 50, 51 and 52 in the DMD gene indicative of Duchenne Muscular Dystrophy.

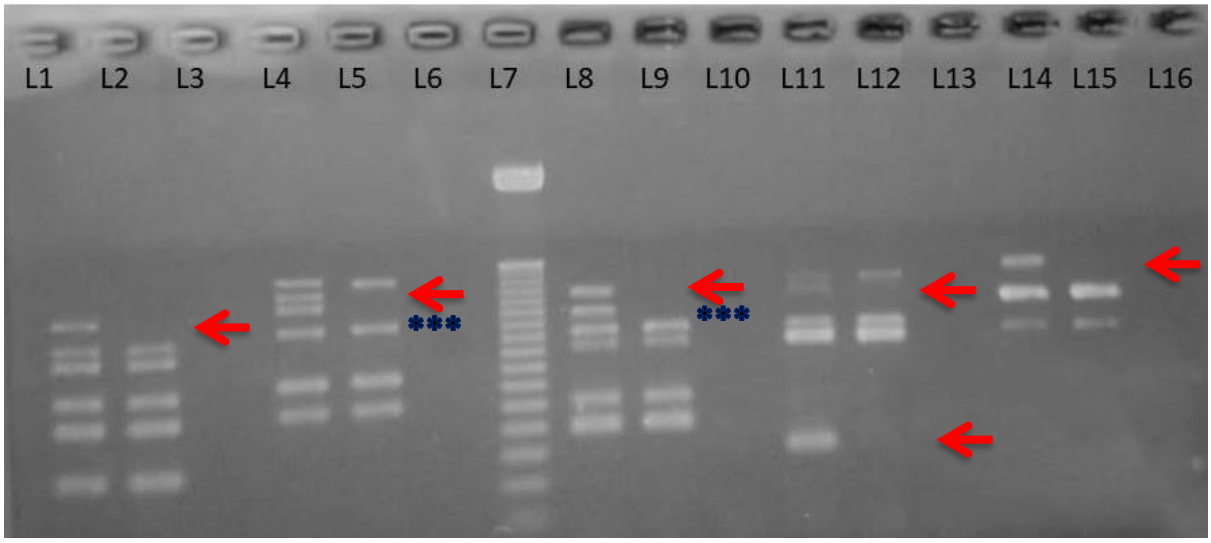

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image

Here,
Arrow (←) are indicating deletion at exon 46, 47,48,50,51 & 52, and

Triple star (***) are indicating nonspecific band detected at exon 45 and 49. Need further evaluation by another technology.

L= Lane (Fig. 1)
L1, L4, L8, L11 and L14 = Control's PCR products
L2, L5, L9, L12 and L15= Patient's PCR products
L3, L6, L10, L13 and L16= Negative PCR products
L7 = Low molecular weight 50bp ladder

L= Lane (Fig. 2)
L1 = Control's PCR products
L2 = Patient's PCR products
L3 = Negative PCR products
L4 = Low molecular weight 50bp ladder

Patient serial No. 20

Test Result: Positive
Interpretation: Dystrophin Gene deletion observed in exon 48-50 indicative of DMD.

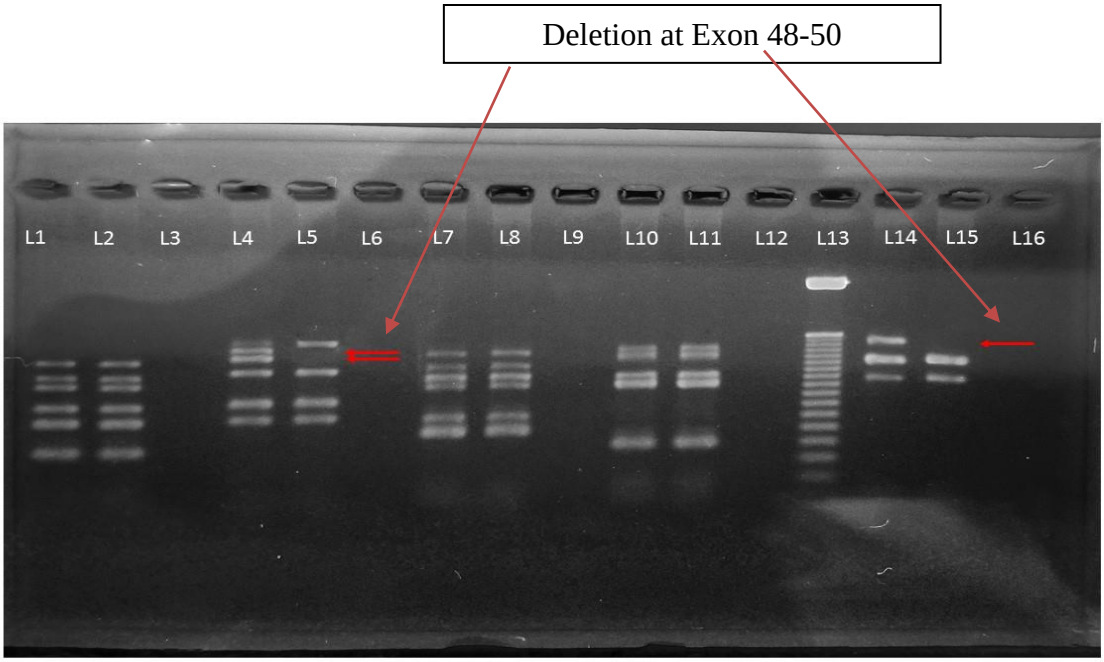

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L8, L11 and L14 = Control's PCR products
L2, L5, L9, L12 and L15= Patient's PCR products
L3, L6, L10, L13 and L16= Negative PCR products
L7 = Low molecular weight 50bp ladder

