MULTIPLEX PCR FOR RAPID DETECTION OF EXONAL DELETIONS IN PATIENTS OF DUCHENNE MUSCULAR DYSTROPHY

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ABSTRACT

Duchenne muscular dystrophy (DMD) is the most common X-linked disorder in children affecting 1 in 3500 males. Since, as of now, we have no treatment for DMD, carrier detection and prenatal diagnosis is the most important preventive strategy. Multiplex PCR helps in rapid detection of hot spot exonal deletions (positive in 65% of cases) as many exons can be identified in a single run. 10 children with characteristic clinical features of DMD and chorionic villus samples of 10 antenatal patients with positive family history were studied. We identified a deletion mutation in exon 49 of the dystrophin gene in a 4 yr old boy referred with signs and symptoms suggestive of DMD using primers for exons 45, 48, 49, 43, 44, 19, 3, 8, 13 and muscle promoter, subjected to multiplex polymerase chain reaction (PCR) and agarose/Nu-Sieve gel electrophoresis. These genetic methods aid in prenatal diagnosis of DMD as well as confirmation of diagnosis in children with signs and symptoms suggestive of the disease.

KEY WORDS

Duchenne muscular dystrophy, multiplex PCR, exons, Hot-spot deletions, prenatal diagnosis

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a lethal and common X-linked muscular disorder characterized by progressive muscular weakness causing death in early twenties. It affects one in 3500 male live births (1, 2). Previous work on this subject entailed many non-specific tests e.g electromyogram, serum creatine kinase and serum calcium (3), and invasive procedures like muscle biopsy (4). The gene coding for the protein dystrophin (deficient in DMD) is one of the largest genes known with a coding sequence of 14 kb, spread over 2400 kb of genomic DNA and contains at least 79 exons (5). DMD gene maps to the short arm of X-chromosome at band Xp 21. DMD is caused primarily by intragenic deletion (65%) or duplications (5%) (6). Southern blotting has also been used for diagnosis but it has many disadvantages as

compared to multiplex PCR (7, 8). Since, as of now, we have no treatment for DMD, carrier detection and prenatal diagnosis is the most important for families which have a history of DMD. Laboratory diagnosis of DMD also poses a challenge for children presenting with signs and symptoms suggestive of the disease. Multiplex PCR is of immense use because, given the appropriate set of primers, the method is specific, sensitive and rapid as many exons can be studied in a single run.

MATERIALS AND METHODS

Peripheral venous samples of 10 children with characteristic clinical features and markedly raised creatine kinase values were taken from department of Pediatrics, Genetic Clinic (AIIMS) for further investigations of DMD. Also chorionic venous samples were studied from 10 antenatal women with family history of DMD who approached us for prenatal diagnosis.

Chorionic villus samples (CVS) were collected at 10-15 weeks of pregnancy. CVS was microscopically dissected for removing the maternal tissue from the sample

Further methodology was the same for both groups.

DNA was extracted using the phenol chloroform extraction method (9). 10 ml of whole blood was lysed

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with 50 ml of lysis buffer (NH₄Cl 0.77 M , KHCO₃ 0.4 M, pH 8.1) and left at 4°C for 4 hours. This was then centrifuged at 5000 r.p.m. for 20 minutes at 4°C and supernatant discarded. 5 ml of 5M NaCl - EDTA (0.5M, pH 8.0) solution was added to make a clear suspension of cells and after addition of 300 µl of Sodium dodecyl sulphate (20 g/100 ml distill. water) and 14 µl proteinase K (2.5 mg/ml distill. water) it was left overnight at 37°C. Phenolic extraction with equal volume of saturated phenol was done 3 times for removing proteins. Chloroform: isoamylalcohol (ratio 24: 1) was used to extract the left over phenol and proteins. The DNA in the aqueous phase was precipitated by 1/10th volume of chilled Na acetate solution and 2.5 volume of chilled absolute ethanol. Using a Pasteur pipette, the precipitated DNA was removed from solution and dried in vacuum. DNA was stored in 500 µl of Tris-EDTA buffer (1M Tris,

0.5M EDTA, pH 8) at 4°C and test put up in 1-2 days.

Polymerase chain reaction was employed for in vitro amplification of the desired DNA segment. Heat stable DNA polymerase (Perkin-Elmer) from the thermophilic bacterium *Thermus aquaticus* and commercially available dNTP's (di-nucleotides Triphosphates) were used. 20-mer polymers obtained from (biosyn@biosyn.com) for exons 45, 48, 49, 43, 44, muscle promoters and exons 19, 3, 8, 13 with similar melting temperatures and near ideal GC content of 50-60% were used.

PCR buffer contained 15 mM MgCl₂, 100 mM Tris-Cl (pH 9.2) 1% gelatin and 50 mM KCl. PCR mixtures contained 10 µl of 10X PCR buffers (1.5 mM), 10 µl of 2 mM dNTP's, primers, 4 µl each of forward and reverse primers, 1 µl Taq polymerase and 10 microlitre of purified DNA in 67 µl water. It was subjected to 30

OLIGONUCLEOTIDE PRIMER SEQUENCES (8,10) FOR DYSTROPHIN GENE - MULTIPLEX PCR

1st group used

EXON	PCR Primer Sequence 5'-3'	Amplified region
Exon 45	F-AAACATGGAACATCCTTGTGGGAC R-CATTCCTATTAGATCTGTGCGCCCTAC	547 B.P
Exon 48	F-TTGAATACATTGGTTAAATCCCAACATG R-CCTGAATAAAAGTCTTCCTTACCACAC	506 B.P
Exon 49	F-GTGCCCTTATGTACCAGGCAGAAATTG R-GCAATGACTCGTTAATAGCCTTAAGATC	439 B.P
Exon 43	F-GAACATGTCAAAGTCACTGGACTTCATGG R-ATATATGTTGTTACCTACCCTTGTCGGTCC	357 B.P.
Exon 44	F-CTTGATCCATATGCTTTTACCTGCA R-TCCATCACCCCTTCAGAACCTGATCT	268 B.P.

