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Optimization of PCR-Multiplex Protocol for Diagnosis of Duchenne Muscular Dystrophy (DMD)

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Duchenne muscular dystrophy (DMD) is an eventually fatal disorder and is characterized by rapidly progressive muscles weakness and atrophy of muscle tissue starting in the legs and pelvis and later affecting the whole body. It is also reported that, it is very common in the males than the females. The gene for DMD, found on the X chromosome, encodes a large protein dystrophin. Dystrophin is required inside muscle cells for structural support; it is thought to strengthen the muscle cells by anchoring elements of the internal cytoskeleton to the surface membrane. Without it, the cell membrane becomes permeable, so that extra cellular components enter the cell, increasing the internal pressure until the muscle cell "explodes" and dies.The subsequent immune response can add to the damage. Due to dystrophin gene disorder DMD occurs at a frequency of about 1 in 3,500 new-born males. In the present study we have optimized Multiplex PCR which is simple, economical and robust compared to standard simple PCR technique. Multiplex PCR technique is the most efficient way of detecting the dystrophin gene mutations. A set of primers are used to detect the mutations. After running the multiplex PCR bands are finally observed in agarose gel electrophoresis with the help of U.V Transilluminator.

Key words: Duchenne Muscular Dystrophy (DMD), Multiplex- PCR, Diagnosis, Dystroglycancomplex (DGC).

Duchenne muscular dystrophy (DMD) is a severe recessive X-linked form of muscular dystrophy characterized by rapid progression of muscle degeneration(Straub *et al.*,1997), eventually leading to loss of ambulation and death(Esa *et al*.,1991) . This affliction affects one in 3500 males, making it the most prevalent of muscular dystrophies (Norwood *et al.*, 2000). In general, only males are afflicted, though females can be carriers (Cooper *et al*., 1988). Rarely females may be afflicted if the father is affected and the mother is also a carrier or if they receive mutation from father and other X is translocated (Dellorusso *et al*., 2002). The disorder is caused by a mutation in the gene DMD, located in humans on the X chromosome (Xp21). The DMD gene codes for the protein dystrophin, an important structural component within muscle tissue (Harper *et al*., 2002).Dystrophin provides structural stability to the dystroglycan complex (DGC), located on the cell membrane (Petrof, 1998).It is the best-known form of muscular dystrophy, due to mutation in a gene on the X chromosome that prevents the production of dystrophin, a normal protein in muscle (Campbell *et al*, 2003).

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DMD typically appears between the ages of two with weakness in the pelvis and upper limbs, resulting in clumsiness, frequent falling, an unusual gait and general weakness (Van Deutekom *et al*., 2003). Some patients also have mild mental retardation (Mendell *et al*., 1995). As DMD progresses, a wheelchair may be needed. Most patients with Duchenne MD die in their early twenties because of muscle-based breathing and heart problems (Law *et al*., 1992). There is no cure for DMD (Brooke *et al*., 1989). Current treatment is directed toward symptoms, such as assisting with mobility, preventing scoliosis, and providing pulmonary therapy (respiratory toilet) (Karpati *et al*., 1993). Gene replacement with dystrophin mini genes is being investigated but no cure appears around the corner (Mendel *et al*., 1989). Simplex PCR uses primer in different tubes and amplify DNA. Therefore in the present paper efforts have been made to optimize the PCR-Multiplex for the diagnosis of Duchenne Muscular dystrophy.

MATERIALS AND METHODS

Collection of serum sample

Serum samples were collected from the patients suffering from DMD disease following the standard protocols.

DNA Isolation & Purification

Cell Lysis: Add 300µl whole blood to a 1.5 ml tube containing 900µl RBC Lysis Solution. Invert to mix and incubate at room temperature for 1 min. Invert again at least once during the incubation. Centrifuge at 13000 -16000 x g for 20 sec. Discard supernatant leaving behind the white cell pellet and about 10-20 µl of the residual liquid. Vortex the tube vigorously for 10 sec to suspend the cells in the residual liquid. This greatly facilitates cell lysis in step 4 below. Now add 300µl of cell Lysis Solution to the resuspended cells and pipette up and down to lyse the cells. Usually no incubation is required, however, if cell clumps are visible after mixing, incubate at room temperature until the solution is homogenous. Samples are stable in Cell Lysis Solution for at least 18 months at room temperature.

Rnase Treatment (optional): Add 1.50µl RnaseA solution to the cell lysate. Mix the sample by inverting the tube 25 times and incubate at 37ºC for 15-60 minutes.

