

# Utility of MLPA in mutation analysis and carrier detection for Duchenne muscular dystrophy

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**CONTEXT:** Multiplex ligation probe amplification (MLPA) is a new technique to identify deletions and duplications and can evaluate all 79 exons in dystrophin gene in patients with Duchenne muscular dystrophy (DMD). Being semi-quantitative, MLPA is also effective in detecting duplications and carrier testing of females; both of which cannot be done using multiplex PCR. It has found applications in diagnostics of many genetic disorders.

**AIM:** To study the utility of MLPA in diagnosis and carrier detection for DMD.

**MATERIALS AND METHODS:** Mutation analysis and carrier detection was done by multiplex PCR and MLPA and the results were compared.

**RESULTS AND CONCLUSIONS:** We present data showing utility of MLPA in identifying mutations in cases with DMD/BMD. In the present study using MLPA, we identified mutations in additional 5.6% cases of DMD in whom multiplex PCR was not able to detect intragenic deletions. In addition, MLPA also correctly confirmed carrier status of two obligate carriers and revealed carrier status in 6 of 8 mothers of sporadic cases.

**Key words:** Dystrophin, Duchenne and Becker muscular dystrophies, multiplex ligation-dependent probe amplification, carrier detection

dystrophies, with an estimated world-wide prevalence of approximately 1 in 3,500 males.<sup>[1]</sup> It is the most severe of the dystrophinopathies, and results from mutations in the *DMD* gene. The *DMD* gene consists of 79 exons, and spans ~2.4 Mb on short arm of X-chromosome. It has at least four promoters and is the largest known human gene.<sup>[2]</sup> The coding portion of the gene is however only about 14 kb long. It codes for the protein dystrophin, which is a membrane-associated protein present in muscle cells. Large deletions are the most common type of mutations in the *DMD* gene, accounting for up to ~65% of DMD cases, while duplications contribute to around 6-10% of the cases. Small insertions, deletions, point mutations and splicing defects, account for the remaining 25-30% of DMD cases.<sup>[3]</sup> Among these sub-exonic mutation sequences, nonsense mutations account for 34%, frameshifts for 33%, splice site mutations for 29%, and missense mutations for 4%.<sup>[4]</sup>

The multiplex PCR techniques described by Chamberlain<sup>[5]</sup> and Beggs<sup>[6]</sup> has been used extensively to detect deletions in DMD. However, the disadvantage with this method is that it only detects deletions in 20-30 out of 79 exons of *DMD* gene. The remaining deletions as well as any duplications cannot be detected by this method. Further, it cannot be used for carrier analysis in female relatives. In the past, Southern blotting, quantitative PCR, and multiplex amplifiable probe hybridization (MAPH) have been used to find out duplications in males and detection of female carriers,<sup>[7-10]</sup> but these approaches has limited success in clinical practice. Recently, a new method, Mutiplex Ligation dependent Probe Amplification (MLPA), based on probe hybridization and amplification of target DNA,

## Introduction

Duchenne muscular dystrophy (DMD; OMIM # 310200) is one of the most common genetic muscular

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has been used for detection of deletions/duplications in DMD gene.<sup>[11]</sup> There are a number of studies, including two from India, emphasizing the utility of MLPA over other methods of mutation detection for DMD.<sup>[12-15]</sup> However, there are very few studies worldwide,<sup>[16,17]</sup> and none from India, regarding the utility of MLPA in carrier detection for female relatives of DMD patients. Here, we present the data regarding the utility of MLPA for both mutation detection in patients and carrier detection in families with DMD.

## Materials and Methods

The study was conducted at a tertiary care hospital in India. The data of all patients referred between July 2009 and March 2011, with a clinical diagnosis of BMD or DMD and a raised level of creatinine phosphokinase, was analyzed. Carrier testing was done in female relatives after detection of mutation in the proband. All blood samples were obtained after taking informed consent from the patients/relatives.

### Molecular analysis

DNA was isolated from leucocytes using standard salting-out protocols and quality and quantity of DNA was checked by nano-drop spectrophotometer. Multiplex PCR using twenty one primers belonging to proximal and distal hot-spot regions was carried out and patients who did not show any deletion by this method were considered for the study.

