ORIGINAL ARTICLE

The Spectrum of Dystrophin Gene Mutations in Duchene Muscular Dystrophy Patients of South-Western Maharashtra in India

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Abstract:

Background: Duchenne Muscular Dystrophy (DMD) is the most common neuromuscular disease of childhood caused by deletion or point mutations in the dystrophin gene. Though the importance of deletion mutations in the dystrophin gene causing DMD have been reported worldwide, no data are available from rural population of Maharashtra. *Objectives:* This study specifically aimed at the investigation of deletion mutations in the DMD gene from the patients from South-Western Maharashtra. *Material & Methods:* Fifty patients with clinically diagnosed DMD were analyzed to screen for intragenic deletions in 21 exons within the DMD gene using the multiplex polymerase chain reaction. *Results:* Amongst the 50 unrelated DMD patients from South-Western Maharashrra we found DMD gene deletions in 47 cases which represent 94 % mutations in DMD patients. Majority of the deletions (85.10%) were located at distal hot spot region that encompasses exons 42-53 and 10.63% of the deletions were located at the proximal hot spot region (exons 2-19). Exons 50, 51, 52 and 53 are most frequently deleted. *Conclusion:* It is important to note that we could be the first to search for the most frequent deletions in the exons of DMD gene in the rural areas of Maharashtra with the help of molecular biology tools.

Keywords: Dystrophin, Duchenne Muscular Dystrophy, Multiplex Polymerase Chain Reaction

Introduction:

Duchenne muscular dystrophy (DMD) is one of the most common genetic muscular dystrophies, with an estimated world-wide prevalence of approximately 1 in 3,500 males [1]. In a typically affected male clinical symptoms of DMD appear around the age of 3 years with progressive failure of muscle growth and wasting, leading to weakness, paralysis and respiratory difficulties. The patient is nonambulatory by the age of 9 or 10 years and usually dies by 20 years of age following cardiac or respiratory complications. DMD is a X-linked recessive disorder, predominantly affecting males and very rarely females [2]. It is the most severe of the dystrophinopathies, and results from mutations in the dystrophin (DMD) gene. The DMD gene consists of 79 exons on short arm of X-chromosome. It has four promoters and is the largest known human gene. The DMD gene encodes for dystrophin protein which is a membrane-associated protein present in muscle cells. In DMD, the deletions result in disruption of

the translational reading frame which prevents the production of a stable dystrophin protein which results in progressive loss of muscle function which results in wheel chair dependence from the age of about 7 years and death around 20 years of age. Large deletions are the most common type of mutations in the DMD gene. Sometimes small insertions, deletions, point mutations, nonsense mutations as well as missense mutations and splicing defects are also responsible for this dystrophy [3]. Approximately 5% of mutations are duplications of large segments of the gene [4] while the rest 35% mutations are point mutations. Partial intragenic deletions account for up to 60% of all cases of this disorder [5].

Various studies have been carried out on DMD at the international level which includes studies in the Estonia where children born and diagnosed between 1977 and 1999 have shown an incidence rate of 11.9 x 10^5 in live born males and point prevalence of 12.7 x 10^5 of the under twenty male population [6]. In a study of the Slovene population the prevalence and cumulative incidence has been estimated to be 2.7 per 100000 and 13.8 per 100,000 respectively [7]. Another study done in the Netherlands has estimated the prevalence rate at birth to be 23.7 x 10^5 male live births yearly [8]. A Japanese study has revealed that the proportion of sporadic cases are 1/3, with the prevalence rate among males 6.7 x 10^5 [9].

A few studies on the molecular and genetic aspects of DMD have been conducted in some parts of India. These studies have used the molecular tools for demonstrating the deletions which are nearly 60% of DMD patients, mainly in the central part of the DMD gene [10-13]. About 79% of these deletions are located in the hot-spot region, between the exons 42-53. Various methods are being used for mutation detection in DMD patients which include multiplex PCR for determining deletions in a set of exons within the deletion 'hotspot' clusters at the 5' end and in the centre of the DMD gene [14-15]. Because of its simplicity, rapidity, reduced cost, and its nonradioactive approach, this technique has proved to be very practical for molecular diagnosis of this disorder in both affected males and female carriers of DMD rearrangements in cases where the disease-causing mutation in the affected male is not known [16].