Protein Precipitation: Cool the sample to room temperature. Add 100µl of Protein Precipitation to the cell lysate. Vortex vigorously at high speeds for 20 sec to mix the Protein Precipitation Solution uniformly with the cell lysate. Centrifuge at 13000-16000 x g for 1min. The precipitated proteins will form a tight dark brown pellet.

DNA Precipitation: Transfer the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a 1.5ml tube containing 300µl of 100% Isopropanol (2-Propanol). Mix the sample by inverting gently for about 50 times. Centrifuge at 13000-16000 x g for 1min. The DNA will be visible as a small white pellet. Discard supernatant and drain tube briefly on clean absorbent paper. Add 1ml of 70% Ethanol and invert tube several times to wash the DNA. Centrifuge at 13000-16000 x g for 1min. carefully use a pipette to remove the DNA (white pellete) from the ethanol, and transfer into another clean tube. Air dries the tube containing DNA in a speed-vac for about 5 sec.

DNA Hydration: Add 100µl of DNA Hydration Solution. Vortex 5 sec at medium speed to mix. Incubate sample at 65ºC for 5 minutes to accelerate rehydration. Vortex 5 seconds at medium speed to mix and pulse spin briefly to collect sample at the bottom of the tube. Store DNA at 4º C. For long time storage, place sample at -20ºC or -80ºC.

The Chamberlain set

In the present study, The Chamberlain set is used to diagnose DMD. Chamberlain set consists of 9 sets of primer pairs to amplify 9 different kinds of exons, found defective in most of the patients. This set consists of the following primer pairs. Chamberlain JS, Gibbs RA, Ranier JE, Caskey CT (1990). Multiplex PCR for the diagnosis of Duchenne muscular dystrophy. In PCR Protocols: A Guide to Methods and Applications (Eds; MA Innis, DH Gelfand, JJ Sninsky, TJ White), Academic Press, San Diego (USA). Pp 272-281.

RESULTS

PCR Trail-1

In the present study, four trails were carried out to optimize PCR Multiplex to diagnose the DMD.The aim of the four trails were to optimize protocol for the diagnosis of DMD.The master mix preparation in all the four trails are presented in the following tables:

Amplified exon	in set	Length (in bp)	Forward /reverse primer	Name
exon 45	a	547	aaacatggaacatccttgtggggac / cattcctattagatctgtcgccctac	ex45-F/R
exon 48	b	506	ttgaatacattggttaaatcccaacatg / cctgaataaagtcttccttaccacac	ex48-F/R
exon 19	c	459	gatggcaaaagtgttgagaaaaagtc / ttctaccacatcccattttcttcca	ex19-F/R
exon 17	d	416	gactttcgatgttgagattactttccc / aagcttgagatgctctcacctttcc	ex17-F/R
exon 51	e	388	gaaattggctctttagcttgtgtttc / ggagagtaaagtgattggtggaaaatc	ex51-F/R
exon 8	f	360	ggcctcattctcatgttctaattag / gtcctttacacactttacctgttgag	ex8-F/R
exon 12	g	331	gatagtgggctttacttacatccttc / gaaagcacgcaacataagatacacct	ex12-F/R
exon 44	h	268	cttgatccatatgcttttacctgca / tccatcacccttcagaacctgatct	ex44-F/R
exon 4	i	196	ttgtcggtctctctgctggtcagtg / caaagccctcactcaaacatgaagc	ex4-F/R

Table 1. Showing Master Mix preparation of first trail

Component	Stock conc.	Final conc.	Final vol (for 1 tube)
DNA	-	-	2.0µl
Buffer	10x	1x	2.0µl
$MgCl_2$	25µM	1.5mM	1.2µl
dNTP's	2.5µM	200mM	1.6µl
Taq	5µl	1µRxn	0.2µl
F. Primer	20µM	0.5mM	0.5µl
R. Primer	20µm	0.5mm	0.5µl
Water	-	-	12µl
TOTAL			20µl

Table 2. The details of thermal cycling profile of Multiplex PCR

STEP	Temperature(°C)	Time (min)	No.of cycles
Initial denaturation	95	3	1
Denaturation	95	0.5	
Annealing	53	0.5	30
Extension	72	0.5	
FinalExtension	72	3	∞
Final storage	20	∞	

Component	Stock conc.	Final conc.	Final vol (for 1 tube)
DNA	-	-	2.0µ l
Buffer	10x	1x	5.0µ l
$MgCl_2$	25µm	1.5mM	3.0µ l
Taq	5µl	1µRxn	0.5µ l
Primer	20 µm	0.5 µm	22.5µ l
Water	-	-	13 µl
TOTAL			50 µl

