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MOLECULAR ANALYSIS OF DUCHENNE MUSCULAR DYSTROPHY CARRIERS IN GUJARATI POPULATION USING MLPA

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ABSTRACT: Duchenne muscular dystrophy (DMD) is caused by mutation in the dystrophin gene. In DMD, only male carrying the mutated DMD gene is affected while female becomes carrier of the disease because of the X-linked recessive characteristics of the disorder. About one-third of DMD cases show *de novo* mutations, while the rest are inherited through carrier others or arise from germlinemosaicism. The recognition of female carriers of the DMD gene is vital in order to know the birth of DMD cases. Multiplex Ligation-dependent Probe Amplification (MLPA) is at the most extensively used method for carrier analysis of DMD gene. This study involved analysis of carrier status of only 15 female relatives of affected males. The MLPA analysis of DMD of these cases was carried to screen whole dystrophin gene for heterozygous exon deletions and duplications. The data revealed 83 heterozygous mutations (82 deletions and one duplication), in 12 cases (80%) out of the 15 carriers analysed. Out of 82 heterozygous deletions, 28 (34.15%) deletions were found in the 5' proximal hot spot region of the DMD gene ranging from exons 1 to 20 and 54 (65.85%) deletions were present in the mid-distal hot spot region of the gene ranging from exons 40 to 55. In 3 cases (20%), no mutation was identified through MLPA and which require further analysis using direct sequencing for point mutations. This study thus would provide the identification of mutation related to deletion/duplication, for genetic counselling of DMD affected families which is the first report in Gujarati population.

Key words: MLPA, DMD disease, Carrier females, DNA analysis, Gujarati population, Mutations.

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INTRODUCTION

Duchenne muscular dystrophy (Mendelian Inheritance in Man MIM 310200) (also knowna Meryon's disease) is the most common X-linked recessive genetic disorder predominantly affecting males and very rarely females (Roberts et al., 1992) with an incidence of 1:3500 live male births (Emery, 1991). It is caused by mutations in the dystrophin gene located at the Xp21 locus. The dystrophin gene contains 79 exons (Prior et al., 2005), leading to a loss of function of the dystrophin protein. Mutations responsible for the disease are deletions or duplications and point mutations. Deletions and duplications account for 60-65% and 5-8% of cases of DMD respectively and in the remaining cases, about 30-35%, are caused by single point mutations (Takeshima et al., 2010).

The vast majority of large deletions detected in DMD gather around two mutation hot spots proximal and mid-distal regions of gene (Koenig et al., 1989; Koenig and Kunkel, 1990), although the reasons for this are uncertain. Unlike the large deletions that gather in just two regions of the dystrophin gene, small deletions and point mutations emerge to be frequently distributed throughout it (Roberts et al., 1994; Gardner et al., 1995).
In DMD males only, carrying the mutated DMD gene is affected while females become carriers of the disorder. About one-third of the DMD cases show *de novo* mutations while the restare inherited through carriermothersor arise from germline mosaicism. Most carriers of Duchenne muscular dystrophy are asymptomatic because of the protective effectof having two X chromosomes. About 5-10% of female carriers shows some degree of muscle weakness and frequently has enlarged calves so-called manifesting carriers (Almeidaet al., 2008; Sakthivel et al., 2013). Manifestingcarriers may have musculoskeletal symptoms (Norman and Harper, 1989) and/or cardiac manifestations, including cardiomyopathy (Politano et al.,1996; Hoogerwaard et al.,1999).
Multiplex PCR (mPCR) was one of the methods which was used for picking up whole exon, single and multiple deletions. Based on these observations, the screening of just 19 exons of the 79 was enough to pick mutations in approximately 65% of all DMD cases or 95-98% of all DMD gene deletions respectively (Chamberlain et al., 1988;Beggs et al., 1990). But the identification of female carriers with deletions/duplications of the DMD gene is a crucial point in order to know/prevent the birth of children affected by this disease only. Diagnosis for these carrier females follows the same ideology as for the affected males. Several biochemical and molecular methods using (CPK) levels have been used to recognize the carrier state (Panigrahiand Mittal, 2001). Multiplex ligation-dependent probe amplification (MLPA) is a variation of the multiplex polymerase chain reaction that permits amplification of multiple targets with only a single primer pair and is easy and fast.