Although several international studies on the genetic and molecular aspects of DMD disorder have been successfully carried out, unfortunately there is paucity of data on molecular aspects such as molecular diagnostics in India. As yet no molecular diagnosis has been regularly implemented in routine use for the identification of mutations in dystrophin gene of this disorder in developing countries including India. Very few studies from different regions of India have reported the deletion mutations in dystrophin gene but no reports are available from rural areas of South-Western Maharashtra. Therefore, in present study we have aimed to characterize the mutations in DMD patients from rural areas of the districts including Satara and Kolhapur

of Western Maharashtra region. Conduction of genetic analysis at molecular levels in the rural areas will be helpful to understand the nature of mutations in the DMD disorder so that genetic counseling can be given to carrier families and specific treatments would be planned to the affected patients. Also, the findings presented in this paper will be useful for implementing the molecular diagnostics for prenatal diagnosis of genetic disorder like DMD in rural hospitals.

Material and Methods:

Patient Samples and Normal Controls:

Fifty samples (n=50) were collected from clinically confirmed DMD male patients aged 2-19 years. The patients were called for different camps organized by Krishna Institute of Medical Sciences in collaboration with Zilla Parishad Kolhapur in a year of 2014. Samples were also collected from patients of Karad region of Satara district. In addition, 50 healthy subjects were used as normal controls. Detailed physical and clinical examinations were carried out including collection of information regarding age, parental consanguinity, birth order, pedigree and reproductive histories of mothers by a pretested proforma.

Genomic DNA isolation from human blood

Two milliliter (ml) of whole blood from patients and normal age matched controls was collected in sterile purple top vacutainer after receiving informed consent. Genomic DNA extraction was carried out from the peripheral blood sample using Purelink genomic DNA extraction and purification Kit (Invitrogen, Life technologies) following the manufacturer's instructions. The quantitative and qualitative analysis of genomic DNA was carried out by Spectrophotometer and agarose gel electrophoresis.

Multiplex Polymerase chain reaction (mPCR)

DMD gene deletions were detected using multiplex PCR reactions. The PCR was first optimized to obtain all possible amplicons as described by Chamberlain et al 1988 and Begg et al 1990 [14-15]. PCR amplification was carried out in 20µL reaction mixture volume containing 1X PCR assay buffer containing 10 mM Tris HCl (pH 8.3), 1.5 mM MgCl2, 50 mM of KCl, 200 µM each dNTP, 1U of Taq DNA polymerase (Merk Millipore) and 0.2 nmole of each primer listed in (Table-1) thereafter 200 nanogram (ng) of purified DNA template of each sample was added to the reaction mixture. The PCR reaction for amplification was carried out in a Master Cycler gradient PCR machine (Eppendorf). The PCR amplification programme was performed as per the following sequence, Initial denaturation at 95ºC for 10 minutes, followed by 30 cycles of denaturation at 95ºC for 1 minute, annealing at 58ºC for 30 seconds, and extension at 72ºC for 1 minute following final extension at 72ºC for 10 minutes. Thereafter the amplification products were analyzed by 2.0% agarose gel electrophoresis in 1X TAE buffer. The gel was stained with ethidium bromide (10mg/ml) and visualized under UV transilluminator and photographed in gel documentation system (Bio-

Rad Laboratories). A total of 21 exons were analyzed, five with multiplex set-1, five with multiplex set-2, six with multiplex set-3 and five with multiplex set-4.

