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Application of Multiplex PCR for Detection of Duchunne Muscular Dystrophy: A Childhood Neuromuscular Disorder

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Abstract

Duchenne muscular dystrophy (*DMD*) is an Xlinked recessive disorder that affects 1 in 3,600 - 6,000 males and is caused by mutation in the dystrophin gene. This is neuromuscular disorder with progressive muscle weakness, which predominantly affect males. Till date no absolute cure of the disease is available in clinical practice. Early diagnosis and timely management are requisite for *DMD* and it enhances the quality of life of patients. Various diagnostic approaches are available but due to accuracy, early detection and non-invasive method molecular tools are most remarkable in recent era. Multiplex PCR has emerged as one of the most convenient tools for screening of *DMD* in terms of its sensitivity, specificity, accuracy, cost effectiveness and time consumption. The current study emphasizes advantages and shortcomings of multiplex PCR with reference to most of the past studies along with its challenges for *DMD* detection in detail. Mutation detection is evidently crucial for diagnosis, but it may also be significant for future therapeutic purposes. Further research is important to elucidate specific mutation pattern in association with management and therapies of proband.

Keywords: Duchenne muscular dystrophy; Multiplex PCR; Dystrophin gene; Mutation; Therapeutics

Abbreviations: DMD: Duchenne Muscular Dystrophy; BMD: m-PCR: multiplex PCR; PCR: Polymerase Chain Reaction; BMD: Becker Muscular Dystrophy; DGC: Dystrophin-Glycoprotein Complex; aCGH: Comparative Genomic Hybridization Microarray; NGS: Next Generation Sequencing; MLPA: Multiplex Ligation-Dependent Probe Amplification; CK: Creatine Kinase; MPS: Massive Parallel Sequencing

Introduction

Duchenne muscular dystrophy is progressive and serious form of childhood muscle wasting with recessive X-linked inheritance pattern, which alone accounts for approximately 80% of all the myopathies [1]. *DMD* is the most common fatal genetic condition in children [2] with the prevalence of about 1 in 3,600–9,337 live male births worldwide [3,4]. It is neuromuscular disease generally caused by protein truncating mutations in large *DMD* gene "dystrophin" at Xp21.2 [5,6]. Dystrophin is a vital protein for myofiber function and muscle-fiber plasma membrane integrity, mutation in this gene diminishes its expression and biological activity [7,8]. Dystrophin gene is the one of the largest of the 30,000 genes and its mutation leads to *DMD* [9]. It encodes proteins in human genome: 79 exons cover 2.6 million bp and protein product is of size 427 KDa [8].

DMD is principally caused by out of-frame deletions or duplications, which lead to complete loss of protein, whereas BMD is mostly caused by in-frame deletions or duplications, which leads to partly functional protein with altered-size [10]. The difference between the severe *DMD* and the allelic, milder Becker Muscular Dystrophy (BMD), occurs due to mutations in the *DMD* gene, which alter the structure or function of dystrophin or prevent any functional dystrophin from being produced. The clinical diagnosis of D/BMD can be confirmed either by the identification of a mutation in the dystrophin gene or by histological analysis of a patient's affected muscle tissue; however, DNA testing has been found to be more acceptable than muscle biopsy owing to its less invasive nature, minimal side effects and reduced costs [11].

Clinically, the disease is characterized by progressive muscle wasting, leading to loss of ambulation by 8–15 y of age and early death due to complications of respiratory, orthopedic, cardiac, and smooth muscle dysfunction problems [4,12,13]. The instance of mutation in *DMD* gene is expected to be either due to inheritance of dystrophin lesions from their maternal side in around two thirds of cases or due to spontaneous mutations in around one third of cases [5,14,15].

The dystrophin protein encoded by *DMD* gene serves as a stabilizing force within muscle structure [16,17]. The muscular dystrophies are varying in age of onset, degree of severity, mode of inheritance and the muscle group that are primarily affected [18,19]. The initial physical symptoms include developmental delays (locomotor, speech and cognitive in some) followed by abnormal gait, enlarged calves, toe-walking and lean backwards to keep their balance, frequent falls and

clumsiness, muscle weakness and difficulty running, jumping, and climbing stairs along with positive Gower's sign [20-22].