Ever since, theoretically, about one third of the DMD mutations are ‘‘de novo’’, the risk of reappearance of the disease in families with a single affected male is connected to the carrier or non-carrier status of the mother of the patient. Hence carrier analysis is necessary to help preventing this disorder. The technique selected to test female relatives must be competent of detecting the mutation in the heterozygous state, i.e. masked by the existence of the corresponding normal allele. Of the quantitative methods available MLPA is extensively used method for carrier analysis of DMD gene. So this technique was used for carrier analysis of DMD gene for carrier study in Gujarati population.

MATERIAL AND METHODS

This study involved analysis of carrier status of only 15 female relatives of affected males. Study samples were collected from Indian Muscular Dystrophy Society (IMDS) Ahmedabad, Gujarat, India. In all of them the diagnosis was established through clinical and Physiotherapeutic examinations. This work was approved by Human Ethical Committee (HEC) of Gujarat University (GUHEC/001/15), Ahmedabad.
Proforma was filled with detailed generic information of patients like age at analysis and onset symptoms, physical attributes etc. Also written consent form was taken in duly filled in English and native language (Gujarati) from the patient’s parent/guardian for collection of blood sample. Before collection of sample, a pedigree was drawn in to comprehend the familial inheritance and to evaluate who was at a higher risk from their close relatives and to counsel them for the same. After filling the proforma with detailed information, blood samples (5 ml) were collected from carrier females in EDTA Vaccutainers of rmolecular analysis. Genomic DNA was extracted using Qiagen DNA extraction kit from peripheral blood from the carrier females. MLPA analysis was carried out by the MLPA kit according to the manufacturer’s instructions (MRC Holland, Amsterdam, Netherlands). The technique screens whole dystrophin gene for heterozygous exons deletions and duplications. The MLPA kit contains one probe for each of the 79 DMD exons of the dystrophin gene and in addition, a probe for the alternative exon 1 (Dp427c). These 80 probes have been divided in two probe mixes. PCR products were analysed on an ABI model 3130 capillary sequencer with the Gene scan software, using Genescan 500 size standards. Individual peaks corresponding to each exon were identified based on the difference in migration relative to the size standards. MLPA ratios (dosage quotient) of below 0.7 or above 1.3 are indicative of a deletion (copy number change from two toone) or duplication (copy number change from two to three), respectively (Chart:1). Carrier result was considered as heterozygous deletion if dosage quotient was 0.35 to 0.65 (Chart: 2) and duplicated if dosage quotient was between 1.35 and 1.55 (Chart: 3).

RESULTS

A total of 15 females from 10 families were included for carrier detection. Of the 15 cases, 8 were mothers, 4 maternal grandmothers, 1maternal aunt, and 2 sisters of affected males. Among the 10 families tested, 4 (40%) showed family history and 6 (60%) were sporadic cases.

MLPA analysis detected mutations in 12 (80%) females and in 3(20%) females (CF9, CF10 and CF12) no mutations were picked up. Among these 12 carrier females, 11 carriers (73.33%) having heterozygous deletions while 1 carrier (6.67%) having heterozygous duplication (Graph 1). Detailed carrier analysis report along with detailed family history of each case was depicted including mutations in index cases (Table 1).

A total of 83 heterozygous mutations (82 deletions and one duplication) in 12 cases (80%) out of the 15 cases (100%) were detected(Graph 2).Out of 82 heterozygous deletions, 28 (34.15%) deletions were found in the 5' Proximal hot spot region of the DMD gene ranging from exons 1 to 20 and 54 (65.85%) deletions were found in the mid-distal hot spot region of the DMD gene ranging from exon 40 to 55 (Graph: 3). Further a total of 24 different heterozygous exon deletions, ranging between 8 and 55, were depicted(Graph: 4).

