"Genetic etiology and Diagnostic strategies for Duchenne and Becker Muscular Dystrophy: A 2012 update."

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Abstract:

Duchenne Muscular Dystrophy (DMD), a type of dystrophinopathy is an X-linked recessive disorder, caused by mutations in the dystrophin gene. Epidemiology and molecular etiology of DMD varies among populations. Since deletions are the most commonly reported mutations in almost all populations, preliminary diagnosis involves detection of deletions. But presence of other mutations, though less common in populations, warrants the need for more comprehensive diagnostic tests. Hence several countries, based on their type of mutational propensity for DMD, have now devised their own strategies and protocols for routine diagnosis of DMD. Most common and convenient technique is multiplex PCR. In India too, development of an integrated strategy consisting of mPCR and several other methods, for the routine diagnosis of DMD is now being considered.

Key words: X-linked recessive disorder, dystrophin gene, molecular etiology, deletions, multiplex PCR

Introduction: Mutations in the dystrophin gene results in a spectrum of muscular dystrophinopathies (Duchenne Muscular Dystrophy (DMD), Becker's Muscular Dystrophy (BMD), Limb Girdle Muscular Dystrophy, etc), of which DMD is the most severe. It is an X-linked recessive disorder with males being affected almost exclusively than females; a characteristic of X-linked recessive disorder. Severity of the disorder makes diagnosis clinically important [1]. Epidemiological studies give a better picture of the incidence of occurrence in the population (table 1). In the West Midlands region of Britain, Duchenne Muscular Dystrophy (DMD) is twice as common as expected in Indians, and is less common than expected in Pakistanis [6]. Sporadic cases, some of which are mosaic cases, have also been reported in some parts of the world [7,8].

Molecular Etiology : Molecular etiology of a genetic disorder helps in designing diagnostic and therapeutic strategies.

International scenario: Deletions are most common (60 to 65% of DMD patients), usually of several kilobases of genomic DNA [9,10]. Depending on maintenance or disruption of the translational reading frame (frame-shift hypothesis), the clinical progression in DMD can be predicted in 92% of cases [11,12]. If a deletion disrupts the translation reading frame of the dystrophin mRNA, then a C-terminally truncated non-functional protein is synthesized resulting in more severe clinical presentation of DMD [13].

A study of 90 unrelated patients, representing more than half the known families in Finland, revealed that deletions were equally common in familial and sporadic disease [14]. The difference in frequency in mosaic cases was observed while comparing the mutation spectra observed in isolated cases of DMD and familial cases [15]. The frequency of deletions of the DMD gene was greater in affected males resulting from a female gametic mutation (75%) than in those resulting from a male gametic mutation (56%) [16].

Table 1: Epidemiological data from different parts of the world

Population	Data
Canadian	1 per 4700 males born between 1969 and 2008 [2]
American	Birth prevalence rate: 1 in 3,500 (2.9 per 10,000) male births [3]
European	$11.99x10^{-5}$ live born males from 1977 to 1990 [4]
Japanese	Incidence rate: $29.2x10^{-5}$ prevalence rate: $6.72x10^{-5}$ [5]

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Mutational Hot Spots:

Deletions are non-randomly distributed occurring mainly (~80%) in the central region (exon 44 to exon 60) and less frequently (~20%) at the proximal (5') region (exons 1 to 19) of the gene which are referred to as the 'major' and 'minor' deletion hotspots, respectively [13,17]. But in Filipino BMD and DMD patients, 5' deletions were more common than central region deletions [18].

A study of 473 patients done in two centers from Brazil and the Netherlands showed that the ratio of proximal to distal deletions was 1:3 in isolated cases and 1:1 in familial cases. From these data the study concluded that proximal deletions probably occur early in embryonic development, resulting in an increased frequency of becoming familial, while distal deletions occur later and have a higher chance of causing only isolated cases [19].

5' hot spot region:

Majority of large deletions initiate at the 5' region of the dystrophin gene, for example: large deletion of introns 2 to 42 [20]. Increased breakpoint frequencies near the 5' end are largely due to large sizes of some introns [13]. Patients with deletions in the amino terminal domain I typically had low protein levels and are very severely affected irrespective of disruption or maintenance of the reading frame, thereby suggesting this domain is functionally critical part of the dystrophin, while loss of just the carboxyl terminus often caused BMD [10]. Yet, several researchers found deletions at 5'end of the gene more common in BMD (the milder version), than in DMD [13,14].

