

Mutation analysis in Saudi Duchenne and Becker muscular dystrophy patients using multiplex PCR

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Abstract

Introduction: In Saudi Arabia, only limited work has been reported on the mutation patterns of Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). This study looks at the spectrum of deletions in the 'hot spot' regions of the DMD gene in Saudi DMD/BMD patients using an enhanced multiplex PCR technique.

Material and methods: Twenty-six exons of the DMD gene were analyzed, in eight unrelated DMD/BMD cases aged 4-19 years, using four different multiplex PCR sets. Each multiplex PCR set amplified a total of six or seven exons. Normal controls were included for validation.

Results: Using an optimized multiplex PCR method, 5 out of 8 DMD/BMD patients showed deletions, while the remaining three had no deletions in regions analyzed. Set 1 detected no deletions in any of the patients, whereas each of sets 2, 3 and 4 detected two, four and three deletions respectively. All of these mutations were located in the distal 'hot spot' region. No deletions were detected in the proximal 'hot spot' region. The normal control samples showed no deletions in any of the 26 exons tested.

Conclusions: In this study, multiplex PCR technology was utilized to demonstrate the frequency of the most commonly found deletions in a limited group of Saudi DMD/BMD patients. The overall distribution of deletion mutations in the distal 'hot spot' region was in accordance with DMD/BMD cases investigated elsewhere. The study also serves as a good starting point for further investigations into the genetic aspects of the Saudi DMD/BMD population.

Key words: dystrophin, Saudi DMD, BMD, deletions, multiplex PCR, DMD gene.

Introduction

Duchenne muscular dystrophy (DMD) is a fatal neuromuscular genetic disease [1] caused by mutations in the DMD gene. It is the most common form of muscular dystrophy (MD) as well as the most frequent muscle disease among children [1, 2], affecting 1 in 3500 live male births. The DMD gene is one of the largest genes known to date, comprising 2.4 mln base pairs. It is structurally complex, comprising 79 exons and 8 promoters [3]. During dystrophin protein production, mutations in this large gene generally result in a disturbance of the open reading frame (ORF). These ORF mutations either result in the production of truncated or degraded protein molecules or can even cause complete absence of dystrophin protein [4, 5]. Intragenic deletions are the most common changes found in the DMD gene, accounting for 65% of dystrophin mutations. The distribution of these

Table I. Four separate multiplex sets optimized for exon amplifications with corresponding fragment size (bp) for differentiation on PAGE

Multiplex set 1		Multiplex set 2		Multiplex set 3		Multiplex set 4	
exon	(bp)	exon	(bp)	exon	(bp)	exon	(bp)
3	410	49	439	48	506	19	459
8	360	12	405	1	419	44	426
7	315	43	357	51	388	45	307
4	196	17	326	9	321	13	238
5	167	52	265	50	271	47	181
60	139	62	191	53	212	46	148
		42	155	6	175		

deletions is not random but they are clustered in the two known 'hot-spot' regions: one near the central part of the gene known as the distal 'hot spot' and the other towards the 5' end known as the proximal 'hot spot' [6]. Point mutations, insertions and nucleotide changes together account for 25-30% [7], while duplications account for 5-10% of these mutations that appear to be evenly distributed throughout the gene [8]. As has been documented by Beggs et al. [9], the clusters of these two hotspots represent the basis for the use of the multiplex PCR technique where by screening 19-24 exons, 98% of all deletions can be identified.

Becker muscular dystrophy (BMD) results from mutations in the same gene and is inherited in the same way but has a much milder presentation than DMD. In BMD, although the distribution of muscle wasting and weakness is similar to that of DMD, the course of the disease is slower and far less predictable. BMD usually presents at an average age of 12 years and death generally occurs in the third or fourth decade, while patients with milder symptoms live longer. The clinical discrimination between DMD and BMD is linked to age at which the patient becomes wheelchair dependant. BMD patients remain ambulant to the age of 16, while DMD patients are wheelchair dependant before the age of 13 years. At present DMD diagnosis is mainly based upon serum creatinine kinase (CK) levels, and this is the only marker for DMD that is routinely used in laboratories across Saudi Arabia. This, together with relevant clinical profile such as disease history and muscle biopsy results, determines the diagnosis and possible course of action. By exploring genetic markers in Saudi DMD/BMD patients, the frequency of mutations can be documented in patients followed by risk assessment in immediate relatives and identification of carriers.