Table 1: Complete carrier analysis results and family history details

Sr. No.	Family No.	Family History	Index Case Id	Mutations in Index case	Carrier Case Id	Carrier Status	Mutations in Carrier	Relationship to Index Case
1.	1	No	DMD24	No mutation detected by mPCR	CF1	Yes	Exon 17 duplicated	Mother
2.	2	No	DMD33	Exon 46, 47, 48,50, 51, 52 deleted	CF2	Yes	Exon 46, 47, 48, 49, 50, 51, 52, 53 deleted	Mother
3.	3	Yes	DMD13	Exon 45, 46, 47, 48,50, 51, 52 deleted	CF3	Yes	Exon 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 deleted	Mother
4.	3	No	DMD 13	Exon 45, 46, 47, 48,50, 51, 52 deleted	CF4	Yes	Exon 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 deleted	Maternal Grand-mother
5.	4	No	DMD43	Exon 46, 47, 48, 50 deleted	CF5	Yes	Exon 46, 47, 48, 49, 50 deleted	Mother
6.	5	Yes	DMD81	Exon 45, 46, 47, 48, 50, 51, 52 deleted	CF6	Yes	Exon 45, 46, 47, 48, 49, 50, 51, 52 deleted	Maternal Grand-mother
7.	5	Yes	DMD81	Exon 45, 46, 47, 48, 50, 51, 52 deleted	CF7	Yes	Exon 45, 46, 47, 48, 49, 50, 51, 52 deleted	Maternal Aunt
8.	6	No	DMD66	Exon 8, 12, 13, 17, 19 deleted	CF8	Yes	Exon 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 deleted	Mother
9.	**7**	Yes	DMD51	No mutations detected by mPCR	CF9	No*	No mutation detected by MLPA	Mother
10.	**7**	Yes	DMD51	No mutations detected by mPCR	CF10	No*	No mutation detected by MLPA	Maternal Grand-mother
11.	**8**	Yes	DMD82	Exon 51, 52	CF11	Yes	Exon 51, 52, 53, 54, 55 deleted	Mother
12.	**9**	No	-	-	CF12	No*	No mutation detected by MLPA	-
13.	**10**	Yes	-	-	CF13	Yes	Exon 8, 9, 10, 11, 12 deleted	-
14.	**10**	Yes	-	-	CF14	Yes	Exon 8, 9, 10, 11, 12 deleted	-
15.	**10**	Yes	-	-	CF15	Yes	Exon 8, 9, 10, 11, 12 deleted	-

*No mutation (deletion/duplication) found

Chart 1: MLPA ratio chart for control female of DMD gene

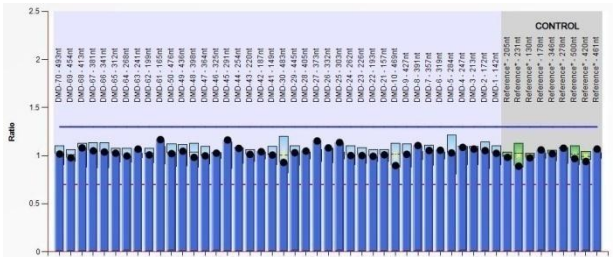


Chart1.a:
Probemix 1 PO34

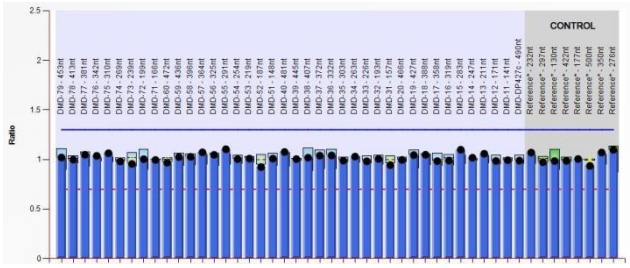


Chart 1.b:
Probemix 2 PO35

Chart 2: MLPA ratio chart for carrier CF8 of DMD gene

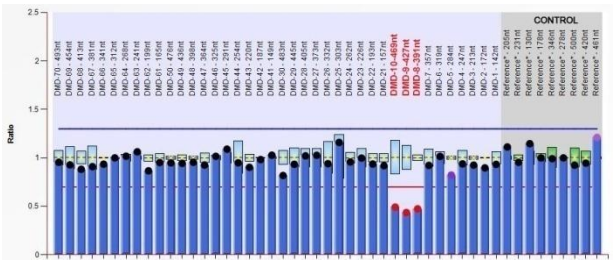


Chart: 2.a:
Sample CF8 showing heterozygous deletion of exon 8-10 inprobemix 1 PO34

