ORIGINAL RESEARCH ARTICLE

Multiplex Ligation Dependent Probe Amplification Based Mutation Analysis of Dystrophin Gene in Nepalese Patients with Duchenne Muscular Dystrophy

Kushal Shrestha[1], Smita Shrestha[2], Mr. Saroj Khatiwada[3], Bishnu Acharya[4], Sulochana Manandhar[5], Rohit Kumar Pokharel[6,7] *

[1]Central Department of Biotechnology, Tribhuvan University, Kirtipur, Nepal.
[2]Central Department of Biotechnology, Tribhuvan University, Kirtipur, Nepal.
[3]Department of Biochemistry, Modern Technical College, Lalitpur, Nepal.
[4]Muscular Dystrophy Foundation-Nepal (MDF-Nepal), Lalitpur, Nepal.
[5]Center for Molecular Dynamics-Nepal (CMDN), Nepal.
[6]Muscular Dystrophy Foundation-Nepal (MDF-Nepal), Lalitpur.
[7]Department of Orthopedics and Trauma surgery, IOM, Tribhuvan University, Kathmandu, Nepal.

Abstract

Duchenne muscular dystrophy (DMD) is X-linked recessive neuromuscular disorders caused due to mutation in dystrophin gene, leading to progressive muscle weakness. This study was done to identify mutation in dystrophin gene in Nepalese patients with DMD using Multiplex Ligation Dependent Probe Amplification (MLPA) assay in Nepal. Twenty one patients from different regions of Nepal, who were clinically diagnosed as DMD were enrolled in the study. Peripheral blood samples were collected in EDTA vials, gDNA was extracted, and deletion mutation in the dystrophin gene was analysed using Multiplex Ligation Dependent Probe Amplification (MLPA) assay.

Exon deletion mutation in the dystrophin gene was observed in 14 (66.6%) out of 21 DMD cases. The most common exon deletion was observed and confined in exon 7-14 and 45-53 of dystrophin gene. The location of deletion in dystrophin gene is apparently non-random with a preponderance found in the hot spot regions. Use of MLPA is useful in detecting copy number changes in DMD proband and suspected carriers in Nepal.

Keywords: Duchene muscular dystrophy, Multiplex ligation dependent probe amplification, Mutation, Nepal.

***Corresponding Author**
Email: pokharel.rohit@gmail.com

Introduction

Duchenne and Becker muscular dystrophies (DMD & BMD) are X-linked recessive neuromuscular disorders with incidence of 1 in 3,500 and 1 in 30,000 live male births, respectively [1]. DMD is characterized by progressive muscle weakness with onset at 3-5 years of age, leading to loss of ambulation by 10-12 years of age without treatment. Respiratory, orthopaedic, and cardiac complications emerge with age, and without intervention, the mean age at death is around 19 years [2].

Both DMD and BMD are caused by mutations in the dystrophin gene (MIM 300377), one of the largest known human genes, spanning about 2.4 Mb of genomic DNA [3]. The most common mutations in the DMD gene are the deletion or duplication of one or more exons [3]. Mutations lead to an absence of or defect in the dystrophin protein, which results in progressive muscle degeneration leading to loss of independent ambulation by the age of 13 years. Variable phenotypic expression relates mainly to the type of mutation and its effect on the production of dystrophin [4].

Muscular dystrophy is diagnosed on the basis of clinical examination, raised creatine phosphokinase (CPK) level, muscle biopsy and dystrophin gene mutation analysis. The genetic tests commonly used to identify dystrophin mutations are multiplex polymerase chain reaction (mPCR), multiplex ligation dependent probe amplification (MLPA), single condition amplification/internal primer, and multiplex amplifiable probe hybridization [5].