2nd Group Used

EXON	PCR Primer Sequence 5'-3'	Amplified region
19	F-TTCTACCACATCCCATTCTTCCA R-GATGGCAAAAGTGTTGAGAAAAAGTC	459
3	F-TCATCCATCATCTTCGGCAGATTA R-CAGGCGGTAGAGTATGCCAAATGAAATCA	410
8	F-GTCCTTTACACACTTTACCTGTTGAG R-GGCCTCATTCTCATGTTCTAATTAG	360
13	F-AATAGGAGTACCTGAGATGTAGCAGAAAT R-CTGACCTTAAGTTGTTCTTCCAAAGCAG	238
PM (Muscle Promotor)	F-GAAGATCTAGACAGTGGATACATAACAAATGCATG R-TTCTCCGAAGGTAATTGCCTCCAGATCTGAGTCC	535

cycles of 45 sec denaturation at 94°C, 30 sec annealing at 55°C and 30 sec, extension at 72°C in the PCR thermal cycler (Programmable thermal controller PTC-100™ from MJ Research Inc. USA).

After amplification, 25 µl of PCR products were run (at 140 volts) on Nu Sieve: Agarose gel (Sea KE M,

LE Agarose, FMC bioproducts) taken in the ratio of 3:1 (4.5 g : 1.5 g) in 150 ml of Tris EDTA buffer(pH 8, 1M Tris, 0.5M EDTA) with added Ethidium Bromide (7.5 µl per 100 ml of gel) for fluorescence of DNA under UV light and Bromophenol blue (5 µl per 25 µl of PCR product) as marker for the run. A 100 base pair DNA ladder was run along with the samples and

NU - Sieve : agarose gel electrophoresis

DELETION OF EXON 49 IN LANE 5

Lane 1. 100 b.p. DNA ladder; Lane 2. Blank; Lane 3. Control showing amplified exons of Group A (ie 45, 48, 49, 43,44); Lane 4. Patient showing normal amplified exons of Group A (i.e. 45, 48, 49, 43 & 44); Lane 5. Affected child showing absence of exon 49, Child had signs and symptoms of DMD; Lane 6. Blank; Lane 7. Control for primer Group B (i.e muscle promoter & exons 19,3,8,13); Lane 8. Patient showing no abnormality for muscle promoter and exons 19,3, 8 and 13); Lane 9. Patient showing no abnormality for exons amplified in Group B (PM, 19,3,8 and 13)

control. Photographs were taken under UV light via Gel Documentation System, USA.

RESULT

Two groups of patients were studied. 10 children referred from the paediatric OPD of AIIMS with markedly raised creatinine kinase levels and characteristic clinical features were investigated. Also CVS samples were studied from 10 antenatal women who approached us for prenatal diagnosis because of family history of the disease. The patients were not related to each other.

Each sample was studied for the commonly deleted exons 45, 48, 49, 43, 44 (group A) and exons 19, 3, 8, 13 and muscle promoter (group B).

We identified a deletion mutation of exon 49 (Fig. 1) in a 4 yr. old boy with characteristic features of DMD and family history suggestive of the disease.

All other paediatric samples did not show any deletion of the exons under study. Also the CVS samples did not show any positivity for deletions of exons under study.

DISCUSSION

Before DNA technology developed, prenatal diagnosis was done by various biochemical markers such as intracellular calcium (11), fetal plasma Carbonic anhydrase and creatinine kinase (12). These markers were unreliable because of overlap between control and DMD fetuses. The technique of restriction fragment length polymorphism (RFLP) was then developed (6). There were various limitations to this method also (13). Though immunochemistry is still the gold standard for diagnosis of DMD (14), fetal muscle biopsy is not advisable for prenatal cases when CVS samples would be less invasive and adequately informative by PCR.

Multiplex PCR is simple to use with significant diagnostic value. It would pick up the common deletion mutations and is ideal for DMD screening. In Asian Indians the common "hot spot" deletions are located mainly in exons 45-51 (15). Here we have used primers in exons 45, 48, 49, 43, 44 in group A and muscle promoter plus exons 19, 3, 8, 13 in group B and diagnosed the deletion mutation in a boy referred for suspected signs and symptoms of DMD. All the other cases were normal for the exonal deletions studied. They require further studies.

A family having one or more children with DMD would greatly benefit from genetic counselling and prenatal diagnosis of the next pregnancy as they can have normal children of both sexes. Since more than 70% of DMD is caused by intragenic deletions and duplications (16, 17) multiplex PCR is of valuable

diagnostic use. For the other negative cases, where deletion is not identified by multiplex PCR, prenatal diagnosis by CA repeat marker can be utilized (18).

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