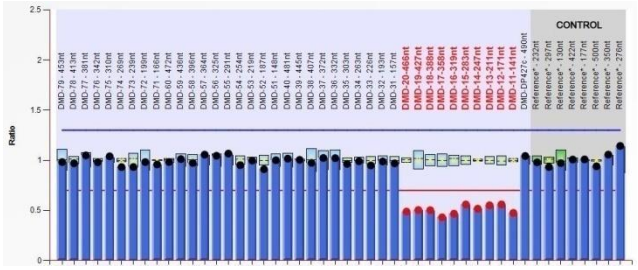


Chart 2.b:
Sample CF8 showing heterozygous deletion of exon 11-20 inprobemix 2 PO35

Chart 3: MLPA ratio chart for carrier CF1 of DMD gene

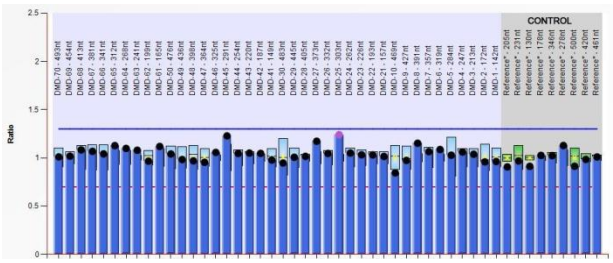


Chart 3.a:
Sample CF1 showing no mutations in probemix 1 PO34

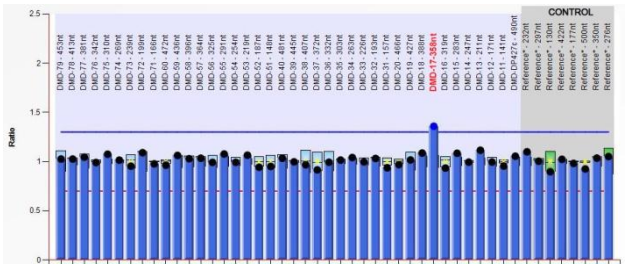
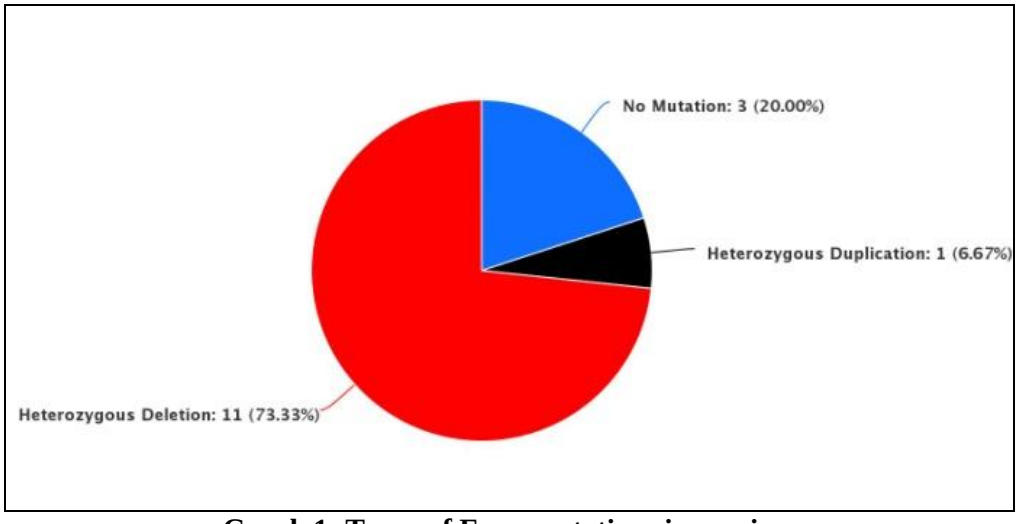
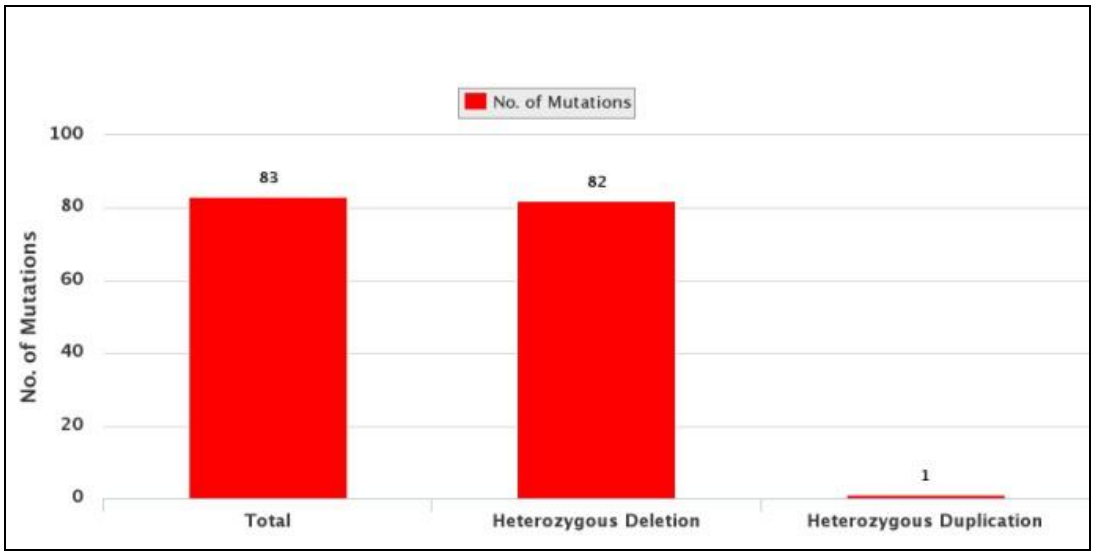


