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MOLECULAR EVALUATION OF DUCHENNE MUSCULAR DYSTROPHY PATIENTS IN GUJARATI POPULATION

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ABSTRACT
Duchenne muscular dystrophy (DMD) is an X linked recessive disorder affecting mostly males with an incidence of 1 in 3600 liveborn boys. The gene for Dystrophin protein is located on X p 21 in males. In this study, 19 clinically diagnosed patients had CK level evaluated and were tested for exon deletions using multiplex PCR. Of the 19 clinically suspected DMD patients, the diagnosis of DMD was confirmed by CK value and/or genetics in 16 patients. The mean age of onset was 5.33 years and the mean age of presentation was 11 years. Delayed motor milestones were present in 17/19 patients (89%) patients. The mean CK value was 11,417 U/l. Of 19 patients, 16 cases showed deletion in at least one exon. Single exon deletion was found in 9 (47%) patients. Distal hotspot Exons 50, 48, 47 and 51 were the commonly deleted exon types and the deletion rates were 17.95%, 15.38%, 12.82% and 10.26% respectively. In this study population in Gujarat, India the deletion rate was 84.21% and were more frequent in the distal end exon. With the availability of genetic analysis, the first investigation of choice in DMD should be genetic analysis before genetic counseling.

KEY WORDS: DMD Patients, Clinical Features, Gene Analysis, Gujarati Population.

INTRODUCTION
Duchenne muscular dystrophy (DMD) is an X linked recessive disorder affecting mostly males with an incidence of 1 in 3600 liveborn boys. The gene affected is the dystrophin gene located on X p 21. This gene codes for dystrophin protein, an important component of the dystroglycan complex of muscles that provides stability to the muscle cells and aids in movement and locomotion. The ailment is a form of muscular dystrophy that leads to progressive muscle degeneration and ultimately untimely/ precocious deathoftheaffected. Deletions are most common (60 to 65% of DMD), usually of several kilobases of genomic DNA. 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.[1, 2]

The most frequent presenting symptoms are motor delay or an abnormal gait. Affected boys may present with difficulty in running or getting up from the ground, frequent falls, or toe-walking.[3] These early symptoms can be seen at the age of 2-3 years. There is progressive muscle weakness, seen in pelvic as well as proximal leg accompanied by the loss of muscle mass. A prominent sign of weakness is the Gower's sign and the lordotic gait while getting up and walking respectively. This problem is accentuated and can lead to application of braces by age of 10 or wheelchair dependence by 12 years. Abnormal bone development is evident as curvature of spine can be witnessed (scoliosis). Death occurs by the age of 25 – 30 years due to respiratory system or cardiac insufficiency.[4]

MATERIALS AND METHODS
Total of 19 referral male patients with DMD were reviewed who had consulted at Indian Muscular Dystrophy Society (IMDS) Ahmedabad, Gujarat, India between the period of 2015-16 for their clinico-pathological condition. Age matched controls were also included in the study. This study was approved by the Gujarat University Institutional human ethics committee (GU HEC 001/2015). Informed consent was given in writing before sample collection by the subjects and the parents of individuals.

PATHOPHYSIOLOGICAL INDICES

Diagnosis was based on age, sex, family history, loss of ambulation, calf hyper trophy, Gower's sign, scoliosis along with age of onset, age of presentation, etc. The age ranged from 5 to 26 years.

Two ml of blood was collected in an EDTA vacuutainer (purple cap) from each patient. A detailed history of the patient was also recorded. DNA was isolated according to John et al.[5] method from peripheral blood lymphocytes by phenol-chloroform extraction method.[6] Multiplex DNA amplifications of the dystrophin gene were carried out according to Chamberlain et al[7] modified by Beggs et al.,[8] (exon: 45, 48, 19, 17, 51, 8, 12, 44, 4, and 46) and Beggs et al [9] (exon: Dp427m, 3, 43, 50, 13, 6, 47, 60, and 52), using two multiplex PCR assays allowing the amplification of 10 and 9 exons respectively. PCR products were resolved on gels or 2.5% Agarose gels, and the gels were analyzed for exonic deletions by the presence or absence of a corresponding band.