Patient serial No. 21

Test Result: Positive
Interpretation: Pathogenic (deletion of exon 1,3,4,6,8,12,16 & 17 was found) (Fig 1)

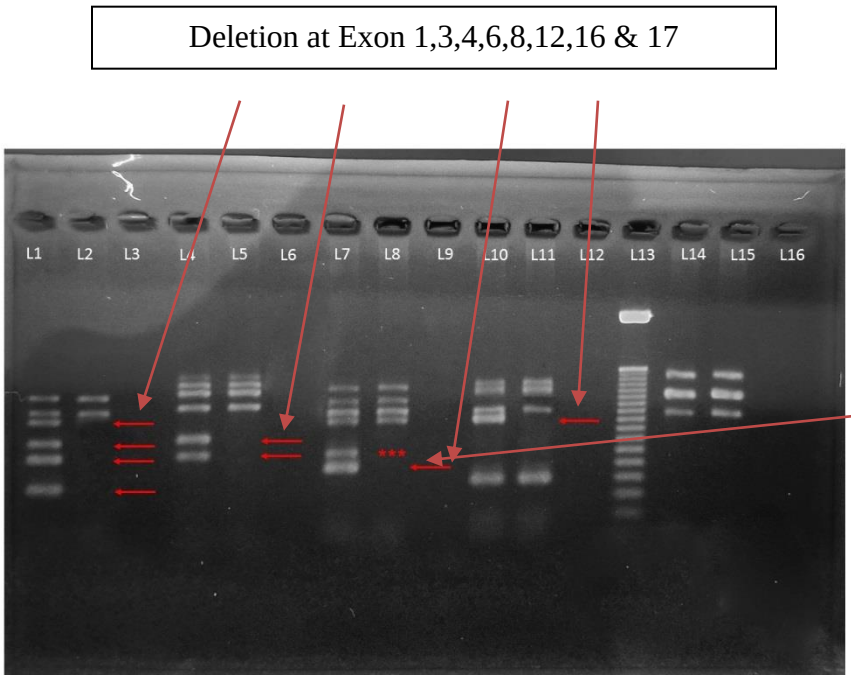

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image

L= Lane (Fig. 1)
L1, L4, L7, L10 and L14 = Control's PCR products
L2, L5, L8, L11 and L15= Patient's PCR products
L3, L6, L9, L12 and L16= Negative PCR products
L7 = Low molecular weight 50bp ladder

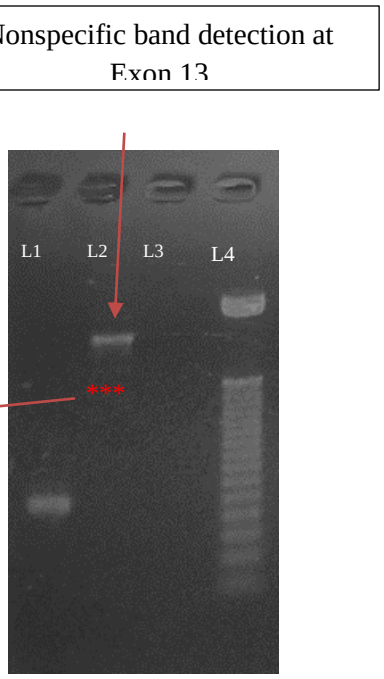

Figure B: nonspecific band detected with Exon 13

L= Lane (Fig. 2)
L1 = Control's PCR products
L2 = Patient's PCR products
L3 = Negative PCR products
L4 = Low molecular weight 50bp ladder

Patient serial No. 22

Test Result: Positive
Interpretation: Pathogenic deletion of exon 51 was found.