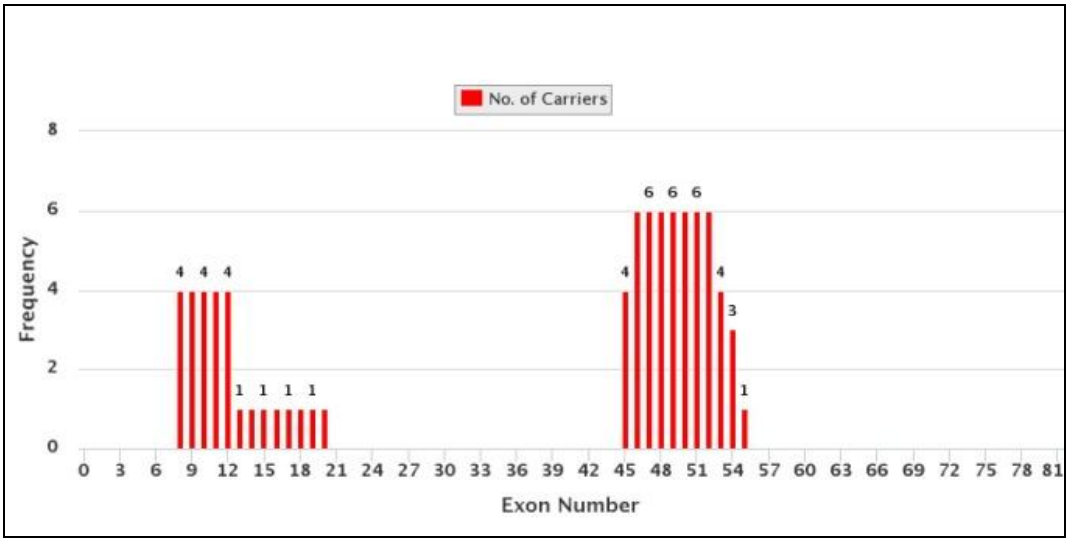
Chart 3.b:
Sample CF1 showing heterozygous duplication of exon 17 in probemix 2 PO35