Mutation analysis of dystrophin gene in DMD/BMD has been performed in several countries [2, 3]. However, no such studies have been performed in Nepal. MLPA, a simple and cheaper genetic test to detect duplication/ deletion mutation, could be a better tool for genetic diagnosis of DMD in Nepalese patients; thereby assisting in timely diagnosis, treatment and management. We conducted present study among clinically diagnosed DMD patients to find the mutation pattern in dystrophin gene using MLPA and test the efficacy of MLPA in identifying mutation in DMD patients in the Nepalese context.

Materials and Methods

The study was conducted among 21 clinically diagnosed DMD patients from different regions of Nepal, who were referred to Muscular Dystrophy Foundation-Nepal (MDF-Nepal). Diagnosis of DMD was done on the basis of clinical presentations and markedly elevated serum creatine phosphokinase (CPK) levels. Proper genetic counselling was done, and consent was taken from each patient and their parents.

Peripheral blood samples (5 ml) were collected from each patient in EDTA vials and transported to laboratory of Central Department of Biotechnology, Tribhuvan University, Kirtipur maintaining cold chain. The samples were stored at -20^0 C until analysis. Genomic DNA was isolated from blood, as recommended by the MRC-Holland, by using the extraction method of the QIAamp DNA mini kit [6]. The absorbance of DNA samples were measured at 230 nm, 260 nm and 280 nm using the UV spectrophotometer to check for purity of DNA samples required for MLPA assay.

A commercial MLPA kit (MRC Holland) with probes of 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) was used to detect DMD deletion/duplication in DMD patients according to the manufacturer's recommended protocol (Amsterdam, Netherlands). 50-250 ng of genomic DNA, in a volume of 5 µL Tris-EDTA, was denatured at 98^0 C for 5 min, cooled down, and then mixed with MLPA P034 or P035 probemix. The mixture was then heated to 95^0 C for 5 min and incubated at 60^0 C overnight for probe hybridization. After 16 hours, ligation was performed with Ligase-65 enzyme at 54^0 C for 15 min and Ligase-65 enzyme was inactivated at 98^0 C for 5 min. Then, PCR amplification was performed with specific SALSA FAM PCR primers (5'-GGGTTCCCTAAGGGTTGGA-3'). After that, the mixture was separated by capillary electrophoresis and then analyzed using ABI-310 Genetic Analyzer [7].

The Genescan analysis software in the ABI-310 Genetic Analyzer analyzed the raw data to quantify the DNA fragments and determine the size of the fragments by comparing them to fragments in a size standard. The electropherograms of test samples were analyzed by comparing with the peak pattern of the male and female reference samples. The novel software Coffalyser.net was used to analyze the data obtained after the capillary electrophoresis run which was further confirmed by visual assessment by overlaying two fragment profiles and comparing the relative intensities of fragments. The relative peak ratio (RPR) of every single exon was plotted to its corresponding bar chart [8]. Absence of peaks corresponding to two or more contiguous exons was taken to represent a genuine deletion. The absence of only one peak in males, corresponding to a single exon, were also recorded which are yet to be investigated further by applying the novel methods as PCR primer flanking the exons in question or by sequencing. The framedness of the mutation in probands were also estimated by using the dystrophin exonic deletions/duplications reading frame checker 1.9 as recommended by MRC Holland. The data from the study was entered into ms excel and analyzed using SPSS version 11.0.

Results

The mean age of DMD patients at the time of study was 11.4 years (age range from 8-18 years). All the patients had very high serum CPK level (median: 6245 U/L, range: 2100-32890 U/L). The genomic DNA extracted from samples had concentration range within 27-59 µg/mL. The ratio of the optical density (OD) of DNA samples at 260 nm and 280 nm ranged from 1.5-2.0 with mean OD 1.76. Among the 21 samples of DMD analyzed by MLPA assay, deletions were observed at various exons of dystrophin gene in 14 samples. Seven samples however did not have any deletion or duplication in the exons. Thus, MLPA tool could detect mutations in about 66.6% (14 of 21 samples). The most prevalent exonic deletion regions were found to be confined in the exon 7-14 and 45-53. Deletion mutations at different exons observed in samples are shown in table 1. Similarly, electropherograms of control sample and test sample after MLPA assay with SALSA probe mix P034 and P035 are shown in **figure 1.**

As indicated by the dystrophin exonic deletions/ duplications reading frame checker 1.9, all the 14 samples with detection of deletion mutation were detected by MLPA assay have out of frame mutations suggesting that the samples were those of DMD patients. But the confirmation of the framedness is yet to be made by gene sequencing, and the 7 samples with negative MLPA result need further genetic testing like sequencing.