Central hot spot region:

The central portion of the dystrophin gene codes for domain III and IV which seem functionally very essential as deletions in these invariably caused DMD. Though, the central region is a preferential site for deletions causing DMD, it includes the distal rod domain (domain II) of the dystrophin molecule (exon 45-exon 53), which can accommodate several in-frame deletions, often resulting in a less severe phenotype, BMD. Conspicuous discrepancy of the protein levels among patients with deletions in the distal portion was attributed to variability in locations of deletion breakpoints relative to intron/exon junctions or alternatively to epigenetic differences that affect the stability of the deleted proteins [10] (Table 2).

Table 2: Review of the deletion patterns in various ethnic groups

Population studied	Mutation analysis
Thai	Most extensive deletions consisted of exon 14 deletions. Most frequently deleted exons were exon 44-52. mPCR detects only 50% of Thai population[21]
Pakistani	Most frequently deleted exons (frequency wise) were 50 (15.11%), 6 (12.79%), 47 (10.46%), 13 (8.13%) and 52 (4.65%) with deletion frequencies [22]
Chinese	DMD exon deletions in local Chinese patients was significantly lower [34.3% (23 patients)] than the commonly quoted 60%. This indicated an ethnic or regional difference in predisposition to DMD exon deletions [23]
American	Out of frame deletion of exon 45 is most common [13]
Asian	Central region is the deletion hotspot in the following 3 Asian populations: Singaporeans (61.9%); Japanese (70.5%); Vietnamese (72.7%) [24]

Scenario in Indian subcontinent:

Studies have provided evidence that in the Indian population too, deletions have been common, about 72% in the western [25] and northern [26] Indian populations. The deletion frequency in Indian population was reported to be much higher (73%) than the American and European population irrespective of the number of patients or the exons analyzed [25] (Table 3).

Why does dystrophin gene have majority of deletion mutation?

- ✓ Large gene size, particularly introns of average size of 35kb may account for the high deletion rate [13]
- ✓ Presence of hyper-mutability elements in the dystrophin gene, such as the THE-1 family of human transposable elements [31]
- ✓ If one assumes unequal crossing-over between the 2 X chromosomes in female meiosis then, deletions, duplications should be generated at equal frequencies. However, duplications occur at a much lower rate than deletions, which hints at mechanisms other than unequal chromatid exchange probably playing an important role in the generation of deletions alone, at this locus. A reason for preponderance of deletions over duplications could be that, duplications may not always be stably inherited and often may undergo spontaneous deletions [32].

Other Mutations reported:

Several other mutations have also been reported, but in small amounts, for example the DMD gene partial duplications account for up to 6 % of DMD and BMD cases. Duplication frequency was reported highest (~80%) near the 5-prime end of the gene, for example duplication of exons 3 and 4 which duplicated of only a part of the actin binding domain, yet caused severe pathological condition, probably since such duplications may lead to a severe disruption of the structure and therefore of the function of this domain.

Table 3: Details of mutations in Indian population :

Indian Population studied	Mutation analysis
Western (Mumbai, India)	Maximum deletions initiated at exon 45 (76.1% of the cases). In a total of 222 patients 84.8% of cases had exon 45-55 deletions [27]
Western (Mumbai, India)	Most deletions occured in exon 44 and exon 51 (central hot spot) region [25]
Eastern (parts of West Bengal, a few eastern states and Bangladesh)	~79% deletions in the central and 17.91% at the proximal (5') hot spot region [28]
Southern	Deletion rate: 73%. Single exon deletion was found in 20.4%. Distal hotspots were Exons 45, 47, 49 and 50 [29]
Southern	Majority of the deletions (78%) at central deletion hot spots mainly exon 50. 22% of the deletions at the 5'deletion hot spot [30]
Northern	Deletion frequency of 73% [26]

Duplications may arise more frequently by an intrachromosomal mechanism than by an interchromosomal mechanism such as unequal crossing-over in meiosis which is consistent with duplication studies in DMD and BMD cases. The differences in the germ-cell development in male and in female or the lack of homologous pairing of the DMD region in meiosis could most likely explain the origination of duplications more often in male than in female [32].

A small number (1/3) of DMD patients with no detectable deletions or duplications have been reported to carry point mutations. Interestingly, DMD is a well-conserved gene despite its large size, in the sense that missense mutations are extremely rare, rather, many of the DMD and the majority of the BMD small mutations lie in noncoding regions of the gene, hence > 95% of point mutations do not disrupt the function of the dystrophin protein. Yet, in few cases point mutations causes premature translational termination resulting in DMD. An important feature of point mutations is that these are unique to the patient and sometimes to his family, hence cannot be used for carrier and/or prenatal diagnosis [33,34].