Table 1: Primers Used for Detection of Exon Deletions of DMD Gene by Multiplex PCR

Group 1		
DMDEX03	[FP]5-tcatccatcatcttcggcagattaa-3'/ [RP]5'-caggcggtagagtatgccaaatgaaaatca-3'	410
DMDEX43	[FP] 5'- tgcaacaccatttgctacc-3'/ [RP] 5'- atcatttctgcaagtatcaag-3'	357
DMDEX43	[FP] 5'-caccaaatggattaagatgttcatgaat/[RP] 5'-tctctctcacccagtcatcacttcatag-3'	271
DMDEX06	[FP] 5'-tggttcttgctcaaggaatg-3' / [RP] 5'-tggggaaaaatatgtcatcag-3'	200
DMDEX60	[FP] 5'-taaatattctcatcttccaatttgc-3'/ [RP] 5'-ttactgtaacaaaggacaacaatg-3'	139
Group 2		
DMDEX47	[FP]5'tgatagactaatcaatagaagcaaagac-3'[RP] 5'acaaaacaaaacaacaatccacatacc-3'	181
DMDEX52	[FP]5'-gtgttttggctggtctcaca-3' / [RP] 5'-catgcatcttgctttgtgtgt-3'	113
DMDEX44	[FP] 5'-gttacttgaaactaaactctgcaaatg-3'/ [RP]5'-acaacaacagtcaaaagtaatttccatc-3'	268
DMDEX48	[FP] 5'-ttgaatacattggttaaatcccaacatg-3'/[RP] 5'-cctgaataaagtcttccttaccacac-3'	506
DMDEX51	[FP] 5'-gaaattggctctttagcttgtgtttc-3' / [RP] 5'-ggagagtaaagtgattggtggaaaatc-3'	388
Group 3		
DMDEX04	[FP] 5'-ttgtcggtctctctgctggtcagtg-3'/ [RP] 5'-ccaaagccctcactcaaac-3'	196
DMDEX08	[FP] 5'-tcgtcttcctttaactttg-3' / [RP] 5'-tcttgaatagtagctgtcc-3'	360
DMDEX19	[FP] 5'-gatggcaaaagtgttgagaaaaagtc-3 / [RP] 5'-ttctaccacatcccattttcttcca-3'	459
DMDEX12	[FP] 5'-ctttcaaagaggtcataatagg-3' / [RP] 5'-catctgtgttactgtgtatagg-3'	331
DMDEX17	[FP] 5'-ttttcctttgccactccaag-3' / [RP] 5'-caccaccaacaaaactgctg-3'	416
DMDEX45	[FP] 5'-ttctttgccagtacaactgc-3'/ [RP] 5'-tctgctaaaatgttttcattcc-3'	547
Group 4		
DMDEX34	[FP] 5'-cagaaatataaaagttccaaataagtg-3'/ [RP] 5'-catgttaatacttccttacaaaatc-3'	155
DMDEX49	[FP]5'-gtgcccttatgtaccaggcagaaattg-3'/[RP] 5'-gcaatgactcgttaatagccttaagatc-3'	439
DMDEX42	[FP] 5'-atggaggaggtttcactgtt-3' / [RP] 5'-ccatgtgaaagtcaaaatgc-3'	102
DMDEX46	[FP] 5'-ccagtttgcattaacaaatagtttgag-3'/ [RP] 5'-agggttaagaagaaataaagttgtgag-3'	139
DMDEX53	[FP] 5'-tcctccagactagcatttac-3' / [RP] 5'-ttagcctgggtgacagtg-3'	212
PCR Control		
Globin	[FP] 5'- tccaactcctaagccagtgc-3' / [RP] 5'-cgatcctgagacttccacactg-3'	804

Statistical Analysis:

Statistical analyses were performed using SPSS version 11 for Windows. Baseline patient characteristics were reported using mean ± SD for continuous variables according to their distribution. A comparative analysis was made using the two tailed student t-test and Chi-square test for comparing the differences in the deletions in different groups. Statistical significance was considered at $P< 0.05$.