Table 1: Exon deletions detected by MLPA assay in DMD patients

Case	Age (years)	Serum Creatine kinase level (U/L)	Exon deletions	Phenotype
1	5	12122	48-50	DMD
2	7	3412	52	DMD
3	6	14467	10-43	DMD
4	8	1287	52	DMD
5	9	11580	45	DMD
6	5	24200	51-53	DMD
7	14	22000	45-50	DMD
8	5	21887	46-49	DMD
9	12	2700	52	DMD
10	11	2100	51	DMD
11	8	4328	12-14	DMD
12	9	3220	51	DMD
13	6	9123	45	DMD
14	7	6245	7-9	DMD

Discussion

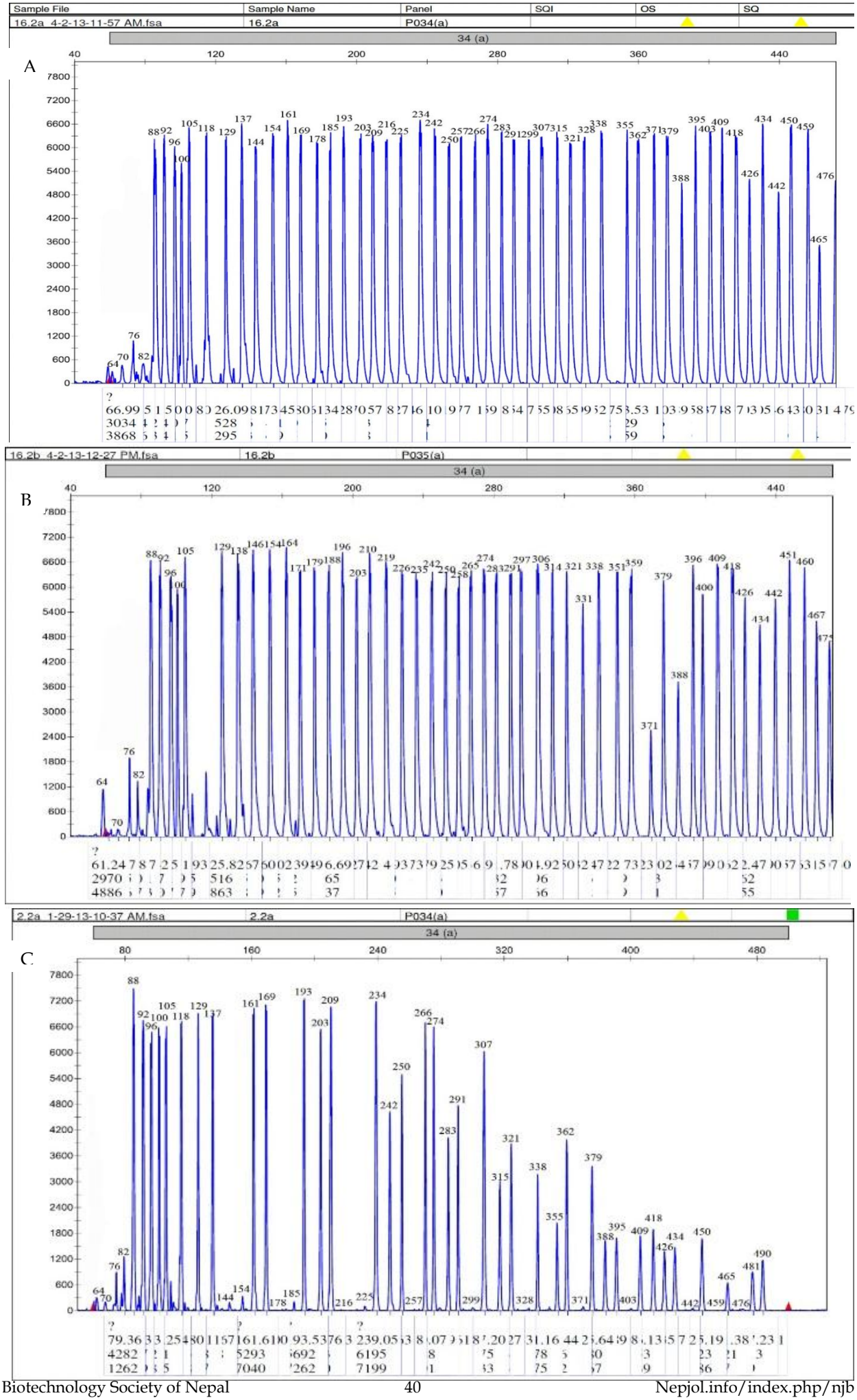
In Nepal, DMD/BMD patients are generally diagnosed late due to the lack of complete understanding of the disease, poor screening of paediatric group for muscular dystrophy and lack of genetic diagnostic facilities in the country. Most of the cases are diagnosed on the basis of history given by the parents, positive clinical signs of DMD and very high CK level in the blood. For the diagnosis at the gene level clients usually go to or the sample is send outside Nepal, which is not accessible and affordable to many of the parents. This study to detect deletion mutation in DMD using MLPA is first genetic pilot study in Nepal.