Thermal Cycling Profile:

STEP	Temperature(°C)	Time (min)	No.of cycles
Initial denaturation	95	3	1
Denaturation	95	0.5	
Annealing	53	0.5	35
Extension	72	0.5	
FinalExtension	72	3	∞
Final storage	20	∞	

PCR Trail- 3: To check with deletions in the given samples

Component	Stock conc.	Final conc.	Final vol (for 1 tube)
DNA	-	-	3.0 µl
Buffer	10x	1x	5.0µ l
$MgCL_2$	25mM	1.5mM	3.0µl
dNTP'S	2.5mM	200mM	4.0.µl
Taq	5µl	1µRxn	0.5µl
Primer	20 µm	0.5 µm	22.5µl
Water	-	-	12 µl
TOTAL			50µl

Thermal Cycling Profile:

STEP	Temperature(°C)	Time (min)	No.of cycles
Initial denaturation	95	4	1
Denaturation	95	1	
Annealing	53	1.5	35
Extension	72	1.5	
Final extension	72	1	∞
Final storage	20	∞	

Thermal Cycling Profile

Agarose Gel Electrophoresis

The gel is prepared by using agarose, 1% agarose (i.e., 0.4g in 40ml of buffer) was used to carry out electrophoresis. By using micropipette inject the stained DNA and 10µl DNA ladder. Close the lid of the electrophoresis chamber and apply power supply at120V for45 mins to1 hour. When the DNA samples and DNA ladder approaches the end of the gel, the power supply is stopped. Now it is possible to visualize the DNA (stained with Ethidium Bromide) under the UV Transilluminator. For all trails the same electrophoresis procedure was followed.

PCR Trail- 2

In this case a primer mix with equal concentrations of all primers of the Chamberlain's set was prepared. Trail-2 is used to check the PCR conditions with a multiplex setup.

Agarose Gel Electrophoresis

The gel is prepared by using agarose, 2.5% agarose (i.e., 1g in 40ml of Buffer) was used to carry out electrophoresis. By using micropipette inject the stained DNA and 10µl DNA ladder. Close the lid of the electrophoresis chamber and apply power supply at120V for 45 mins to 1hour. When the DNA samples and DNA ladder approaches the end of the gel, the power supply is stopped. Now

it is possible to visualize the DNA(stained with Ethidium Bromide) under the UV Transilluminator.

Agarose Gel Electrophoresis

The gel is prepared by using agarose, 2.5% agarose (i.e., 1g in 40ml of Buffer) was used to carry out electrophoresis. By using micropipette inject the stained DNA and 10µl DNA ladder. Close the lid of the electrophoresis chamber and apply power supply at120V for 45 mins to 1hour. When the DNA samples and DNA ladder approaches the end of the gel, the power supply is stopped. Now it is possible to visualize the DNA (stained with Ethidium Bromide) under the UV Transilluminator.

Agarose Gel Electrophoresis

The gel is prepared by using agarose, 2.5% agarose (i.e., 1g in 40ml of buffer) was used to carry out electrophoresis. By using micropipette inject the stained DNA and 10µl DNA ladder. Close the lid of the electrophoresis chamber and apply power supply at120V for 45 mins to 1hour. When the DNA samples and DNA ladder approaches the end of the gel, the power supply is stopped. Now it is possible to visualize the DNA (stained with Ethidium Bromide) under the UV Transilluminator.

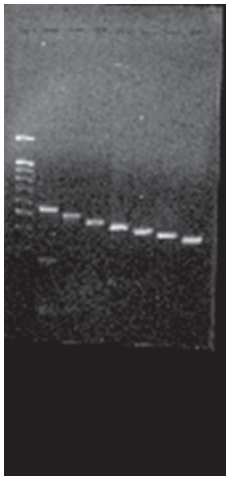

Fig. 1. In trail-1 amplifications are seen for all primer sets

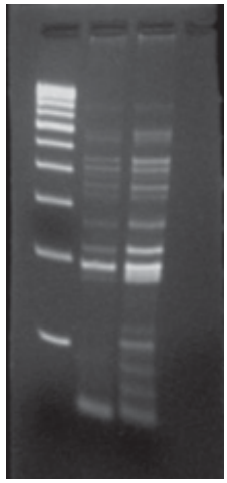

Fig. 2. Amplifications are seen for the particular primer sizes

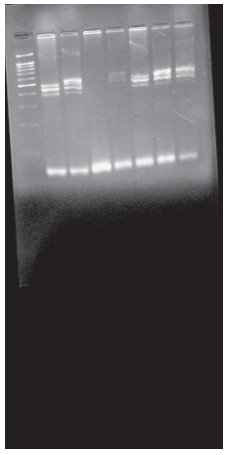

Fig. 3. In the second line, 3rd and 4th bands are missing, which correspond to exon 12 and exon 8 respectively. In the third line, 9th band is missing, which corresponds to exon 45 and it indicates that the patient is suffering with DMD problem