In addition to muscle pathology, intellectual impairment of varying degree is present in about 30% of all *DMD* patients. *DMD* affected boys are usually wheelchair bound by the age of 13 years due to eventual weakness [4]. Gradually all the muscles of the body including hearts and lungs muscles become very weak and non-functional which leads to the early death in their 3rd decade of life [4]. Along with clinical symptoms, serum creatine kinase (CK) level is also elevated. Since last two decades, analyses of both the *DMD* gene and dystrophin protein have improved the diagnosis of *DMD* [5,23,24] (**Figure 1).**

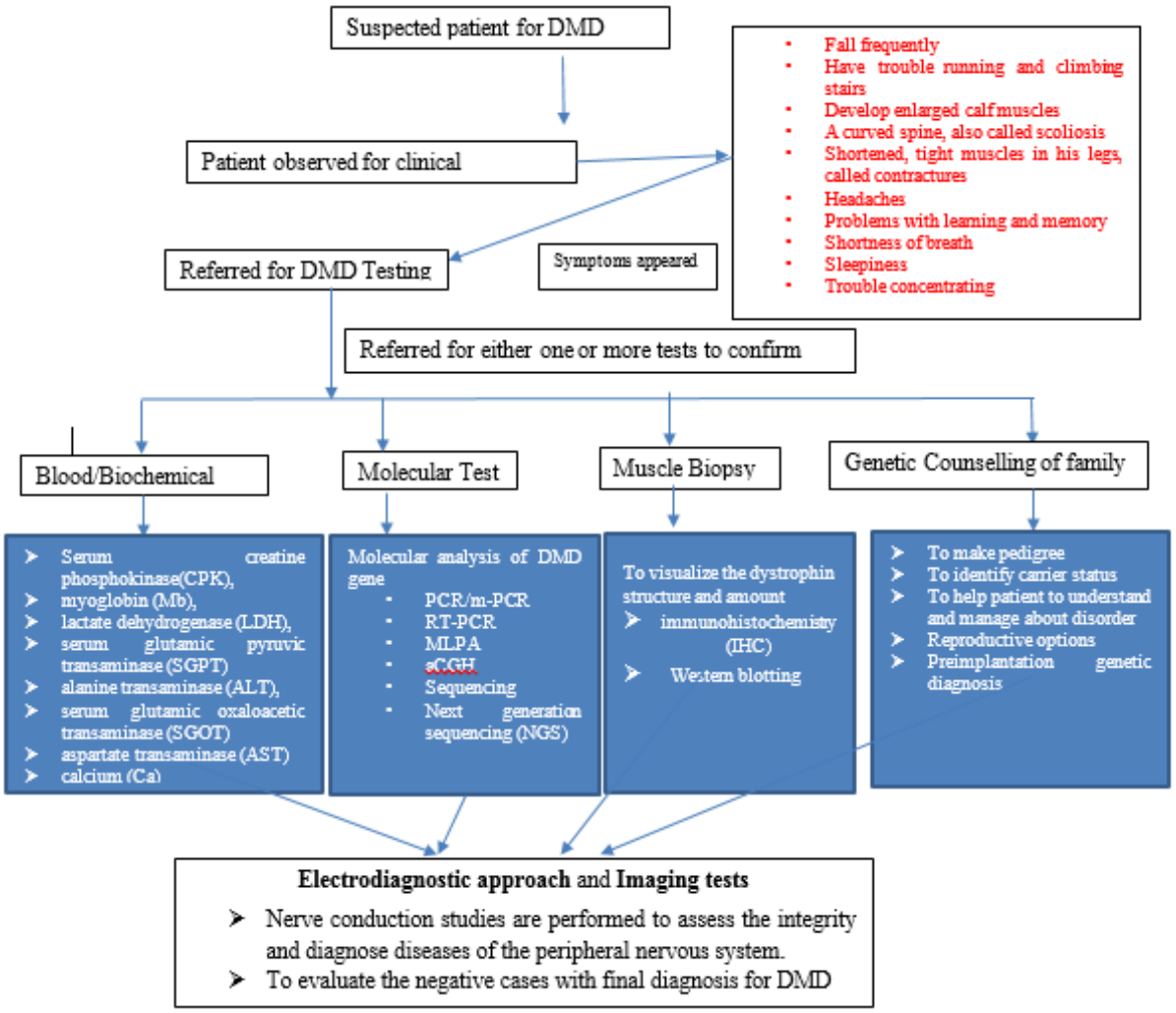

Figure 1 Step-wise diagnostic strategies for determination of *DMD*.