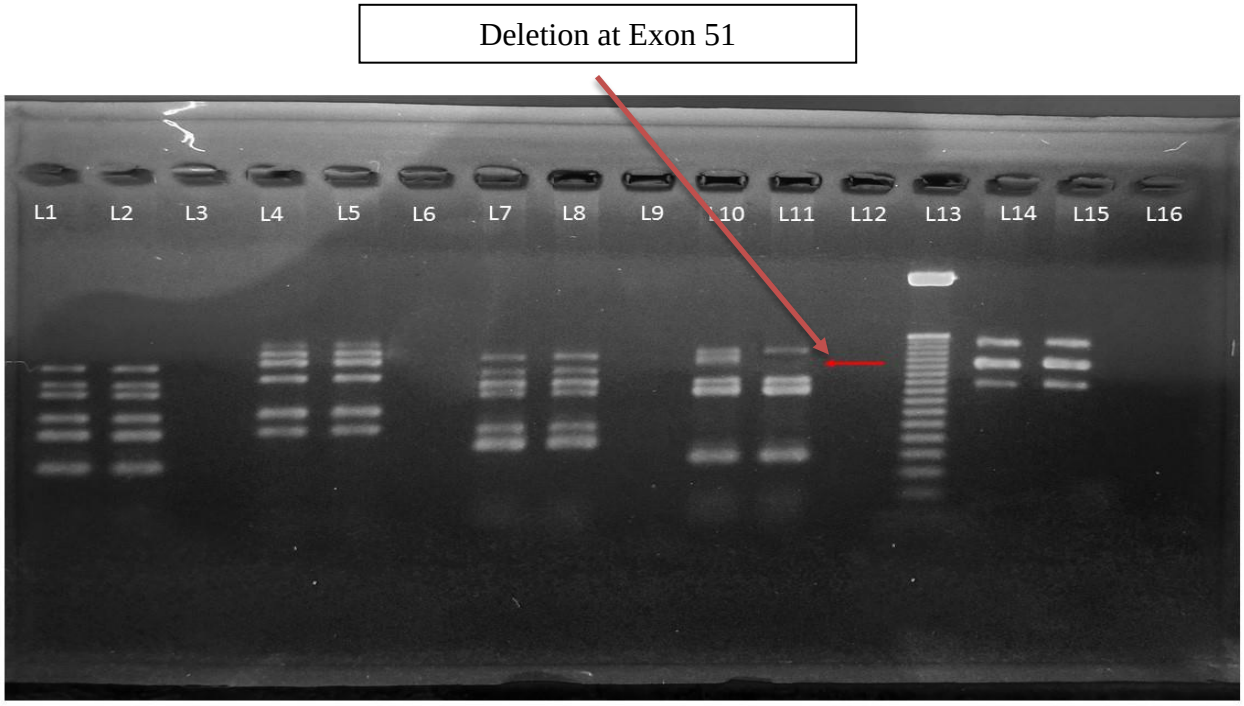

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L7, L10 and L14 = Control's PCR products
L2, L5, L8, L11 and L15= Patient's PCR products
L3, L6, L9, L12 and L16= Negative PCR products
L13 = Low molecular weight 50bp ladder

Patient serial No. 24

Test Result: Positive
Interpretation: Pathogenic deletion of exon 12-13, 16-17, 19, 32, 34, 41-44 was found.

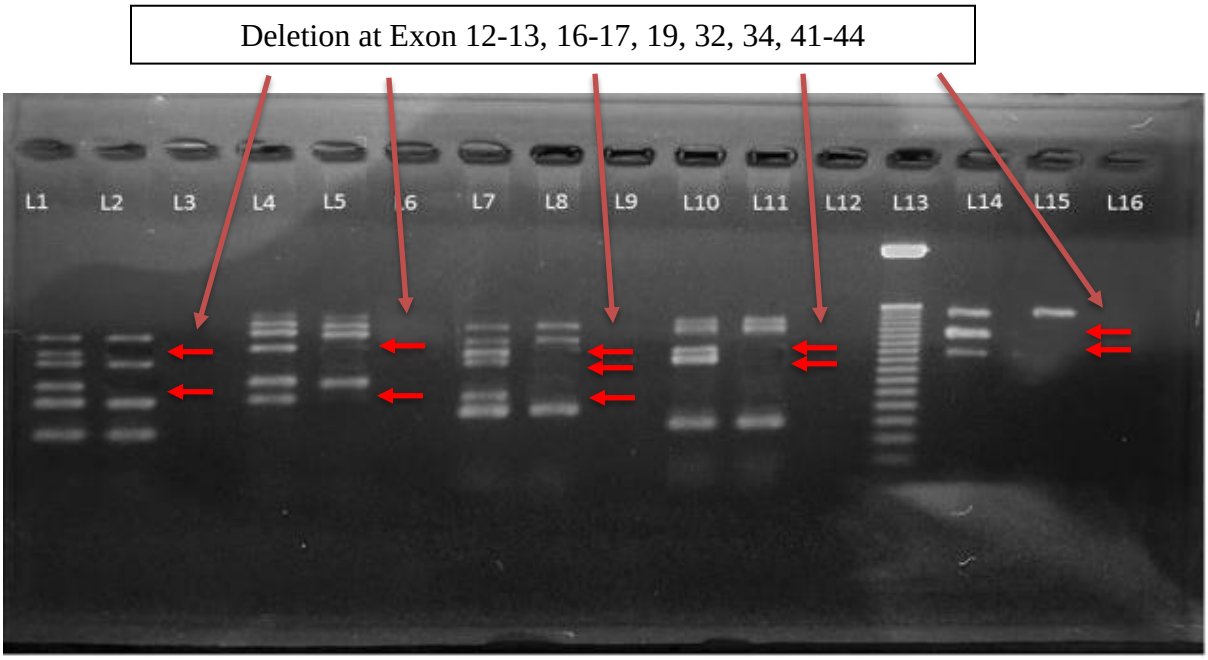

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L7, L10 and L14 = Control's PCR products
L2, L5, L8, L11 and L15= Patient's PCR products
L3, L6, L9, L12 and L16= Negative PCR products
L13 = Low molecular weight 50bp ladder

Patient serial No. 26

Test Result: Positive
Interpretation: Pathogenic deletion of exon 52 was found.