Among the 21 samples of clinically diagnosed DMD patients, deletion of dystrophin exon was found in 14 samples (66.6%). However, 7 samples (33.3%) did not show any deletion or duplication. Thus, MLPA was efficient in accurately confirming mutations in about 67% of all the cases. A number of studies have found MLPA assay to be highly sensitive diagnostic assay to identify mutation in DMD and BMD patients [9]. In the study to evaluate the efficacy of MLPA technique in comparison with the traditional multiplex PCR assay in detection of exon deletions and duplications of the DMD gene by Lai et al., MLPA was able to detect all the known deletions and duplications; it detected four additional mutations that had been missed by multiplex PCR [10]. In a study in China amongst 70 DMD/BMD patients, MLPA detected exonic deletions in 42 patients (60%), exonic duplications in 7 patients (10%) and 21 patients (30%) showed normal results [11].

In the present study, only exons deletion was observed. No exon duplication was found. We found most prevalent exonic deletion regions to be confined in the exons 7-14 and 45-53. One, 3, 4, 6 and 44 exons deletion was observed in 7, 4, 1, 1 and 1 patients respectively. No novel mutations were identified in this study. In a study in Iranian DMD/BMD patients, 30.9 % of patients had single exon deletion, while group and contiguous exon deletions were identified in 41% of the patients [12]. The most numerous exon deletions included exons 45-50, and two exons 3-5 and 41-43 duplications (1.4 %) was observed in a BMD and a DMD patient, respectively [12]. In a study in Polish DMD/BMD patients, Zimowski et al. identified 110 deletions, 22 duplication (in one patient two different duplications were detected) and 2 point mutations. Deletions involved mainly exons 45-54 and 3-21, whereas most duplication involved exons 3-18 [13]. It has been found that deletions account for approximately 60-65% of mutations and duplications for 5-10% in DMD/BMD. The remaining cases are mainly point mutations (30-35% of the cases). Although deletions encompass all 79 exons, two deletion hotspots (exons 45-55 and exons 2-19) are recognized [14].

Identification of exon deletion is important to design gene therapy by exon skipping method that is an emerging therapy for DMD that can transform DMD into BMD is based on the recovery of reading frame induced by alternative splicing of antisense oligonucleotides [15]. More patients may benefit from individual exon skipping therapies following a comprehensive understanding of the correlation between genotypes and phenotypes under the guidance of large-scale genetic epidemiological studies [16]. Similarly identifying disease at earlier stage could help in better management of patients and thus better life quality.

