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Original Article

Detection of Duchene muscular dystrophy carriers with quantitative fluorescent polymerase chain reaction

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Abstract

Aim: Duchenne Muscular Dystrophy (DMD) is an X-linked, progressive, lethal neuromuscular disorder affecting 1/3500 live-born males. Mutations occur in the dystrophin gene, which is located at Xp21.2. Partial gene deletions occur in two "hot-spot" regions, and can be responsible for up to 60-65% DMD cases, while 5-10% of the cases are caused from clustered gene duplications. Mutations can be inherited from female carriers (2/3) or be de-novo mutations (1/3). Deletions can be easily detected in affected males via multiplex PCR or MLPA. On the contrary, determining the status of female carriers is difficult. The aim of this study is to optimize the gene-dosage method using quantitative fluorescent PCR.

Material and Methods: Fluorescently labeled primers are used for amplification and automated detection of amplicons and are designed in multiplex format. The primers contain eighteen exons located within "hot-spot" regions. A promoter region and STR markers are also included in the test as internal controls and for linkage analysis. This is followed by a PCR automated genetic analyzer for the detection of PCR products. This study includes twenty-four families, each with a previously diagnosed member.

Results: Results showed the same correlation as was previously reported in nineteen patients, whereas three patients had an extra exon deletion and one patient had one less exon deletion than previously reported. In nineteen families, the mothers were carriers, and in five families, the mothers were not carriers.

Conclusion: As a conclusion for carrier screening in DMD patients, quantitative fluorescent PCR is a fast, reproducible and robust method can be used for detection of deletions.

Keywords: Duchenne muscular dystrophy; quantitative fluorescent PCR; carrier screening.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked neuromuscular disorder with a prevalence of 1 in 3500 live-born males, and a reported 15.9-19.5 cases per 100, 000 live male births in the USA and UK respectively. Duchenne muscular dystrophy is caused by pathogenic variants of dystrophin gene which encodes a 427-kDa rod-shaped cytoskeletal protein (1-4). Approximately 65% of DMD patients have deletions, 7-10% have duplications, and 25% have point mutations in the dystrophin gene. These mutations can either be together or alone. One-third of all DMD cases are from de-novo mutations with no family history. Both deletions and duplications are preferentially clustered in two areas: the amino-terminal (exons 3–7) and the central (exons 44–55) regions. Because of this phenomenon, these exons are referred to as "hot-spot points" (4-6).

Conventional PCR methods can detect exon deletions in the dystrophin gene of affected males, but these methods cannot provide detection for women who are carriers. Carrier status in family members is frequently determined by haplotype analysis, dosage analysis with Southern blots, fluorescence in situ hybridization, use of gel electrophoresis to separate PCR products, or other semi-quantitative methods. Also, multiplex ligation-dependent probe amplification (MLPA) methods have been used for detecting mutations in the dystrophin gene (2-5, 7-10). For fluorescently-labeled protocols, laboratories should prepare different PCR reaction set-ups. There is no single-tube approach available for detection of deletion/

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duplication mutations in hot-spot regions. This study's aim is to establish a relative quantitative fluorescent PCR technique to screen for exon deletion/duplication mutations in hot-spot regions of the dystrophin gene using one streamlined approach. We combined two traditional conventional multiplex PCR reactions in a single PCR tube, and we included an STR marker as an internal standard for reaction controls.

MATERIAL and METHODS

Selection of samples and DNA isolation

In this study we included twenty-four families who had a family member diagnosed at clinical and molecular levels as having DMD at Istanbul University, Istanbul School of Medicine, Neurology Department and Genetic Diagnosis Center. We retrospectively analyzed the conventional multiplex PCR results of index cases and also prospectively analyzed the relatives' samples for estimation of carrier status. We screened fifty-one cases in twenty-four families for DMD-exon deletions and carrier status. Detailed information about the patients and their deletion sites are given in Table 1.23. Patients who had exon deletions in the dystrophin gene, plus twenty-four mothers, two sisters and two cousins of index cases, were included in the study, but we could not obtain a blood sample from one index case. We also included sixty healthy female DNA samples for control and for statistical and validation analysis. Informed consent was obtained for each patient accepted by the local Istanbul University, Cerrahpasa School of Medicine Ethics Committee.

We used InstaGene Matrix (Bio-Rad) kit for DNA isolation and performed DNA isolation from the blood samples according to the manufacturer's instructions. For detection of PCR linearity and detection of concentration, we randomly selected twelve DNA samples quantified by real-time PCR. For this test, we used the Quantifiler Real-Time PCR kit (Applied Biosystems). With this method, we can estimate the actual mid-log phase of the samples, which is essential for gene-dosage analysis. We defined Cycle 33 as the mid-log phase for our samples (Figure 1). The rest of the DNA samples' concentrations were measured by UV spectrophotometer. The DNA samples were stored at - 20°C until use.