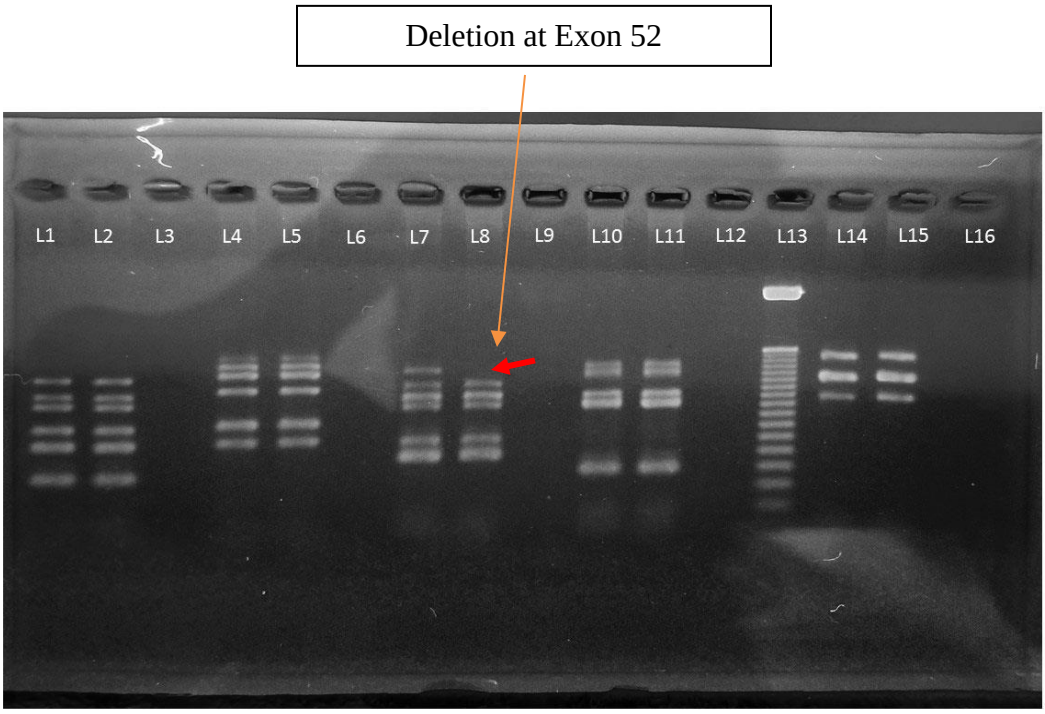

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L7, L10 and L14 = Control's PCR products
L2, L5, L8, L11 and L15= Patient's PCR products
L3, L6, L9, L12 and L16= Negative PCR products
L13 = Low molecular weight 50bp ladder

Patient serial No. 27

Test Result: Positive
Interpretation: Pathogenic deletion of exon 50 and 52 was found.

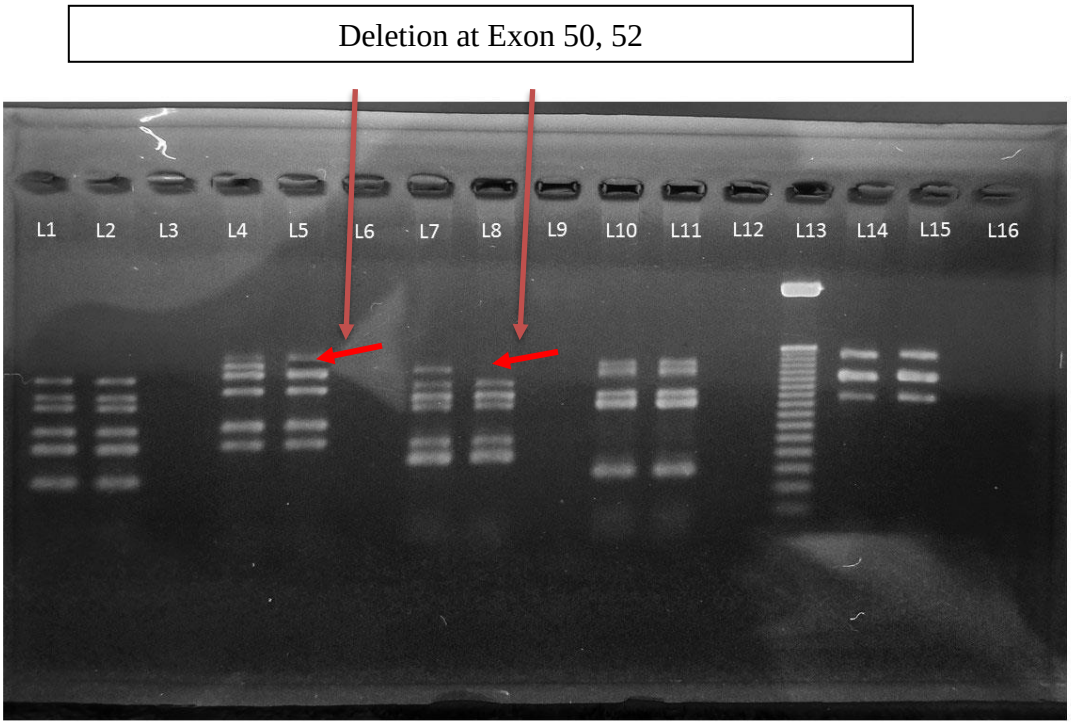

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L7, L10 and L14 = Control's PCR products
L2, L5, L8, L11 and L15= Patient's PCR products
L3, L6, L9, L12 and L16= Negative PCR products
L13 = Low molecular weight 50bp ladder

Patient serial No. 28

Test Result: Positive
Interpretation: Pathogenic deletion of exon 45-47 was found.