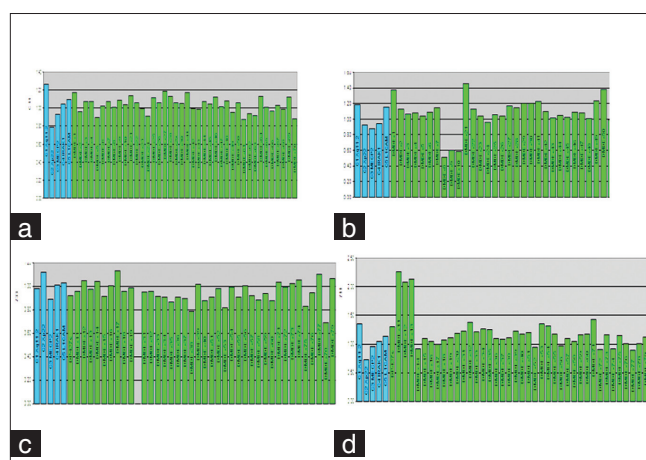
MLPA analysis was carried out according to the manufacturer's instructions (MRC Holland, Amsterdam, Netherlands). Briefly, 200–500 ng DNA was denatured and hybridized overnight at 60°C with the SALSA probe mix P034 (DMD exons 1–10, 21–30, 41–50 and 61–70) and P035 (DMD exons 11–20, 31–40, 51–60 and 71–79). Samples were then treated with DNA Ligase for 15 min at 54°C. The reaction was stopped by incubation at 98°C for 5 min. Finally, PCR amplification was carried out with the specific fluorescent labelled PCR primers. Amplification products were electrophoresed on an ABI PRISM 3100 Genetic Analyzer. The data obtained was analyzed by spreadsheet P034 and P035 provided by National Genetic Reference Laboratory Manchester. Patient

result was considered as deleted if odd ratio was  $<1:19$  and duplicated if  $>19:1$ . Carrier result was considered as heterozygous deletion if dosage quotient was 0.35 to 0.65 and duplicated if dosage quotient was between 1.35 to 1.65 [Figure 1].

## Results

A total of 217 DMD cases were investigated for dystrophin gene mutation by multiplex PCR. Deletion mutations were identified in 161 (74.2%) cases by multiplex PCR and 56 cases (25.8%) did not show any deletion. Out of these 56 patients, five patients were not investigated further due to poor DNA quality. The remaining 51 cases were investigated by MLPA. MLPA helped in identification of deletions/duplications in 12 cases (23.5%) [Figure 1]. The deletions and duplications detected by MLPA were checked by reading-frame checker ver.1.9, available at the Leiden database ([www.dmd.nl](http://www.dmd.nl)) and correlated clinically with patient phenotype. The details of mutations identified are shown in Table 1. Mutations were not identified in rest 39 cases (76.5%). These cases could probably account for point mutation or some other muscular dystrophy.

A total of ten females related to probands with known DMD mutation were included for carrier detection. Two mothers were obligate carriers and eight mothers were from families with sporadic cases. MLPA analysis detected mutation in 8 females (80%) including



**Figure 1: (a) Normal MLPA peaks, (b) Heterozygous deletion in exons 8-10 in a carrier female, (c) Hemizygous deletion in exon 21, (d) Hemizygous duplication in exons 11-13**

the two obligate carriers. The details are shown in Table 2. Two mothers did not show the mutation found in the respective probands, which could probably be due to occurrence of new mutation in the proband or gonadal mosaicism in the mother. Out of these, four mothers presented for antenatal testing by MLPA. Prenatal testing using MLPA detected one fetus to be affected and three to be normal for DMD.