Primers and PCR Set-Up

One of our goals in this work to develop an assay was to allow the screening of exon deletion/duplication mutations in hot-spot regions of the dystrophin gene within a single-tube format. For detection of exon deletions in DMD cases, mainly two multiplex PCR assays are available: the Chamberlain set and the Beggs set (11,12). While these two multiplex PCR assays cover the hot-spot regions of the dystrophin gene, their primer sets have large Tm differences which do not allow multiplex PCR amplification within a single-tube. We rearranged these primers' Tm values by adding or removing suitable nucleotides at the 5' end of the primers using Primer Express Software v3.0 (Applied Biosystems). After redesigning the primers, the forward primers were labeled with fluorochromes 6-carboxyfluorescein (FAM), or hexachloro-6-carboxyfluorescein (HEX) for automated post-PCR analysis. At the same time, primers of Amelogenin gene and X22, DXS1236 STR markers were added to the primer set as internal standards for controlling the reactions and calculations in each multiplex PCR. These primer pairs are described in Table 2, and the distribution of Tm values are given in Figure 2. The primers were ordered from TIB MOLBIOL (Germany).

Multiplex PCR amplification was performed using 10x GoldST*R Buffer (Promega). Each reaction was carried out in a 0.2 ml thin wall tube with a total volume of 25 µl and a mix containing 2.5 µl of 10x buffer, 5 µl of primer mix (10mM each primer, stock), 12 µl of ddH2O and 0.5 µl AmpliTaqGold (Applied Biosystems) taq polymerase enzyme. The master mix was aliquoted in tubes into which 5 l (1-10 ng) of template DNA was added. The PCR thermal cycling was done on a GeneAmp 9700 (Applied Biosystems) thermal cycler, with incubation at 95°C for 10 min, 33 cycles consisting of denaturation at 95°C for 45 sec, annealing at 57°C for 1 min, and elongation at 72°C for 1 min 20 sec. We performed a final extension at 60°C for 60 min.

Capillary Electrophoresis and Data Interpretation

For separation of PCR products, gel electrophoresis was used. The programming was as follows: 15 KV at 60°C for 30 min on an ABI Prism 310 Genetic Analyzer, using the POP-4™ polymer. We used GeneMapper 4.0 (Applied Biosystems) software for analysis of amplification peaks. If we could not detect a peak belonging to a particular exon, we reported this situation as a deletion. For carrier screening, capillary electrophoresis data were converted to Excel (Microsoft), and the Pr (Peak Ratio) values were calculated with the formula below to control for non-carrier female samples (PrC) and possible carrier female samples (PrU= Peak Ratio Unknown) with an index case in the family:

Pr = (Peak area of exon / Sum of all peak areas) * 100

After calculation of PrC, we calculated the normal distribution of PrC values for each exon peak with Gaussian Distribution Analysis. We called this value PrCa (peak ratios for control avarice). For this purpose we used an Excel macro downloaded from http://www.vertex42.com/ExcelArticles/mc /Files/NormalDistribution.xls. We divided PrU into PrCa for an estimation of carrier status, and we calculated the diagnosis index (DI) for each suspected case. Hypothetically, DI (the ratio between PrU and PrCa for each exon) should be 0.5 for a single gene copy (deletion on carriers), 1 for a double gene copy (normal individual) and 1.5 for a tree gene copy (duplication).

Results

Our re-designed primer sequences are given in Table 1. The final mean Tm was 59.35°C (minimum 58.20°C and maximum 60.00°C) with a standard deviation of 0.44°C. Deletion mutations could be efficiently determined by the

absence of the exon peak of interest (Figure 3). In this study, we investigated twenty-three index cases with different deletion mutations in hot-spot regions. These cases were from twenty-four families (Table 1). With one family, we could not obtain blood sample from the index case but obtained a blood sample from his mother. We could completely detect previously reported deletion mutations in nineteen index cases. In four of the twenty-three index cases, we detected additional deletions; in Family 3, we detected an Exon 13 deletion, in Families 4 and 12, we detected Exon 49 deletions as additional deletions. In Family 10, we detected deletions for Exons 48-52, whereas we didn't find a deletion on Exon 47, which had previously been reported. All additional deletions were neighbor to previously deleted regions, so we could conclude that our results overlapped with previous patient results. After our gene-dosage analysis of the twenty-four families, nineteen (79%) mothers were found to be carriers, and five (21%) mothers (in Families 1,3,11,12 and 14) were not carriers. We investigated four female relatives of index cases (two sisters and two cousins). For the given mutations (Table 2), we defined the sister of the index case as a carrier and the maternal female cousin as a non-carrier in Family 2, the sister of the index case as a carrier in Family 4, and the maternal female cousin as a carrier in Family 6.