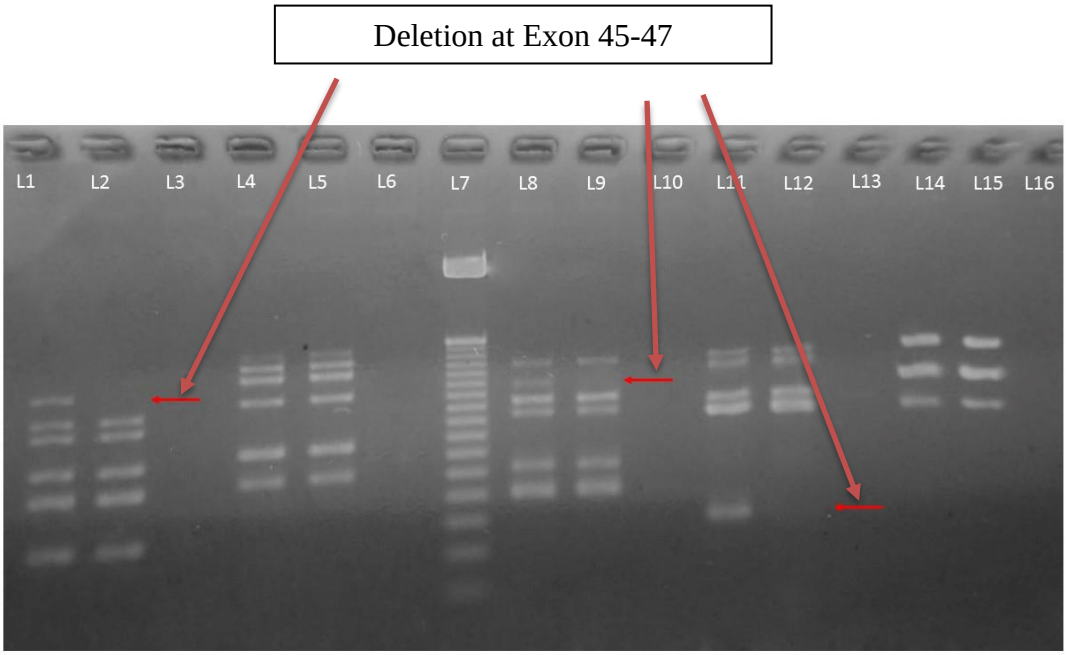

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L8, L11 and L14 = Control's PCR products
L2, L5, L9, L12 and L15= Patient's PCR products
L3, L6, L10, L13 and L16= Negative PCR products
L7 = Low molecular weight 50bp ladder

Patient serial No. 29

Test Result: Positive
Interpretation: Pathogenic deletion of exon 46,47 and 48 was found.

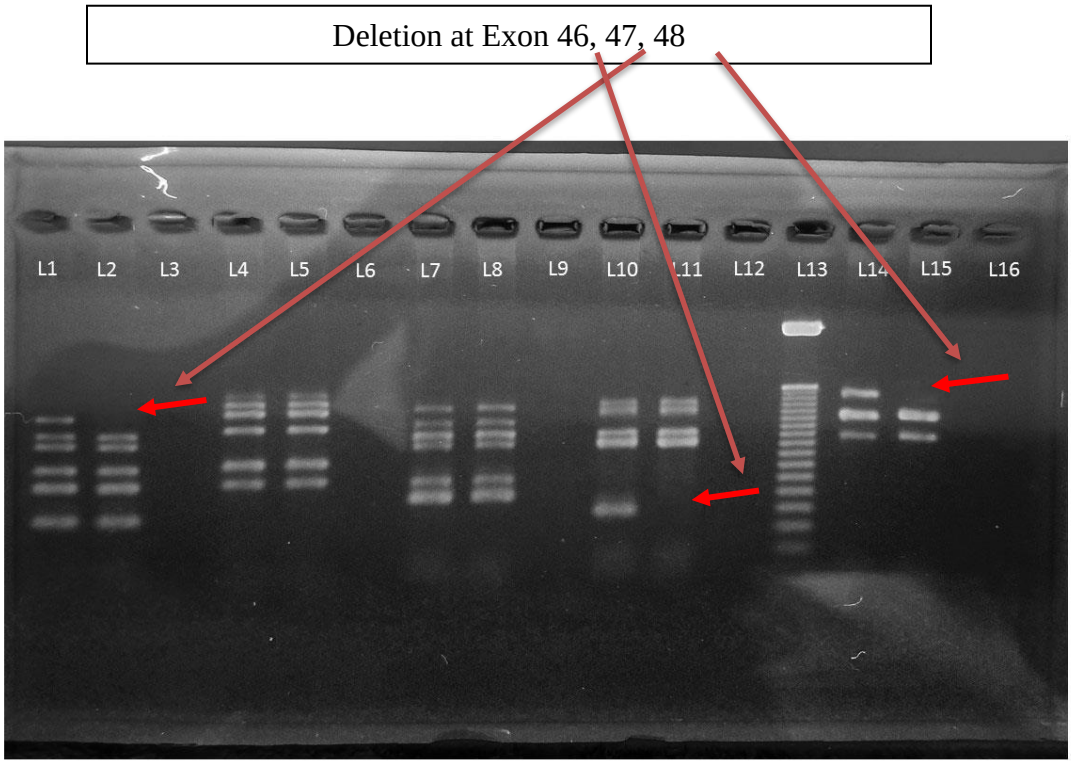

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L7, L10 and L14 = Control's PCR products
L2, L5, L8, L11 and L15= Patient's PCR products
L3, L6, L9, L12 and L16= Negative PCR products
L13 = Low molecular weight 50bp ladder

Patient serial No. 30

Test Result: Positive
Interpretation: Pathogenic deletion of exon 52 and 53 was found.