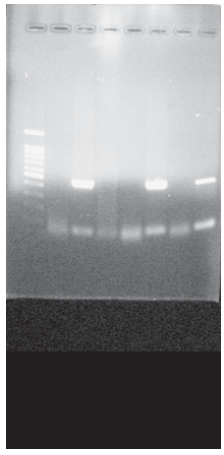

Fig. 4. In the second line, 8th and 9th bands are missing, which correspond to Exon 48 and Exon 45. In the third line, 5th band is missing which corresponds to Exon 51.It indicates that the patient is suffering with DMD problem

DISCUSSION

Duchenne muscular dystrophy (DMD) is a severe recessive X-linked form of muscular dystrophy characterized by rapid progression of muscle degeneration(Straub *et al.*,1997), eventually leading to loss of ambulation and death(Esa *et al*.,1991) . This affliction affects one in 3500 males, making it the most prevalent of muscular dystrophies (Norwood *et al.,* 2000). In general, only males are afflicted, though females can be carriers (Cooper *et al.,* 1988). Females may be afflicted if the father is afflicted and the mother is also a carrier (Dellorusso *et al.,* 2002). The disorder is caused by a mutation in the gene DMD, located in humans on the X chromosome (Xp21). The DMD gene codes for the protein dystrophin, an important structural component within muscle tissue(.Harper *et al*.,2002)Dystrophin provides structural stability to the dystroglycan complex (DGC), located on the cell membrane(Petrof ,1998).

It is caused by a defective gene for dystrophin (a protein in the muscles) (Scott *etal*., 1981). However, it often occurs in people without a known family history of the condition (Sutherland *et al,* 1981). Because of the way the disease is inherited, males are more likely to develop symptoms than are women (Scott *etal*., 1981). The sons of females who are carriers of the disease (women with a defective gene but no symptoms themselves) each have a 50% chance of having the disease (Bakker *et al.,* 2000). The daughters each have a 50% chance of being carriers (Abbs, 1996).

Duchenne muscular dystrophy occurs in approximately 1 out of every 3,600 male infants (Chamberlain *et al.,* 1988). Because this is an inherited disorder, risks include a family history of Duchenne muscular dystrophy (Chamberlain *et al*., 1990). The muscular dystrophies associated with defects in dystrophin range greatly from the very severe Duchenne muscular dystrophy (DMD) to the far milder Becker muscular dystrophy (BMD) (Gelfi, *et al.,* 1995). DMD and BMD result from different mutations in the gigantic gene that encodes dystrophin (Holding, *et al.*, 1993). The DMD gene contains 79 exons spanning at least 2,300 kb (Lee, *et al.*, 1998). Deletions cause deficiencies in 1 or more of these, which may explain why mental retardation and cardiomyopathy sometimes accompany DMD, for there are clear cut differences in clinical presentation(Abbs, *et al.,*1992) depending on what the deletions remove from the dystrophin gene(Anonymous, 1992). The DMD gene encodes a 3,685-amino acid protein product (Ausubel *et al.,* 1994). From its amino acid sequence, dystrophin is similar to pectin and other cytoskeletal proteins .It is rather like an I-beam with globular domains at each end, joined by a rod-like segment in the middle (Beggs *et al*., 1990).

In the present study, Multiplex PCR technology is used for the diagnosis of Duchenne Muscular Dystrophy. DNA was isolated and purified from whole blood sample by using well defined DNA Purification protocol. The primers used for this experiment are the chamberlain set of primers. After the multiplex PCR samples are run in Agarose gel electrophoresis and bands are observed in UV Transilluminator. If bands are formed for the given sample then it indicates that there is no deletion in the particular gene. If bands are not formed with the set of primers then there is a chance of deletion in the gene.

In the present study it has been observed that the given unknown samples collected from DMD patient's shows deletions for exons 8,12,45,48 &51.Therefore, the present DMD patient samples have mutations on the above mentioned exons.

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