Splicing mutations that cause exons skipping, producing a semi-functional mRNA or disruptions of exonic splicing enhancers has also been reported in some cases of DMD [35,36].

Evolution of the molecular based diagnosis:

Several diagnostic tests other than molecular based diagnosis such as biochemical test (CK test), EMG, Skeletal muscular biopsy, Western blotting, etc, existed long before the DNA based diagnosis was established [1]. All of these have their own drawbacks, some of which can be overcome by DNA based diagnosis such as, it replaces the general discomfort of the invasive muscle biopsy test and is also cost effective [13].

With the knowledge of the molecular etiology of DMD, the DNA based diagnosis was established [9,37]. Since deletions were the major cause, a number of scientists concentrated on detection of deletions alone, initially. This was done primarily by using the entire dystrophin cDNA probe [38] and by using cDNA probes of exon-containing *Hind* III fragments [14]. Due to large number of exons and large size of the gene, deletion diagnosis by southern blotting and RFLP analysis, had several major limitations and hence their use in routine diagnosis was not feasible [9]. A higher frequency of deletion indicates that there is a preferential deletion of exons in DMD and BMD [39] and hence can be used for diagnostic purpose. Based on the observation that the dystrophin gene has 2 deletion hot spots, primer sets for diagnosis of DMD using multiplex polymerase chain reaction (mPCR) were designed and 54% of the samples or 79% of the deletions could be detected using those sets of primers [9]. One of the earliest studies on Indian population used the 2 out of the 9 Chamberlain multiplex primer set to screen deletion in clinically diagnosed DMD patients and suggested the use of the remaining sets of primers, for an effective prenatal and carrier diagnosis in the Indian population [40].

Subsequently, other oligonucleotide primer sequences that could amplify additional 8 exons and a muscle promoter of the dystrophin gene in a single mPCR were described. These primers along with the existing primer set offered detection of about 98% of deletions in patients with DMD or BMD. The primers could amplify most of the exons particularly in the deletion hot spot region, allowing determination of deletion endpoints and prediction of mutational effects on the translational reading frame [41].

Thus, the original 6-exon Chamberlain-set was modified to 9-exon and ultimately into a 10-exon set [9]. An additional 9-exon Beggs-set, [37] was developed to increase the total number of deletions detected and to define the borders of the deletions in the deletion 'hotspot'. Still latter, a 'Basic Protocol' describing three complementary mPCR assays that detect 26 dystrophin gene exons was accepted. All these set of primers are available on the Leiden Muscular Dystrophy data pages, a DMD database website: http://www.dmd.nl web site [42] and all these sets of primers have been found suitable for detection of mutations in DMD gene by many scientists. At least one of these exons are reported missing in >95% of deletions [43].

The two types of polymorphisms that are useful for mutation analysis are CpG dinucleotide [44] (resulting from Base substitution) and $(CA)_n$ polymorphism [45,46]. The $(CA)_n$ repeats were the first ideal 5' polymorphic markers described for this region of the dystrophin gene [45].

An update on the Diagnostic strategies:

A sect of Indian scientist, opine that with the availability of genetic analysis, the first choice of investigation in DMD should be genetic studies and muscle biopsy should be considered when genetic tests are negative or unavailable [29].

As the primer sets for mPCR were formulated, Beggs and Kunkel (1990) were among the first to suggest a molecular diagnostic protocol in the form of flowchart [41].

Since the mPCR analysis proved to be a sensitive (detecting almost 98% of deletions), rapid and reliable method in establishing the deletions in the gene, for all populations in general [47,30,13] initially, scientists relied exclusively on the mPCR for developing a diagnostic test for DMD. But currently several reports claim modification in the basic mPCR protocol increasing the efficiency of mutations detection, which have been summarized in table 4.

Table 4: Modifications in the MultiplexPCR protocol :

	Modified mPCR protocols	**Observations**
Japanese	quantification using conventional duplex PCR and real-time PCR	Preimplantation genetic diagnosis coupled with gender determination proved a better option for carriers of duplication mutation [48]
Canadian	Modified quantitative mPCR assay designed to detect deletions/duplications in all exons of the gene and the brain promoter followed by direct sequencing of the coding region and intron/exon boundaries	Detected pathogenic mutations in 98% (106/108) total patients [49]
Netherlands population	'Alternate Protocol': a modification of the Basic Protocol for radioactive detection of duplications in males and deletions in carrier females.	Efficient for detection of duplications and deletions in carriers [43]
Indian	Quantitative mPCR	It was found better suited for carrier diagnosis in the female relatives of BMD/DMD patients with identifiable deletions [50]

Several other methods and strategies have been devised to include detection of the small deletions or insertions, splicing mutations that account for ~30%-35% [46] of DMD mutations so as to improve the sensitivity of the diagnostic test (table 5).