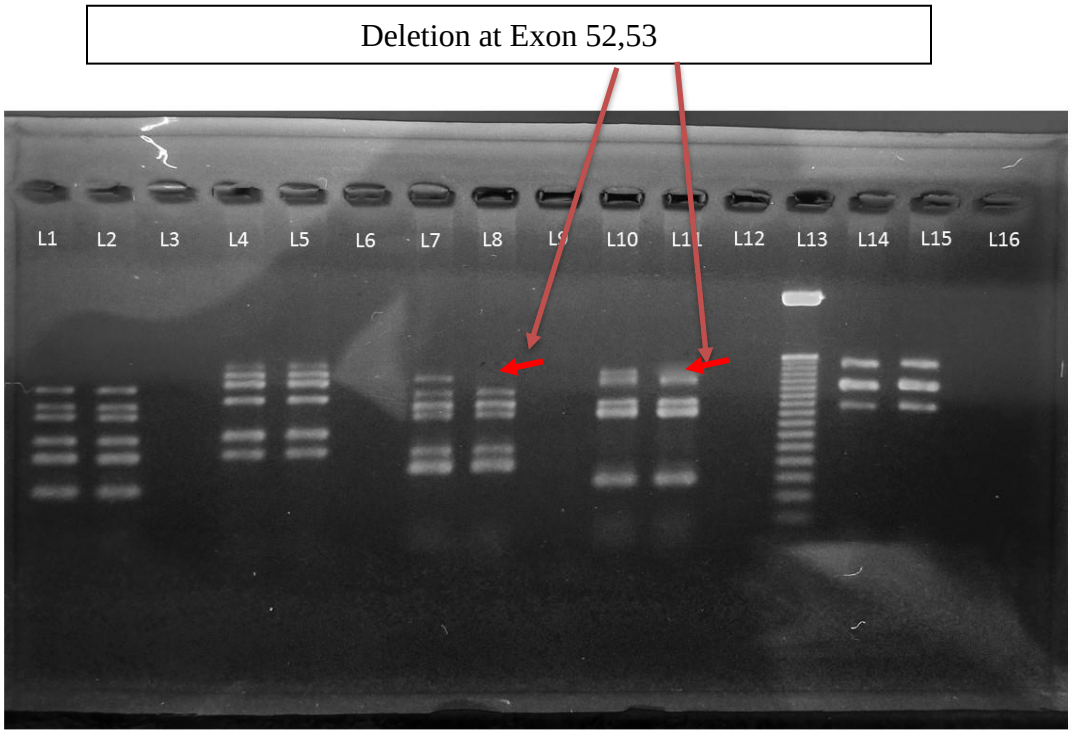

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L7, L10 and L14 = Control's PCR products
L2, L5, L8, L11 and L15= Patient's PCR products
L3, L6, L9, L12 and L16= Negative PCR products
L13 = Low molecular weight 50bp ladder

Patient serial No. 31

Test Result: Positive
Interpretation: Pathogenic deletion of exon 46,47,48,51,52 and 53 was found.

