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

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Abstract

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder that affects 1 in 3,600 - 6,000 males and is caused by mutation in the dystrophin gene. This is a 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 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).

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).

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).

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).
Use of multiplex PCR for detection of DMD

PCR is one of the most auspicious methods; 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 exon 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
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.

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

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<td>84</td>
<td>Taiwan</td>
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<td>270</td>
<td>-</td>
<td>Complementary DNA (cDNA) and chromosome analysis</td>
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<td>EMG, MLPA</td>
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<td>30</td>
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<td>China</td>
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<td>105, 86, 34</td>
<td>Singapore, Japan, and Vietnam</td>
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<td>Malaysia</td>
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<td>7 exons (43-46) (49-51)</td>
<td>SCPK</td>
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<td>DMD</td>
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<td>Western India (Mumbai)</td>
<td>32</td>
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<td>-</td>
<td>MLPA</td>
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<td>Southern hybridization</td>
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<td>47</td>
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