Knowing the exact mutation in a proband, today one can determine the possibility of a life span upto their 4th decade [**5**]. In *DMD*, 65% accounts for deletion, 5-8% for duplication and the remaining for point mutation and small insertions [**25**]. The most common cause of death is cardiac and respiratory failure but improvements in health care, use of steroids and assisted ventilation have extended their life span [**26**].

Approximately, 60% of dystrophin mutations are large insertions or deletions that lead to the frame shift errors, whereas approximately 40% are point mutations or small frameshift rearrangements [**27,28**]. Around one-third of the *DMD* patients originate through new mutations while the rest are inherited through carrier mothers or arise from germinal mosaicism. For confirmation of *DMD* 3 major parameters will be observed that are as follows:

Patients have muscle weakness and large calves. The muscle enzyme in the patient's blood called CK is very high. This means the muscle cells are breaking down. DNA blood test (genetic test) showed a change in patient's dystrophin gene, if the genetic test is normal, then muscle biopsy is preferred to perform to visualize dystrophin pattern in the muscle cells.

Dystrophin gene

This gene was identified in 1987, and protein produced by it is also called as dystrophin. This gene is one of the most

complex and largest genes identified till date with 2.4 kbp size and 79 exons [29]. The location of the gene is at Xp21.2 of human X-chromosome. This is the largest gene covering 2.5 mega bases [30].

This gene contains up to eight alternate promoters. Three promoters located at the 5' region of dystrophin gene and gives full length transcription product of size 14 kb mRNA [31]. The gene encodes a large protein with 3685 amino acid residues [**32**] containing an N-terminal actin-binding domain and multiple spectrin repeats (**Figure 2A**).

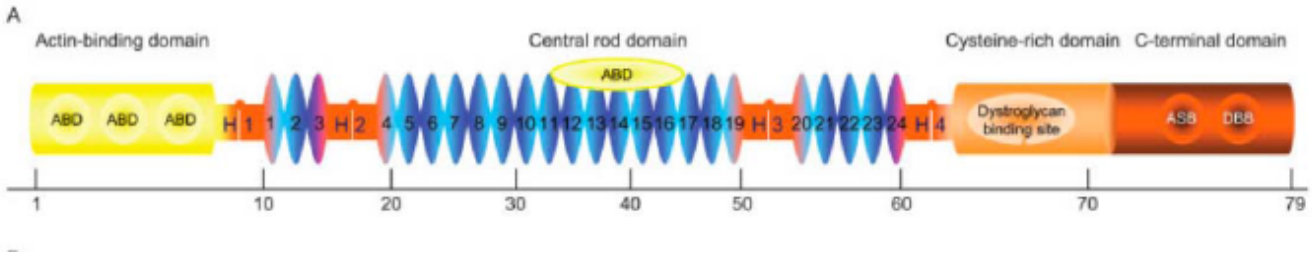

Figure 2A Schematic representation of dystrophin protein and its domains (Adapted from Aartsma-Rus et al. [45]).

The encoded protein forms a component of the dystrophin-glycoprotein complex (DGC), which bridges the inner cytoskeleton and the extracellular matrix. Dystrophin protein is principally expressed in skeletal, cardiac and smooth muscles and at lesser extent in brain [33] (**Figure 2B**).

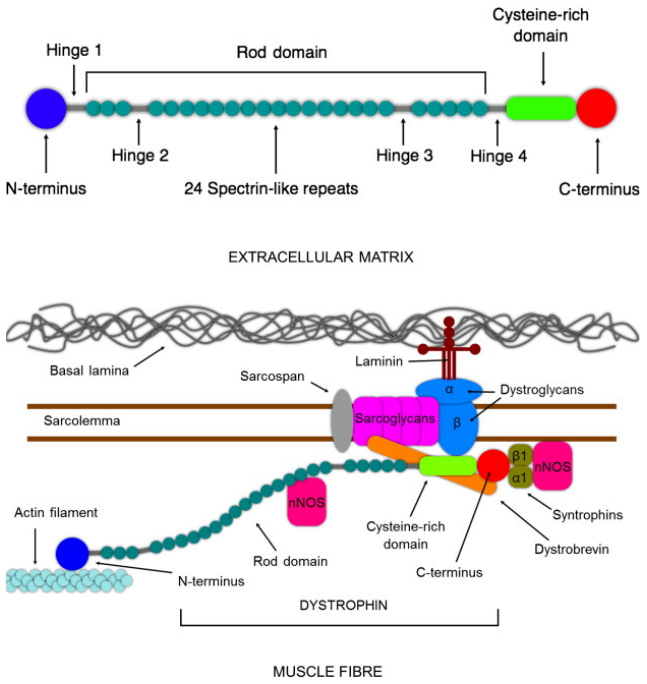

Figure 2B Dystrophin and the dystrophin-associated glycoprotein complex (DAGC). (Adapted from Douglas and Wood, [105]).