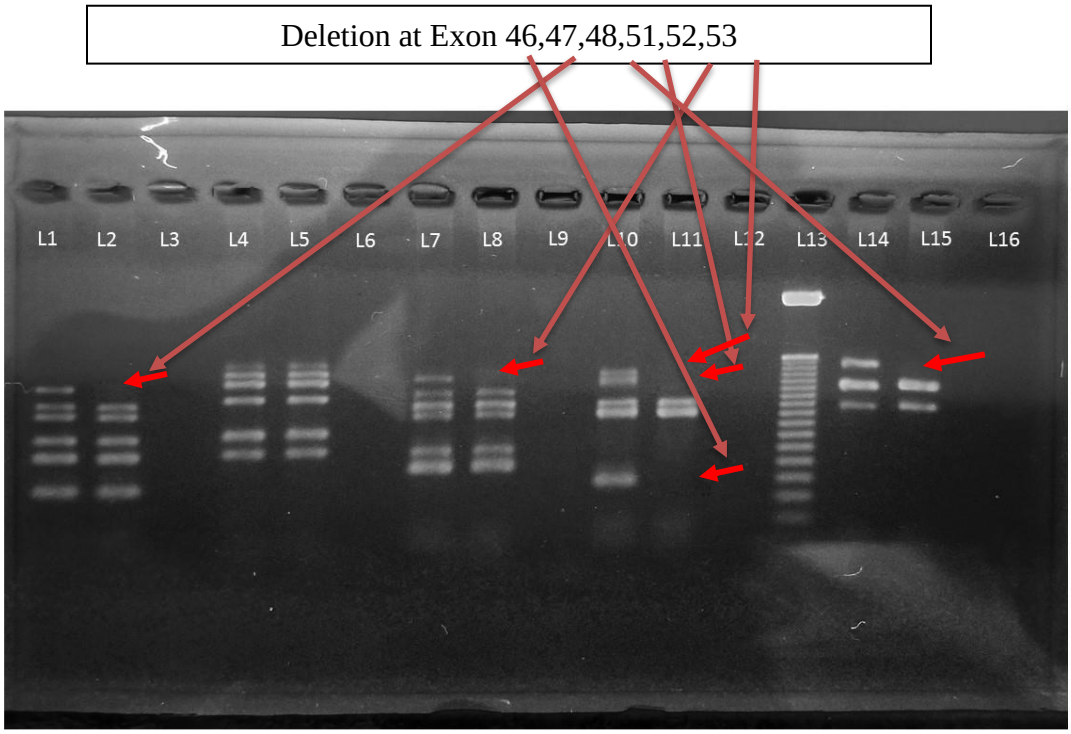

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L7, L10 and L14 = Control's PCR products
L2, L5, L8, L11 and L15= Patient's PCR products
L3, L6, L9, L12 and L16= Negative PCR products
L13 = Low molecular weight 50bp ladder

Patient serial No. 32

Test Result: Positive
Interpretation: Pathogenic deletion of exon 48 was found.

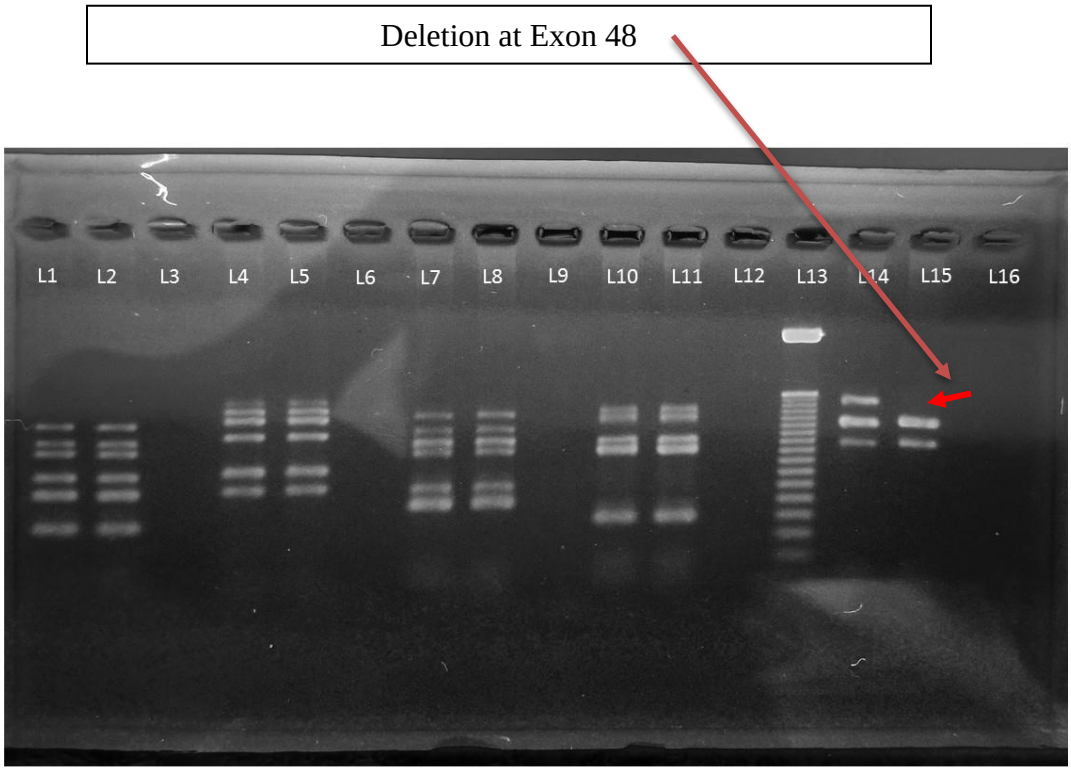

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L7, L10 and L14 = Control's PCR products
L2, L5, L8, L11 and L15= Patient's PCR products
L3, L6, L9, L12 and L16= Negative PCR products
L13 = Low molecular weight 50bp ladder

Patient serial No. 33

Test Result: Positive
Interpretation: Pathogenic deletion of exon 47 and 48 was found.