Ethics and biosafety:

The study protocol was approved by Institutional Ethics and Biosafety Committee of Krishna Institute of Medical Sciences for the use of human subjects in research. Informed consent was obtained before collecting the blood samples and confidentiality of results was maintained.

Results:

Molecular analysis of DMD gene mutations was carried out by the mPCR to identify the pattern of exon deletions in the dystrophin gene from DNA samples from 50 DMD patients. Out of 46 unrelated patients of DMD, thirty four (68%) belonged to Hindu religion and sixteen (32%) to Muslim religion. The mPCR procedure targeting 21 exons was first standardized and then subsequently tested on test samples and normal controls. In first reaction mixture the primers for multiplex set-1, whereas in second reaction the primers for multiplex set-2, in the third reaction mixture multiplex set-3 primers and in fourth reaction mixture multiplex set-4 primers (Table -2) along with common internal control primers were added in the reaction as an indicator of status of PCR reaction which produced 804 bp fragment. A total of 47 (94.3%) cases showed intragenic deletions in the dystrophin gene and the localization of these deletions. All deletions were found clustered in the two deletion-prone regions including distal and proximal end of the promoter of the dystrophin gene and exon deletions were observed in 47 out of 50 patient samples, whereas the remaining three samples did not show any deletions for all of the four multiplex sets tested.

Table 2: Multiplex Sets Optimized for Exon Amplifications with Corresponding Fragment Size (Bp) for Differentiation on 2% Agarose Gel

Multiplex set						Total no. of exons
Multiplex Set-1						
Exon	3,	43,	50,	6,	60	5
bp	410	357	271	200	139	
Multiplex Set-2						
Exon	48,	51,	44,	47,	52	5
bp	506	388	268	181	113	
Multiplex Set-3						
Exon	4,	8,	12,	17,	19, 45	6
bp	196	360	331	416	459 547	
Multiplex Set-4						
Exon	34,	42,	46,	49,	53	
bp	155	102	139	439	212	5

Among the 47 cases, majority of deletions (72.34%) were located at the distal hot spot region that encompasses exons 45-53 where as 14.30% of the deletions were located at the proximal hot spot region (exons 2-19) and one patient mutation covered both hot spots including exon 6 region to exon 60. It is interesting to note that deletions in South –Western Maharashtrian patients are most frequent in the region from exon 50 to exon 53.

The most frequent exon deletion in overall deletions were 52 (25.53%), followed by 51 & 53 (17.02%), and 50 (12.76% (Table-3). Single exon deleted were (exon 2-19) in 9 patients (18.00%), and in 38 patients (76.00 %) multiple exonic deletions were seen where as 3 patients (6.00%) did not show exon deletions.

The distribution of deletions in the samples is summarized in the Fig. 1(A-D). The mean age of onset was 1.72 + 0.69 years (range 0.8-3 years) and the mean age at presentation was 6.24 + 176 (range 4-16 years). All boys presented with progressive proximal muscle weakness particularly of the lower limbs and the majority (75.3%) complained of muscle pseudohypertrophy. Thus the number of exons deleted in patients varied from each other except two who pairs of brothers from two different Jain families showed similar pattern of the deletions.

Table 3: Frequency Distribution of Deletions in 50 Patients Suffering From DMD with Most Common Deletions

Exon No.	Percent Frequency of Each Deletion
52	25.53
53	17.02
51	17.02
50	12.76
49	08.51
48	08.51
47	06.38
45	06.38
46	04.25
42	04.25
34	04.25
60	04.25
44	02.12
43	02.12
19	02.12
17	02.12
12	02.12
8	02.12
6	02.12
4	02.12
3	02.12