CPK ANALYSIS

The patient's reports of creatine phosphokinase (CPK) or creatine kinase (CK) was done using fully automated biochemical analyser Cobas Integra 400 system from Roche.

RESULTS

In the study, sixteen out of nineteen DMD patients had deletions (84.21%). Three DMD cases did not show deletions. All the cases complained of lower limb weakness, Gower's sign (17/19), frequent falls, calf hypertrophy (17/19) and lordosis. The clinical details are described (Table 1).

CPK VALUES

The mean CPK value was 11,417 U/l which was higher than normal closely matched groups (38 U/l to 174 U/l) (Table 1).

GENETIC FINDINGS

Deletion pattern was higher with respect to other exons in 8, 44, 45 and 50 (12.5%) followed by others (Figure 1). There were total of 39 deletions in all the patients. The data showed that 81.25 % (13/16) of the deletions were located at the 3' downstream hot-spot region (exons 41-60, distal rod domain). The patient deletion percentage was 84.21% (16/19) and others (1-19) are detected at proximal region (Table 2). The distal exon deletion (46, 47, 48, 50, 51) obtained by Chamberlain and Beggs have been presented in Figure 2.

Table 1: Clinical symptoms of DMD patients.

FEATURES	DMD (N=19)
Mean Age of Onset (yrs)	5.33
Mean Age of Presentation (yrs)	11
Consanguinity	4
Familial	2
Gower's Sign	17
Calf Hypertophy	17
Scoliosis	3
State of Ambulation :-	
a. Own	12
b. Wheelchair bound	7
CPK value (Mean)	11,417 U/l

Normal Range of CPK: 38 U/l to 174 U/l
Patient Range of CPK: 21.88U/l to 22,098 U/l

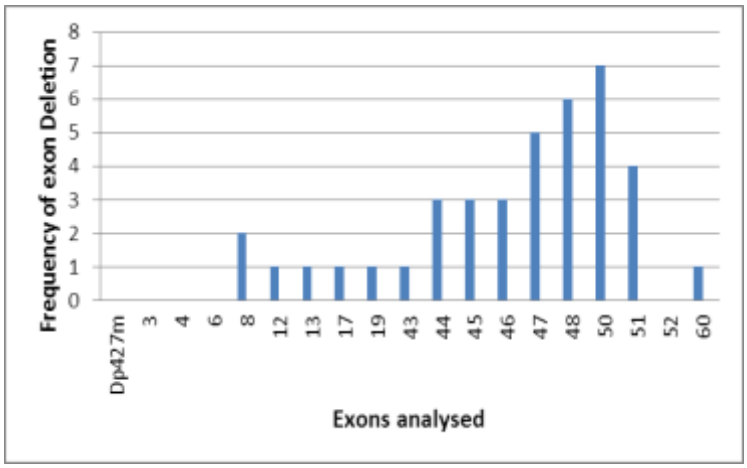

Figure 1: Frequency of exon deletion in DMD probands.

Table 2: Deletion pattern followed by phenotype in DMD probands

Deletion	No. of Probands	Deletion Percentage	IN/OUT Frame	Phenotype
del 8	2	12.5	out	DMD
del 12-48	1	6.25	out	DMD
del 44	2	12.5	out	DMD
del 45	2	12.5	out	DMD
del 46-50	1	6.25	out	DMD
del 46-51	1	6.25	out	DMD
del 46-60	1	6.25	out	DMD
del 47-48	1	6.25	in	DMD
del 48-51	1	6.25	in	DMD
del 50	2	12.5	out	DMD
del 50-51	1	6.25	in	DMD
del 51	1	6.25	out	DMD