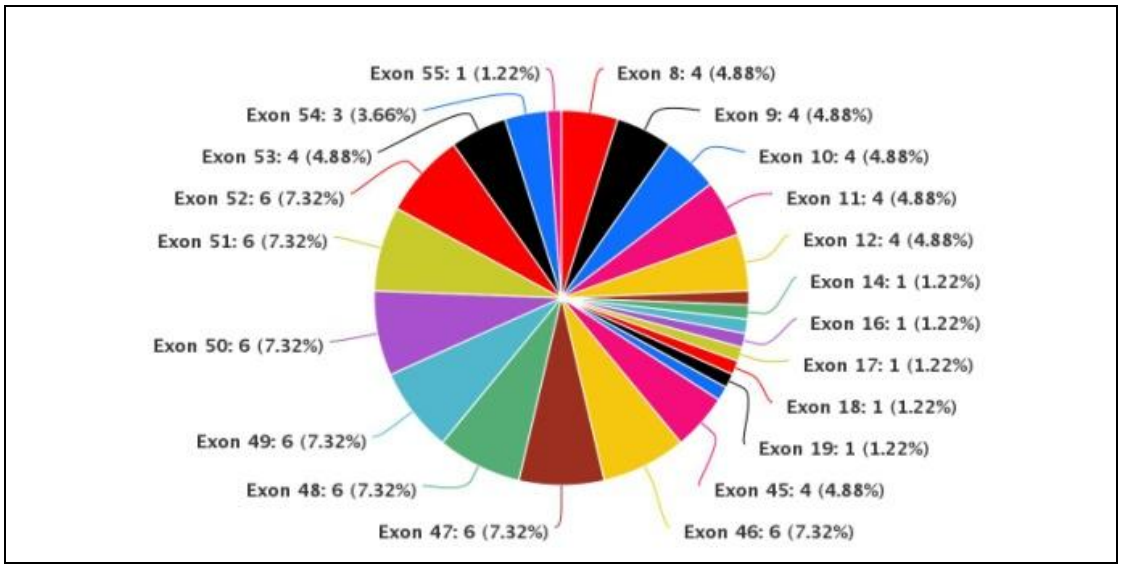
Graph 1: Types of Exon mutations in carriers



Graph 2: Bar chart representing type heterozygous mutations incareers



Graph 3: Column chart showing frequency of heterozygous exon deletions in carriers 1-79



Graph 4: Distribution of heterozygous deletions of Exons (frequency and percentage) of total deletions in carriers

DISCUSSION

Molecular testing is becoming the primary diagnostic method for DMD (Yan et al., 2004). Several methods have been devised for DMD carrier analysis by different groups with each carrying their own advantages and disadvantages. MLPA was one such method which was used for carrier analysis. As per our knowledge is concerned, we are the first to document the detection of female carriers of deletions/duplications of the DMD gene applying this MLPA approach in Gujarat.

Our study include molecular and biochemical analysis of total 101 DMD patients, 20 female carriers and 70 age and sex matched healthy controls (Rashmi, 2016). Out of this only a total of 15 females from 10 families were included for carrier detection. Among the 10 families tested, 4 (40%) showed family history, 6 (60%) were sporadic (Table:1). Thus our study is in accordance with the studies of previous authors who reported to have higher percentage of de novo mutations in DMD patients (Murugan et al., 2013; Yang et al., 2013). MLPA analysis detected mutations in 12 (80%) females while in 3(20%) females no mutations were picked up. Further we found higher mutational rates than that of other studies carried out by Pikó et al. (2009 ; 45%), Dastur et al. (2011 ; 50%), Yang et al. (2013 ; 50.75%) who studied 95, 30 and 400 female relatives of DMD patients respectively and lower than the observations was made by Gatta et al. (2005) accounting (100%) who studied 12 female relatives. However our mutational results are in accordance with the study of Verma et al. (2012) in India who reported 80% mutations in 8 carrier females from their MLPA analysis. It has also been observed that about 2/3 of the mothers of sporadic cases are carriers of mutations. We also found 2/3 carrier cases of our families showed sporadic (60%). Of these 12 carrier females, 11 carriers (73.33%) had 82 heterozygous deletions while 1 carrier (6.67%) possessed only one heterozygous duplication. Out of 82 heterozygous deletions, 28 (34.15%) deletions were found in the 5' Proximal hot spot region of the gene ranging from exons 1 to 20 and 54 (65.85%) deletions were in the mid-distal hot spot region of the gene ranging from exon 40 to 55 in accordance with above previous studies carried by MLPA analysis in female carriers (Pikó et al. 2009; Murugan et al., 2013). These mutations viz. deletions or duplications could be correlated with clinical indices of this disease in our study and is also related to percent frequency of mutations existing in each exon of the gene (Graph 4). However in 3 cases (20%), no mutation was found who had positive family history for DMD gene which could be further detected by Direct Sequencing technology.

CONCLUSION

Due to lack of data on the molecular analysis of DMD carriers in the Gujarati population, this study has been undertaken on molecular analysis of the DMD gene in carrier females with reference to deletion and duplication mutations. The outcomes derived from the data collected in this study would provide a platform for genetic counselling, management and prevention of DMD in Gujarati population. This study will further need to be extended for carrier analysis by direct sequencing to detect point mutations in those cases where this MLPA fails.

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CONFLICT OF INTEREST: None declared

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