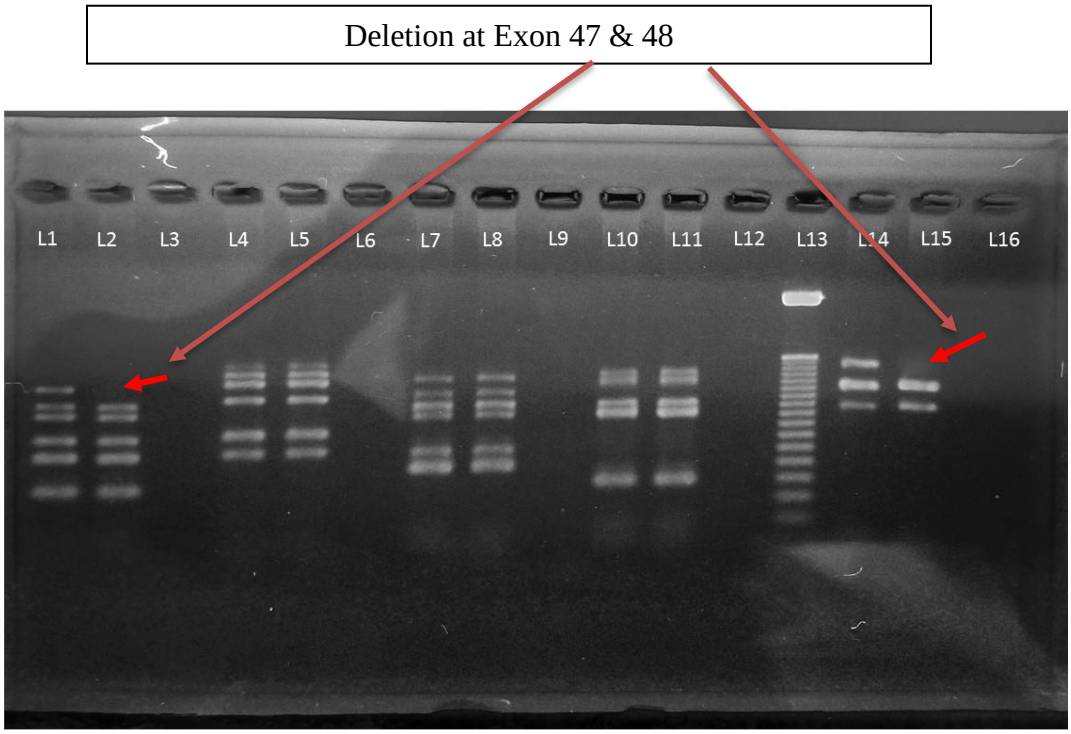

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L7, L10 and L14 = Control's PCR products
L2, L5, L8, L11 and L15= Patient's PCR products
L3, L6, L9, L12 and L16= Negative PCR products
L13 = Low molecular weight 50bp ladder

Patient serial No. 34

Test Result: Positive
Interpretation: Pathogenic deletion of exon 53 and 60 were found.

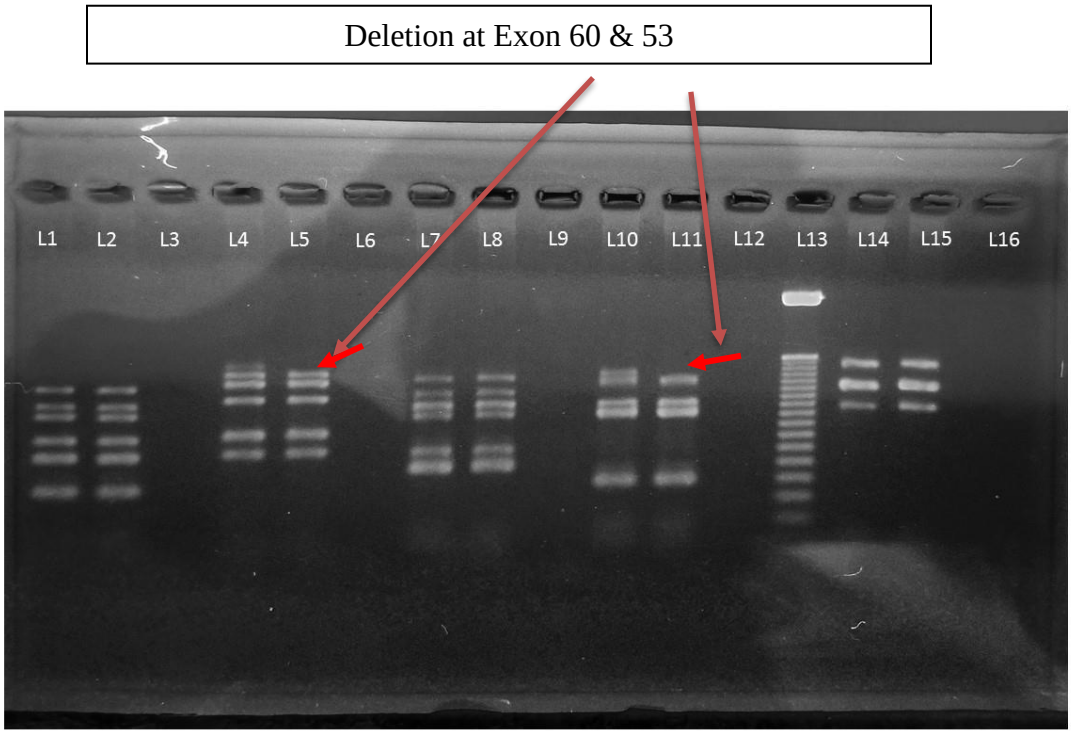

Figure A: DMD gene corresponding multiplex PCR gel electrophoresis image.

L= Lane
L1, L4, L7, L10 and L14 = Control's PCR products
L2, L5, L8, L11 and L15= Patient's PCR products
L3, L6, L9, L12 and L16= Negative PCR products
L13 = Low molecular weight 50bp ladder