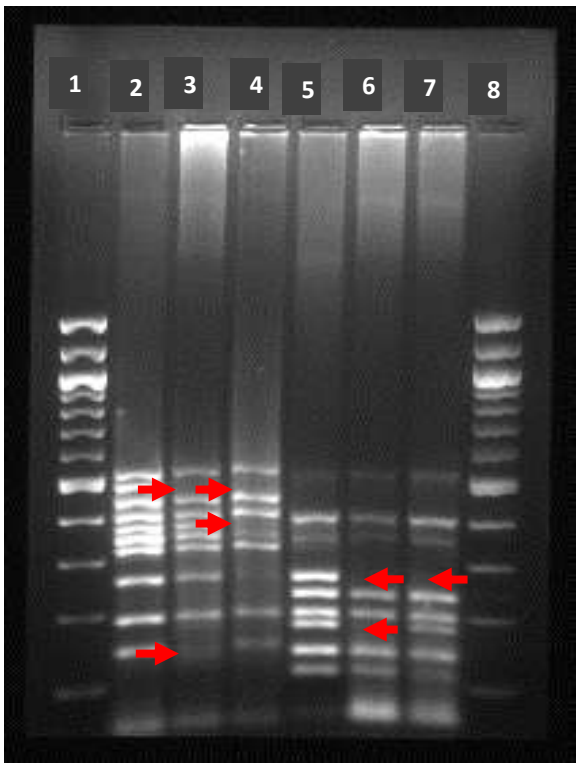

Fig 2: Gel image showing the electrophoresis of m-PCR product of Dystrophin gene amplified by 2 sets of primers in DMD case no. 17 & 18.

LANE 1: Ladder (100 bp)
LANE 2: Positive control (Chamberlain set) exon: 45, 48, 19, 17, 51, 8, 12, 44, 4, 46
LANE 3: DMD 17 proband, Chamberlain set; exon: 45, **48**, 19, 17, 51, 8, 12, 44, 4, **46**
LANE 4: DMD 18 proband, Chamberlain set; exon: 45, **48**, 19, 17, **51**, 8, 12, 44, 4, 46
LANE 5: Positive control (Beggs set) exon: Dp427m, 3, 43, 50, 13, 6, 47, 60, 52
LANE 6: DMD 17 proband, Beggs set; exon: Dp427m, 3, 43, **50**, 13, 6, **47,** 60, 52
LANE 7: DMD 18 proband, Beggs set; exon: Dp427m, 3, 43, **50**, 13, 6, 47, 60, 52
LANE 8: Ladder (100 bp)

DISCUSSION

DMD is one of the genetic anomalies, which is present in males and transferred from maternal side. It is identified at age of 2-3 years after birth with clinical symptoms of the disease. In our study, 19 DMD cases were registered from our society, whose blood was drawn after investigations based on CK values and other clinical symptoms. DNA sample was extracted and these samples were used for gene analysis using Beggs and Chamberlain techniques for deletion analysis. These were used for 80-90% exon deletions of the gene. The data revealed that 16 patients (16/19) have been identified with 39 deletions. These included that 13/16

had 81.25% located in exons of 41-60 of distal rod region.

Maximum numbers of deletion (7) was detected in exon 50 following exon 48 (6) and 47 (5) in central rod region in our study. Same results were noticed by earlier workers in Gujarat[2,10, 11] and other parts of India [12, 13] for DMD patients to support our data. Previous studies noted that the most common exons deleted were exon 51, 52, and 45 in their studies.[14, 15, 16] Comparatively, our Gujarati population had deletions in 47, 48, 50 and 51 exons being maximum in exon 50 as cited above in support of our earlier data.[2][11]

According to the reading frame rule, out of sixteen, thirteen male probands had OUT-frame deletions justifying DMD type. But, other three had IN-frame mutations like that of BMD type that showed DMD symptoms according to reading frame rule.[17]

Other factors of DMD disease like socio-economic status, other biochemical markers in addition to CPK values need proper understanding of it. [10] In depth study of gene analysis using MLPA and NGS will be done for exact understanding of the disease and then call for counseling if necessary in affected family. [11]

CONCLUSION

In this study cohort, in Gujarat, DMD analysis points out exon deletion is nearly similar comparatively with other studies globally including India. Further, NGS technology is called for proper understanding of disease for genetic counseling in Gujarat.

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CONFLICT OF INTEREST

No conflict of interest is expressed by any authors.

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