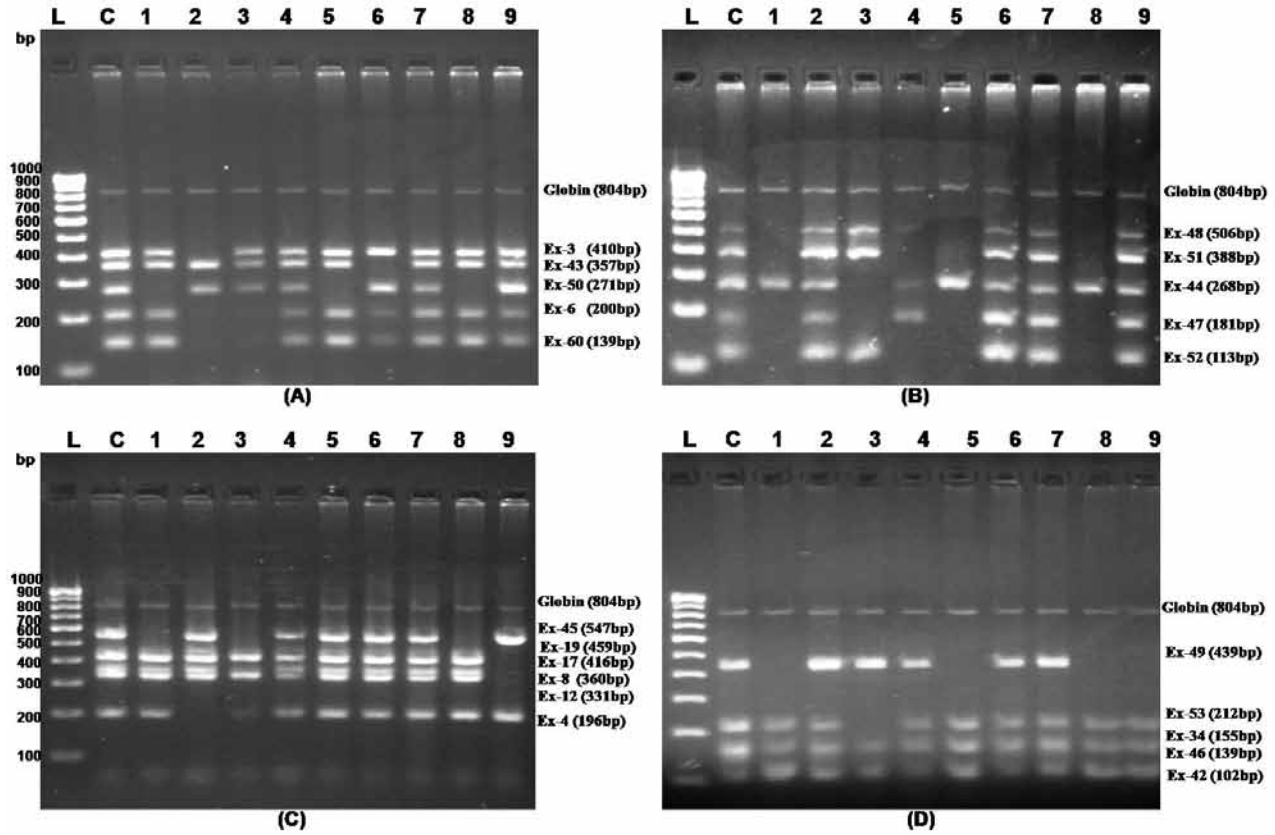

Fig.1: Representative Ethidium Bromide Stained Agarose Gel Images showing Multiplex PCR Detecting Deletion Mutations of 21 Hotspot exons of DMD Gene Grouped in Four Multiplex Sets including (A) Exon (Ex)-3, Ex-43, Ex-50, Ex-6, Ex-60 (B) Ex-48, Ex-51, Ex-44, Ex-47, Ex-52 (C) Ex-45, Ex-19, Ex-17, Ex-8, Ex-12, Ex-4 and (D) Ex-49, Ex-53, Ex-34, Ex-46, Ex-42 from the Patients of South-Western Maharashtra using a Set of Primers. The Numbers at the Right Indicate the Amplified exons with Size in Base Pair. Lane L- 100 bp DNA Ladder, Lane C- Sample from Normal Control, Lanes 1-9 showing PCR Product as an Indication of Presence or Absence of exon Deletion in Different Patients. Internal Control (Globin) of Fragment Size 804 bp was Included in Each Reaction to Ensure the Effective Amplification.