Mutation analysis of *DMD*

Mutation studies of the dystrophin gene are focused on detecting deletions or duplications of one or more exons, and multiplex PCR that amplifies selected deletion-prone exons has been used as the most efficient method of mutation detection [34,35]. Mutations in dystrophin protein disrupt the open reading frame and prevent the full translation of its protein product, in ~65% of cases [30,36].

Mutations within the dystrophin gene are mainly distributed within two hotspot regions which map towards the central region (encompassing exon 43-51) and 5' proximal region (encompassing exon 2-19), small deletions and point mutations appear to be evenly distributed across the gene [37]. Depending on mutation type, the expression as well as function of any protein vary, and dystrophin deficiency leads to *DMD*/BMD [38-41].

Large deletions spanning one or more exons are identified as the cause of the disease in about 65%–70% of *DMD*/BMD cases; the remaining has point mutations, mainly nonsense and frame-shift mutations (30%) or duplications (6%) [42,43] (**Figure 3**).

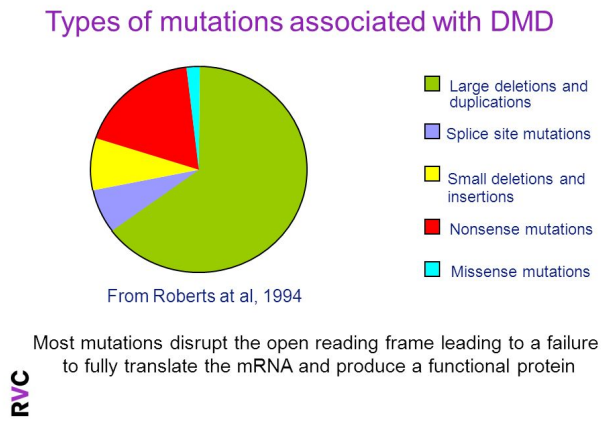

Figure 3 Types of mutations associated with *DMD* (Adapted from Roberts et al., [106]).

Mutations are either inherited from asymptomatic female carriers (~70%) or de novo (~30%) [44]. Thus, in these gross rearrangements, the reading frame rule explains the clinical difference between *DMD* and BMD at the molecular level [45]. Generally, in case of *DMD* mutations reading frame is disrupted, whereas in case of BMD it is maintained. Therefore, most of the *DMD* mutations create premature stop codons, which presumably results in the expression of truncated proteins that lack the dystrophin C-terminus [46].

Use of multiplex PCR for detection of *DMD*

PCR is one of the most auspicious method; it has been used for detection of various genetic disorders such as *DMD*, β-thalassemia, cystic fibrosis, PKU, MCADD, sickle cell anemia, hemophilia etc. [34,47-50], particularly multiplex PCR assay [51]. In all the clinical laboratories, PCR and other sequence-based detection methods are being used progressively more and considered as one of the most important research tools. For DNA based diagnosis of *DMD* and BMD, multiplex PCR (m-PCR) has established as one of most important tools. Multiplex PCR is a common enrichment technique for targeted massive parallel sequencing (MPS) protocols.

MPS is widely used in biomedical research and clinical diagnostics as the fast and accurate tool for the detection of short genetic variations [52]. More than two sets and up to ten sets of PCR primers are selected for Multiplexing to amplify those regions of the gene that are susceptible to deletion in *DMD*/BMD. In these disorders, 70% of cases are due to a dystrophin gene rearrangement, usually partial deletion, which can be detected by Southern blot analysis using cDNA probes [53]. Southern blotting can reveal large dystrophin gene deletions and duplications in males; however, Southern blotting is time-consuming, requires hazardous reagents, and is limited to only relatively large deletions/duplications [54].

M-PCR amplification is very useful technique for diagnosis of various disorders including *DMD*. Major advantages of m-PCR include, step-by-step protocol development, critical parameters such as cost and time deduction for experimentation, more accuracy and efficiency, marked detection process specially in case of mutation analysis. Therefore, its application was studied and reported by many scientists [55,56].