## Discussion

We have evaluated the clinical utility of MLPA for mutation detection, carrier identification, and antenatal diagnosis. We identified 11 patients with exonic deletions and one with exon duplication. Thus, use of MLPA resulted in identification of 12 additional cases (5.6%) with deletion/duplication, which were not detected by multiplex PCR. Most of the studies have reported detection of 5-8% additional cases by MLPA; hence, our results are in accordance with published literature.<sup>[14,15,18,19]</sup>

Clinical phenotype depends upon the exact location of mutation in gene and not upon the size of deletion or duplication. An out-of-frame or frameshift deletion, irrespective of size, is expected to produce a severe phenotype, whereas an in-frame mutation is expected to result in a milder phenotype, namely Becker muscular dystrophy. Large in-frame deletions removing up to 35 exons in the central rod domain have been described for mild BMD.<sup>[20-23]</sup> Reading-frame checker ver1.99 identified nine deletions as out-of-frame and three as in-frame deletions.<sup>[24]</sup> In one case, we found a large deletion of exon 1 to 52 resulting in DMD phenotype. Patient 2

and 6 had an in-frame deletion, but they showed DMD phenotypes. Reading-frame rule cannot be applicable for 9% cases as reported in Leiden database. There are 7% cases having in-frame deletion resulting in severe phenotype, whereas 2% having out-of-frame mutation, but resulting in BMD phenotype, as described in Leiden database. However, due to its inherent disadvantages, the reading frame rule cannot be used always to predict the phenotype.

Antenatal diagnosis using MLPA identified three fetuses to be unaffected and one to be affected. MLPA is the only method which can be used for prenatal diagnosis when the deletions/duplications are not detectable by multiplex PCR. Hence, we can provide accurate antenatal diagnosis and genetic counseling for 5.5% additional families by using MLPA for detection of mutation.

Carrier analysis is another major advantage of MLPA over conventional multiplex PCR. Out of the ten females analyzed for carrier status following detection of deletion/duplication in proband, eight females were detected as carriers for mutation by MLPA. Two females were obligate carriers. Six of eight mothers of sporadic cases of DMD were found to be carriers by MLPA. It has been observed that about two thirds of mothers of sporadic cases are carriers of mutation. Detection of carrier status is important for counselling the family regarding future pregnancies. Previously, Southern hybridization and semi-quantitative PCR based methods were used for carrier analysis but they are technically cumbersome and accuracy of results was also questionable. Now, MLPA is an easy method to detect carriers if mutation is known in the proband. Moreover, MLPA can also be used for determining positive carrier status even when

**Table 1: Mutations identified in patients by MLPA, which were not detected by multiplex PCR**

Patient number	Mutation detected in exons	In frame/Out of frame	Clinical presentation (DMD/BMD)
1	Deletion 20-22	Out of frame	DMD
2	Deletion 10-44	In frame	DMD
3	Deletion 3-7	Out of frame	DMD
4	Deletion 55	Out of frame	DMD
5	Deletion 56	Out of frame	DMD
6	Deletion 24-25	In Frame	DMD
7	Deletion 1-52	Out of frame	DMD
8	Deletion 8-10	Out of frame	DMD
9	Deletion 11	Out of frame	DMD
10	Deletion 11 (CVS)	Out of frame	DMD
11	Deletion 20-29	Out of frame	DMD
12	Duplication 11-13	In frame	BMD

**Table 2: Mutations identified in carrier females by MLPA**

Carrier no.	Obligatory carrier	MLPA result	Proband mutation	Carrier status with MLPA
C1	Yes	Del 8-12	Del 8-12	Confirmed
C2	No	No deletion	Del 47-49	Not carrier
C3	No	Del 45-47	Del 45-47	Confirmed
C4	No	Del 44-48	Del 44-48	Confirmed
C5	No	Del 8-12	Del 8-12	Confirmed
C6	No	Del 47-52	Del 47-52	Confirmed
C7	No	No deletion	Del 48-52	Not carrier
C8	No	Del 46-50	Del 46-50	Confirmed
C9	No	Del 8-9	Del 8-9	Confirmed
C10	Yes	Del 10-44	Del 10-44	Confirmed

Del- Exon deletion

the proband is deceased, although negative result cannot rule out carrier status.

Although no definitive therapy is available for DMD at this point of time, accurate detection of mutation is helpful in genetic counselling as well as prenatal diagnosis. The newer therapies like antisense oligonucleotide therapy are mutation specific and require the knowledge of mutation to select proper oligo for the patient. Therefore, MLPA can be used with confidence for identifying duplications and deletions in DMD patients as well as for carrier detection.

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