Discussion:

The importance of deletion mutations in the dystrophin gene causing DMD have been reported worldwide [17-20]. The frequency of intragenic deletions varies in different populations. In American studies intragenic deletions are reported in 55-70% of cases [22], whereas in Asian and European studies much lower frequency of deletions were observed. In Europe, several regions of abrupt changes in gene frequencies have been identified, most coincident with linguistic boundaries [22]. The most common deletions coincided with Egyptian DMD patient group [23]. The deletion frequency in Indian population was reported to be much higher than the American and European population [24]. The proportion of deletions in different populations of India shows a wide variation in the distribution. The reported frequency of deletion in various parts of India ranges from 62 % to 74 % [25-26]. In the present study the rate of intragenic deletions in dystrophin genes is very high (94.3%). Total of 73% of deletions in DMD patients was observed in North Indian population [10]. The percentage of cases having large intragenic deletions in the dystrophin gene in Eastern India is 65.7% [13] which is slightly lower than that reported in the North India [10]. The prevalence of DMD in South Indian population is less compared to North Indian population [27-28]. There is a single study from Maharashtra addressing mutation analysis of DMD gene [29]. Studies on the molecular and genetic aspects of DMD conducted in Northern and Eastern parts of India used the multiplex PCR protocols for demonstrating the deletions mainly in the central part of the DMD gene [10-12]. These studies focus mainly on establishing multiplex PCR method for the detection of deletion mutations and to a lesser extent, their distribution in the 'hotspot' regions of the DMD gene.

In this study, we have successfully applied mPCR system for the detection of twenty one deletions in hot spots region of DMD gene commonly found in Indian population with the help of four groups of primers (Table-1). Amongst these twenty one deletions five common deletions showed prevalence in the patients of DMD and the remaining mutations were probably infrequent to be seen among the limited sample size. The most common deletions found in this study were common with those of [10, 13, 28-29] but differed from those reported by other researchers [6, 17-20]. The most prevalent mutation identified in all studies is Ex-53 showed highest prevalence whereas (6-60) mutation had the lowest level among all heterozygous DMD exon deletions were in this study. The distribution of most appropriate DMD exon deletion profiles also are widely studied at regional level and it is postulated that the majority of mutations originate from major states of South, North and East regions [10, 13, 27-28]. It is seen that distribution of deletion in the dystrophin gene varies from population to population. In various parts of Indian subcontinent the occurrence of most of the deletions (~79%) is seen in the central hot spot

region (exon 44-53) [24] and 18% at the proximal of hot spot region in the eastern parts of India [13]. It is well seen that 73% of deletions occur in the central hot spot region of DMD gene in the Southern parts of India [27-28]. In this study we have seen much lower rate of deletions in the hot spot region of 25.5%.

Due to lack of data on the molecular aspects of DMD in the Western region of India, this study has been undertaken on molecular analysis of the DMD gene with special reference to deletion mutations. Thus the data on molecular genetic testing from the population of low income community of Western Maharashtra regions provide a specific exon deletion profile of DMD gene from South-Western Maharashtra region. The outcomes derived from the basic data collected in the present study would provide a platform on which future health care planning for the prevention DMD in rural Maharashtrian communities and sub-populations can be undertaken. This study will further be extended to carrier and prenatal analysis of female relatives of DMD patients. Also a baseline database of these diagnosed patients and families will be established with an intension to identify the primary support services to be provided to the families carrying DMD disorder.

Conclusion:

This study provides the pattern of dystrophin gene mutations from rural population which will open a new avenue for implementation of molecular diagnostics for prenatal diagnosis and prevention of genetic disorder by proper counseling in rural areas of Maharashtra.

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