M-PCR also have some drawbacks which hinders during optimization such as poor sensitivity or specificity and/or preferential amplification of certain specific targets depending on different cases. The m-PCR utilizes more than one primer sets and at times it gives spurious amplification products other than the desired target due to primer dimers formation [57,58]. Therefore, the scientist's primarily focussing to minimize nonspecific interactions during optimization of m-PCR [**59**].

In *DMD* gene, m-PCR is utilized for amplification of most commonly deleted and hotspot region exons. Various centers are being using PCR multiplex sets of Chamberlain et al. [**34**] and Beggs et al. [**35,60**] and Kunkel et al. [**61**] and also their modifications. However, these assays do not cover complete gene with all 79 exons as well as promoters, thus end point of all deletions cannot be characterized. With the implementation of m-PCR, 95-98% of deletion mutation can be detected in male patients only [**62**].

Exon skipping can be used as an important tool for detection of *DMD* gene and dystrophin mutations. To provide the advantage a large proportion of *DMD* patient's exon 51 was chosen as the target for first clinical trial and its removal would benefit the patients [**63**]. Synthesis of partially functional dystrophins through antisense oligonucleotide (AON)-mediated exon skipping is a therapeutic approach and exon 51 was firstly targeted through the United States regulators [Food and Drug Administration (FDA)] approval [64].

Deletion of one or more exons are common in *DMD* causing mutations, cases arising from exonic duplications, nonsense mutations, splice motif detects, micro-insertions or deletions may be more amenable to exon skipping [63].

M-PCR has been widely used in D/BMD for deletion detection, however it is time-consuming and difficult to cover all of the exons. Besides, approximately 10% patients with duplication mutations may be misdiagnosed [65]. In 2002, multiplex ligation-dependent probe amplification (MLPA) was invented by Schouten [66], which possesses the capacity to quantify all 79 exons in only 2 reaction sets and facilitates the diagnosis of D/BMD. With MLPA, identification of deletion, duplication as well as point mutation of whole 79 exons are easy and effective. Murugan et al. [67] utilized m-PCR as well as MLPA for mutation detection in 150 cases. With m-PCR, deletions were identified in 103 patients whereas MLPA identified deletions, duplications and nine additional mutations in patients.

Thus, this study reported MLPA as cost-effective and precise tool for diagnosis of *DMD* in a developing country like India. Verma et al. [68] reported MLPA as most appropriate tool for intragenic deletions as well as carrier status detection over m-PCR. With the advancement of technologies, the recent therapeutics like antisense oligonucleotide therapy are mutation specific and require the knowledge of mutation to select proper oligo for the patient.

For the detection of genomic rearrangements of the *DMD* gene custom designed oligonucleotide array-CGH platform is a reliable. Gaudio et al. [69] reported the efficacy of array-CGH platform in detecting submicroscopic copy-number changes involving the *DMD* gene, as well as providing more precise breakpoint identification at high-resolution and with improved sensitivity.

Thus, microarray-based genomic analysis has revolutionized cytogenetics [54,70,71]. With development of various tools and techniques, still molecular tool m-PCR is most convenient and appropriate method for the detection of approximately 98 per cent of deletions, which accounts for 65 per cent of all

mutations [34,35]. Earlier studies which have been used m-PCR for D/BMD detection are as discussed in **Table 1** [72-106].

Table 1 Deletion pattern in *DMD*/BMD patients of different regions including India.