The objective of our study was to exploit multiplex PCR technology in identifying and reporting the spectrum of deletion mutations in our

clinically diagnosed Saudi DMD/BMD cases by screening the 'hot spot' regions of the 79-exon DMD gene. Furthermore, the detection spectrum of these deletions was expanded by adding new exons 5, 7, 9, 46 and 62, amounting to 26 exons in total.

Material and methods

The twenty-six primers used to detect deletions in the DMD gene were divided into four multiplex PCR sets according to their size, as shown in Table I.

Eight blood samples were collected from unrelated DMD/BMD male patients aged 4-19 years. The patients were referred to us from three general hospitals in Western Saudi Arabia. The diagnosis was established by clinical assessment, family history and elevated serum CK level, as illustrated in Table II. Serum CK levels were also observed in blood samples collected from immediate relatives of the patients.

In addition, 36 healthy subjects were used as controls for validation purposes. Three to four ml of whole blood was collected using a sterile technique and sterile tube filled with EDTA as anticoagulant factor. DNA was extracted from whole blood samples using the Qiagen™ DNA extraction kit. DMD gene deletions were detected using the four multiplex PCR reactions. A total of 26 exons were analyzed, six with multiplex set 1, seven with multiplex set 2, seven with multiplex set 3 and six with multiplex set 4, as shown in Table I with corresponding fragment size in bp.

The reaction mixture was made in a volume of 50 µl containing 1 × home made PCR buffer, 10 mM dNTPs, 1 M MgCl₂, 200 ng/ml of each primer, 5000 U/ml Taq DNA polymerase (Amersham biosciences, USA) and 200 ng/ml of genomic DNA of each sample. The amplification was carried out as a 'hot start' followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 70°C for 2 min. Cycling

Table II. Patients’ profile with subsequent serum CK values

Patient	Age (years)	CK concentration IU/l	Number of sisters	Number of brothers	Other affected siblings	Family history of DMD/BMD
1	8	3000	4	1	–	No
2	9	3000	2	5	–	No
3	19	900	1	1	–	No
4	15	2800	1	1	–	No
5	4	32 000	–	–	–	No
6	6	6000	–	–	–	No
7	6	20 000	1	0	–	No
8	11	3700	–	–	–	No

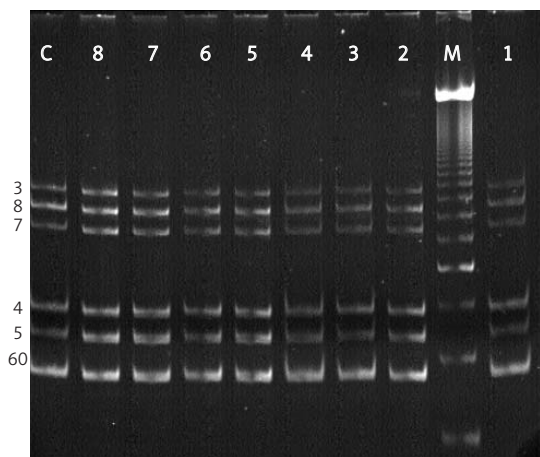


Figure 1a. Multiplex set 1 detects 6 exons
All of the six exons are amplified in the 8 DMD patient and normal control samples; therefore no exon deletions are observed
lane C – normal control
lane M – 50 bp DNA ladder marker
lanes 1, 2, 3, 4, 5, 6, 7 and 8 – DMD patient samples

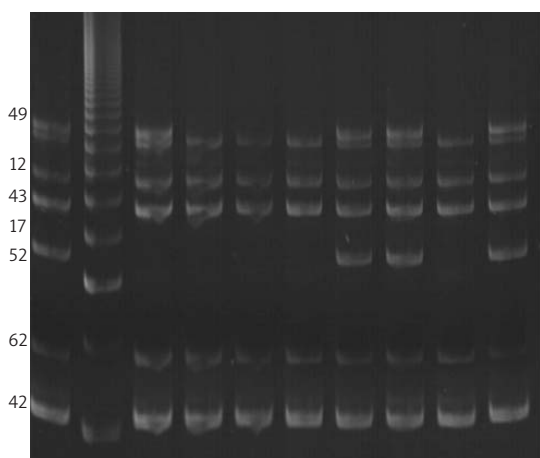


Figure 1b. Multiplex set 2 detects 7 exons
Patient samples 2, 3, 4, 5 and 8 have exon 52 deleted. Samples 3, 4, 5 and 8 also have exon 49 deletion. In samples 1, 6 and 7 no deletions were found, similar to the normal control
lane C – normal control
lane M – 50 bp DNA ladder marker
lanes 1, 2, 3, 4, 5, 6, 7 and 8 – DMD patient samples

was concluded with a final extension at 70°C for 5 min. The PCR products were analyzed on 5% polyacrylamide gel and visualized under UV light after ethidium bromide staining.