This was the first genetic study on DMD in Nepal. The location of deletion in dystrophin gene is apparently non-random with a preponderance found in the hot spot regions in Nepalese population as well. Use of MLPA is useful in detecting copy number changes in DMD proband and suspected carriers.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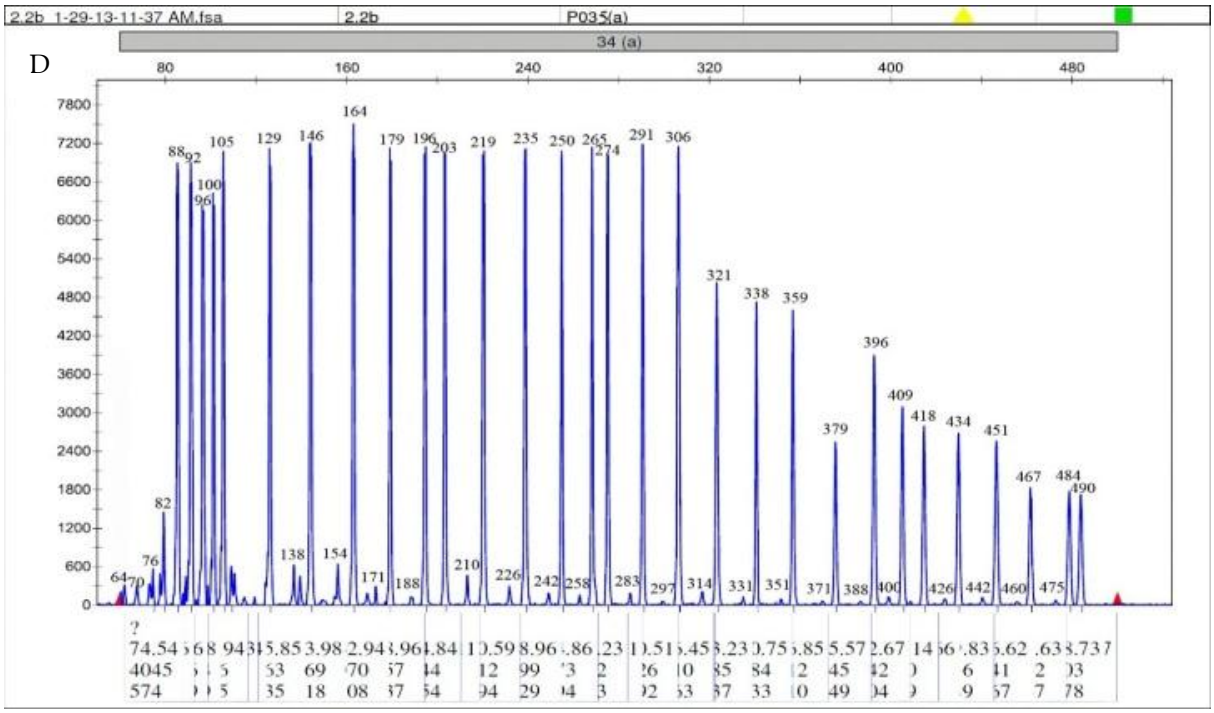

Figure 1. Electropherogram of control sample and test sample after MLPA. **Fig. 1A** and **Fig. 1B** are for normal control sample with P034 and P035 respectively. **Fig. 1C** and Fig. **1D** are for DMD patient with deletion in dystrophin gene with P034 and P035 respectively.

Competing Interest

None

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Author's Contribution

KS, SS and RKP designed the study and collected samples. KS, SS, SK, BA and SM engaged in sample collection and conducted laboratory analysis. SK performed statistical analysis and wrote the manuscript. KS, SS and RKP revised the draft. All authors read and approved the final version of the manuscript.

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