S. No.	Disorder	No. of patients	Region of study	No. of exons studied	No. of patient with deletion	Longest deletion	Additional tests done	Age of patients (Years)	Max frequency of exon deleted	Deletion Frequency	Reference
1	DMD/BMD	15	Saudi Arabia	26	12	9 exons (45-53)	SCPK	Feb-19	49 & 50 (13.34%)		[72]
2	DMD/BMD	70	Eastern India (Kolkata)	31	46	12 exons (42-53)	SCPK	Jan-16	48 & 49	65.70%	[73]
	DMD/BMD	108	Kolkata	42	67	38 exons (11-48)	SCPK, Carrier Detection	Mar-15	50	62.05%	[74]
3	DMD/BMD	72	Moroccan	18	37	11 exons (43-52)	Western Blotting & immunofluorescence	Apr-32	46 & 47	65%	[75]
4	DMD	69	Northeast India	17	49	9 exons (44-52)	SCPK	02-Sep	50 (14.38%)	74%	[76]
5	DMD/BMD	15	Western Saudi	9	6	-	SCPK	May-19	51 (20%)	40%	[77]
6	DMD/BMD	22	Delhi	27	12	9 exons (44-52)	Southern hybridization		45	54.54%	[78]
7	DMD/BMD	88	Gujrat	26	65	8 exons (45-52)	SCPK, LDH, Myoglobin	-	50	73.86%	[79]
8	BMD	347	Mumbai	32	222	11 exons (45-55)	SCPK	Dec-13	45 (76.1%)	89.10%	[80]
9	DMD	10+10	New Delhi	10	1	4 exons	SCPK	-	49	5%	[81]
10	DMD	180	New Delhi	22	90	8 exons (45-52)	MLPA	-	45	50%	[82]
11	DMD	25	Western India (Mumbai)	15	18	-	serum creatine kinase (CK) and electromyography	-	44 & 51	72%	[83]
12	DMD	101	South India	27	44	-	SCPK	-	50	73%	[84]
13	DMD	66	South India	19	41	-	-	-	50	62.10%	[85]
14	DMD	160	Northern India	27	103	7 exons (45-51)	-	-	-	64.40%	[86]
15	DMD	100	Western India	19	-	-	-	-	-	74%	[42]
16	DMD	74	Iran	19	38	-		-	-	51.30%	[87]
17	DMD/BMD	29	Korea	20	21	12 exons (44-55)	SCPK, MLPA, Carrier detection	0.4-13	45	72.40%	[88]

18	DMD	84	Taiwan	19	11	16 exons (45-60)	PCR, DHPLC	-	-	13%	[89]
19	DMD	442	Japan	19	270	-	Complementary DNA (cDNA) and chromosome analysis	-	-	61%	[25]
20	DMD/BMD	29	Iran	29	29	-	EMG, MLPA	7.49-21.58	-	100%	[90]
21	DMD/BMD	-	-	26	-	-	-	-	-	-	[91]
22	DMD/BMD	110	North West Iran	24	63	8 exons (44-51)	SCPK	-	50	57.30%	[92]
23	DMD/BMD	123	Serbia, Belgrade	18	71	9 exons (45-53)	MLPA	-	50	57.70%	[93]
24	DMD/BMD	63	Hong Kong	18	44	9 exons (51-60)	MLPA	-	50	69.80%	[94]
25	DMD	128	Johannesburg, South Africa	24	40		MLPA	-	-	31%	[95]
26	DMD/BMD	150	Chennai	30	103	10 exons (45-54)	MLPA	Mar-36	49 and 50	68.70%	[67]
27	DMD	211	Pakistan	18	86	-	-	-	50	40.75	[96]
28	DMD/BMD	167	China	-	-	-	-	-	-	61.7	[97]
29	DMD/BMD	1053	China	-	-	20 exons (3-22)	MLPA	-	-	86.40%	[98]
30	DMD	105, 86, 34	Singaporean, Japan, and Vietnam	-	-	-	-	-	50, 49 & 50, 51	40.00% 51.20% 40.00%	[99]
31	DMD/BMD	202	Thailand	31	99	14 exons	MLPA	-	-	49%	[100]
32	DMD	20	Malaysia	7	14	7 exons (43-46) (49-51)	SCPK	0.5-10	49, 50 and 51	60%	[101]
33	DMD	20	Western India (Mumbai)	32	0	-	MLPA	02-Sep	-	-	[102]
34	DMD/BMD	121	North India	28	88	22 exons (1-22)	Southern hybridization	1.5-5	45	73%	[103]
35	DMD	50	South Western Maharashtra	21	47	11 exons (42-53)	-	Feb-19	52	94%	[104-106]

Conclusion

DMD and BMD are both caused by mutations in the dystrophin (*DMD*) gene. Genetic screening and confirmation of the mutation is important for patients because it has implications for disease prognosis, genetic counselling and evaluating each patient's eligibility for emerging genetic therapies. With advancement of molecular tools, the diagnosis of *DMD*/BMD has become more convenient and accurate. Multiplex PCR is one such most important molecular tool which is most commonly used sensitive method has significantly increased detection of small dystrophin gene mutations and made it possible to diagnose approximately 90% of patients with Duchenne muscular dystrophy by DNA analysis. These findings, combined with cost savings and safety issues, provide compelling reasons to consider DNA analysis as

the initial diagnostic test for the suspected dystrophin-deficient patient. Thus, with use of m-PCR efficient as well as early detection of *DMD*/BMD is possible which also provides patient's timely management and care to enhance their quality of life.

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