Results

These deletion patterns in the eight Saudi DMD/BMD patients are illustrated in Figure 1 (a), (b), (c) and (d), corresponding to the four multiplex sets respectively. Exon deletions were observed in five out of eight patients’ samples, and the remaining three samples did not show any deletions for all of the four multiplex sets tested.

The distribution of deletions in the five samples is summarized in Table III. These deletions were confined to the distal hot spot region of the DMD gene that includes exons 45 to 53. The number of exons deleted in a given patient varied from three in patient #2, to at least eight exon deletions observed in patients #3, 4 and 8 respectively, as demonstrated in Figure 2. For all of the eight patient samples, no deletions were detected in the proximal hot spot region of the DMD gene, which includes exons 1-19.

Discussion

The incidence of DMD/BMD in Saudi Arabia has never been reported, which could be due to the lack of a functional national registry for this disease. This emphasizes the need for establishing a national registry to allow proper estimation of DMD/BMD status in Saudi Arabia.

DMD is a fatal neuromuscular genetic disease and it is the most common form of MDs, frequently occurring in children across the world [10], and yet studies on DMD/BMD in the Saudi population, particularly at the molecular level, are very few. This makes our study, primarily focused on molecular investigation of the DMD gene, with special reference to targeting known mutations using the multiplex PCR technique, of interest.

With respect to the location of the deleted exons, our patients were categorized into two groups. In the

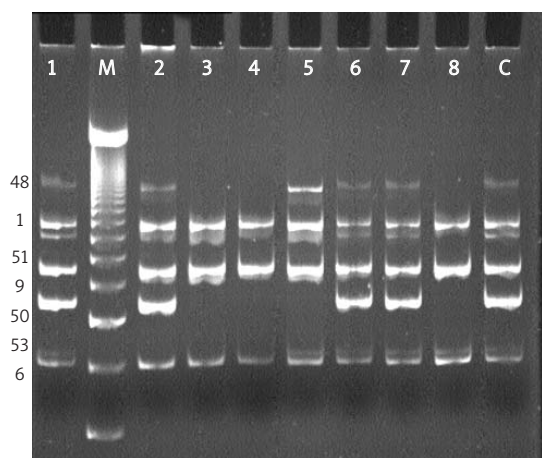


Figure 1c. Multiplex set 3 detects 7 exons
 Patient samples 3, 4 and 8 have exon 48 deleted, whereas samples 2, 3, 4, 5 and 8 also have exon 51 deleted. Patient samples 3, 4, 5 and 8 also have exon 50 deletion, whereas exon 53 is deleted in samples 2, 3 and 4. Samples 1, 6 and 7 were found to have no deletions coinciding with normal control
 lane C – normal control
 lane M – 50 bp DNA ladder marker
 lanes 1, 2, 3, 4, 5, 6, 7 and 8 – DMD patient samples

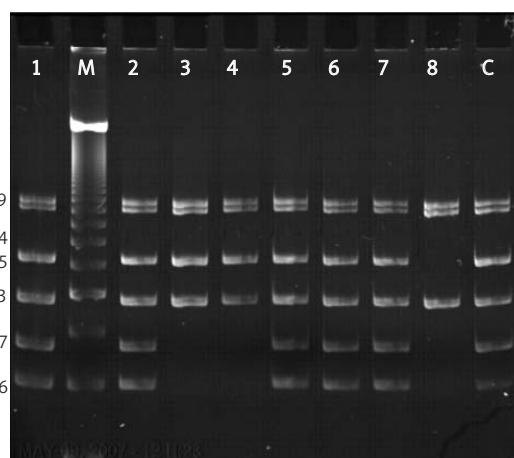


Figure 1d. Multiplex set 4 detects 6 exons
 Patient samples 3, 4 and 8 have exons 46 and 47 deleted; however, patient 8 also has exon 45 deleted. Samples 1, 2, 5, 6 and 7 were found to have no deletions coinciding with normal control
 lane C – normal control
 lane M – 50 bp DNA ladder marker
 lanes 1, 2, 3, 4, 5, 6, 7 and 8 – DMD patient samples

first group, representing 5 out of 8 cases, the deletions were restricted to the distal hot spot region, with frequency of deletions in each patient varying from three to at least eight (see Table III). In a population of 8 patients, it is not a surprise that no deletions were found in the proximal hot spot assuming that the deletion frequencies are comparable to those observed in the Western world [8, 11, 12].