However, many of these techniques have limitations. For example, multiplex amplification probe hybridization (MAPH), though simple and effective, requires more input DNA and is technically laborious procedure. FISH, CA-repeat marker analysis and exon specific quantitative PCR are valuable tools to confirm known rearrangements in carriers but are not effective to screen patients directly.

Further, methods such as detection of virtually all mutations- SSCP (DOVAM-S), and SCAIP are time-consuming, laborious, and do not detect duplications accurately. Also, carrier testing in females is often difficult when a related affected male is unavailable [54]. Similarly, PTT technique for carrier diagnosis too, has practical limitation.

Table 5: Diagnostic Strategies in various populations :

Population	Diagnostic Protocol	Discussion
Indian	High resolution NMR based analysis of serum lipids of the DMD patients	Ratio of concentration of free-cholesterol to cholesterol-esters was significantly higher in DMD as compared to healthy subjects. This data could be of diagnostic importance for DMD, especially in when genetic diagnosis fails [51]
American	Multiplex ligation-dependent probe amplification (MLPA) followed by Single condition amplification/internal primer sequencing (SCAIP)	Deletions/duplications which are not detected by MLPA undergo SCAIP analysis for detection of point mutations, thus more sensitive [52]
Chinese	MLPA combined with mPCR and/or short tandem repeat-based linkage analysis	MLPA detected 10 mutations missed by mPCR. The protocol diagnosed 70-80% of all referred cases [53]
American	high-resolution comparative genomic hybridization plus microarray-based	Most sensitive and rapid method of diagnosis [54]
European	fluorescence multiplex quantitative PCR followed by Conformation sensitive capillary electrophoresis	Applicable to any large size gene, especially with heterogeneous, unknown mutations [55]
Italian	Log-PCR	Noninvasive, sensitive, cost-effective protocol, detecting up to 85% of total gene mutations. Assay time estimated is 6 hours [56]
Swedish	Interphase fluorescence in situ hybridization (FISH) analysis on single nuclei from blastomeres for detecting deletions	Detects carrier embryos along with affected and unaffected embryos [57]
Netherlands population	semiautomated denaturing gradient gel electrophoresis scan along with PCR spanning 95 amplicons	Subtle changes within the coding and splice site, carriers without large deletions or duplications and 15 unique mutations were detected [58]
Multicentric	Denaturing high performance gel	86 amplicons of dystrophin gene were

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[American and Netherlands population]	electrophoresis and direct sequencing	screened [59]
Indian	Single strand Conformation Analysis and Heteroduplex Analysis followed by DNA sequencing	Of the 50 clinically confirmed unrelated non-deletional D/BMD patients 3 were observed and confirmed for the point mutations [46]
French	protein truncation test (PTT)	Detects of the disease-causing mutations in more than 90% of the patients with a significantly higher efficiency than DNA-based strategies. Identifies mutations in non deletion sporadic cases [60]

PTT is much difficult to implement and RNA sample from a muscle biopsy (preferably) is not always available. Additionally, PTT on lymphocytes RNA though possible is difficult to perform [60].

Moreover, even though most of these methods increase the sensitivity, have economic constrains preventing their application in routine diagnosis. A UK based report, evaluated economic feasibility of some molecular tests for DMD such as reverse transcription-polymerase chain reaction (RT-PCR) and a protein truncation test to determine point mutations. Using mathematical model to estimate costs and clinical benefits, this study found these tests to be expensive [61].

A recent study (2010), in DMD/BMD patients from India showed MLPA as a useful tool for verification of absence of deletions and duplications in all 79 exons claiming that the extent of the deletions and duplications could be more accurately defined by MLPA [62]. Another recent study (2011) put forth an algorithm of mPCR and MLPA based diagnosis for the Indian population, which was less invasive and cost-effective. This conclusion was based on retrospective and prospective analysis performed on 150 male patients [63]. Nevertheless, the economic viability of many of the above discussed protocols for the Indian population is yet to be evaluated.

Conclusion :

It is well evident from the mutation studies of the dystrophin gene, that deletions are the most common of all the mutations. Yet searching for deletions alone does not ensure a foolproof diagnosis of DMD. Hence several countries, based on their type of mutational propensity for DMD, have now devised their owe strategies and protocols for routine diagnosis of DMD. Multiplex PCR identifies majority of mutations and is relatively economical for most populations. In India too, development of an integrated and comprehensive strategy consisting of mPCR and several other methods, for the routine diagnosis of DMD is the need of the hour.

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