The second group (3 out of 8) showed no deletions in either region. These cases possibly represent the low frequency of DMD/BMD patients with undetectable deletions [13]. One possible explanation could be that deletion mutations may exist in these patients but occur in other regions of the gene [8]. However, it is also possible that other

types of mutations such as point mutations, insertions or nucleotide changes and duplications may be responsible for the disease in these patients [14, 15]. Therefore it is highly recommended to target the whole DMD gene, and the most suitable technique to accomplish this task is the multiplex ligation dependant probe amplification assay (MLPA). This method has the capability to interrogate gene dosage at multiple target loci using sets of fluorescent probes [16], where genetic variations are detected by ligation of these site-specific fluorescent probes followed by a PCR amplification step for up to 45 target sequences at one time [17]. As a result, all 79 exons of the DMD gene can be scanned for deletions in a single assay.

Table III. Deletions distribution in five out of eight Saudi DMD/BMD patients. All of these mutations occurred in the distal 'hot spot' region (exons 45-53) of the DMD gene

Patient Sample #	Exon								
	45	46	47	48	49	50	51	52	53
2							x	x	x
3		x	x	x	x	x	x	x	x
4		x	x	x	x	x	x	x	x
5					x	x	x	x	
8	x	x	x	x	x	x	x	x	
Times deletion observed	1	3	3	3	4	4	5	5	3

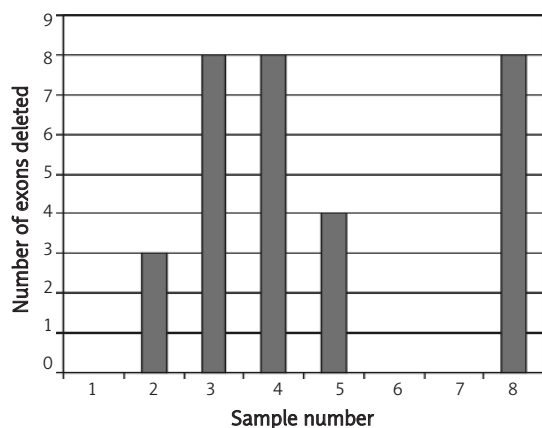


Figure 2. Number of deleted exons in the five DMD/BMD patients, whereas no deletions were detected in the remaining three patients

Although with respect to distribution of deletions our study was confined to a small group of patients, the findings were in agreement with a solitary study published by Al-Jumah on a much larger group of DMD/BMD patients in Saudi Arabia [11]. Comparison with data reported in various countries also show similarities, such as India with 61% [18], Thailand and Greece with 63% each [19-21], Turkey with 59% [22], USA with 65% [10] and Egypt where reported DMD gene mutations vary from 50 to 67% [23]. However, deletion pattern differences do exist from one population to another or even within the same population. A recent study in China by Lo [24] demonstrated 34.3% exon deletions in local Chinese patients, significantly lower than the commonly quoted 60%. This may be suggestive of ethnic and regional differences or genetic variability in predisposition to DMD/BMD.

DMD is an inherited X-linked recessive disease and the mode of transmission is primarily from a carrier mother to her son. The life expectancy is very low since effected males die at or before adolescence [23]. Carriers are defined as having one of the X chromosomes with a positive mutation in the DMD gene locus while the other X chromosome performs normal function. However, in rare instances some females with positive DMD phenotype have been reported, where they had X-autosome translocations [7].

Studies have shown that *de novo* mutations in the DMD gene can cause DMD/BMD to occur in families without prior disease history [1, 25, 26], where all the family members of the affected one present normal serum CK levels. This means that the brothers of the patient are not affected and even the mothers and sisters may not carry DMD/BMD mutations, since it has been reported that 70% of the carrier females show slight elevations in serum CK levels.

In conclusion our study attempts to shed some light on the genetics of DMD/BMD in a limited number of Saudi patients with particular relevance to deletion mutations in their DMD gene. In Saudi Arabia, as in many other countries, this neurodegenerative disorder has extensive implications and places a heavy burden on both the affected ones and their families, where most patients are wheelchair bound after the first decade. The progress in genetic technology and the application of new approaches for DNA analysis, such as multiplex PCR and MLPA, allow rapid molecular-based diagnosis of this disease. However, in the absence of effective therapeutic intervention, such methods may currently be helpful in guiding the patients and their families about the pattern of inheritance, determining female carriers and opening possibilities of prenatal diagnosis in pregnant women with previous DMD/BMD children.

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