For detection of carriers we used gene-dosage testing, but we also included two STRs (X22 and DXS1236) and amelogenin gene amplification primers in our test. DXS1236 amplification failed during our optimization tests, so we excluded DXS1236 from our multiplex setting. We added these regions to our test for a second control of deletion carriers (we used total peak areas in this study; we planned these markers as back-up controls). Our second purpose was to control maternal cell contamination and sex definition with these markers in prenatal samples. After our validation study with sixty healthy female samples, we found that the average peak ratio was 88.75%. The X<X min distribution rate was 10.27%, indicating PCR failure (due to the PCR inhibitor, insufficient DNA etc.). These tests can be repeated with suitable DNA samples. Thus, we conclude that our test can reflect 99.02% of normal samples, meaning that we can detect carriers with 99.02% accuracy.

Table 1. Re-designed primers

Exon	Direciton	Sequences	Tm oC	Amplicon Size(bp)
PM	F	CTAGACAGTGGATACATAACAAATGCATG	60	518
	R	GTAATTGCCTCCCAGATCTGAGTC	59.1	
3	F	TCCRTCATCTTCGGCAGATTA	59.3	401
	R	CGGTAGAGTATGCCAAATGAAAATC	59	
4	F	CGGTCTCTCTGCTGGTCAGTG	59.6	189
	R	AGCCCTCACTCAAACATGAAGC	59.4	
6	F	CCACATGTAGGTCAAAAATGTAATGAA	59.2	204
	R	ATGTCTCAGTAATCTTCTTACCTATGACTATGG	60	
8	F	TTTAGGCCTCATTCTCATGTTCTAATTAG	59.5	366
	R	CTGTCCTTTACACACTTTACCTGTTGAG	59.6	
12	F	TGATAGTGGGCTTTACTTACATCCTTC	59.1	331
	R	AAAGCACGCAACATAAGATACACCT	59.2	
13	F	TTGGCTTGGAATGGTTTTAGGTT	59.7	153
	R	CTTGAAGCACCTGAAAGATAAAATGTT	59.2	
17	F	ACTTTCGATGTTGAGATTACTTTCCC	59	413
	R	AGCTTGAGATGCTCTCACCTTTTC	59.2	
19	F	ATGGCAAAAGTGTTGAGAAAAAGTC	58.8	456
	R	CTACCACATCCCATTTTCTTCCA	58.8	
43	F	TGTCAAAGTCACTGGACTTCATGG	60	346
	R	GTGTTACCTACCCTTGTCGGTCC	59.6	
44	F	TCTTGATCCATATGCTTTTACCTGC	59.5	264
	R	CATCACCCTTCAGAACCTGATCT	58.2	
45	F	ATGGAACATCCTTGTGGGGAC	59.5	543
	R	CATTCCTATTAGATCTGTCGCCCTAC	59.4	
47	F	GTTGTTGCATTTGTCTGTTTCAGTTAC	59.1	180
	R	GTCTAACCTTTATCCACTGGAGATTTG	58.6	
48	F	TTGAATACATTGGTTAAATCCCAACAT	59.6	506
	R	TCCTGAATAAAGTCTTCCTTACCACAC	59.1	
49	F	CCCTTATGTACCAGGCAGAAATTG	59.7	436
	R	GCAATGACTCGTTAATAGCCTTAAGATC	59.9	
50	F	CACCAAATGGATTAAGATGTTCATGA	59.9	267
	R	TCTCTCACCCAGTCATCACTTCATAG	59.7	
51	F	GAAATTGGCTCTTTAGCTTGTGTTTC	59.7	387
	R	GGAGAGTAAAGTGATTGGTGGAAAAT	58.9	
52	F	AATGCAGGATTTGGAACAGAGG	59.1	104
	R	CGATCCGTAATGATTGTTCTAGCC	59.8	
60	F	AAATTGCGCCTCTGAAAGAGAAC	59.9	134
	R	AGAAGCTTCCATCTGGTGTTCAG	58.5	
	F			
	R			

Table 2. Results of patients and cases in this study

Family Number	Previously Reported Deletions	Sample No	Sample Decription	Result of Current Method
1	del8_50 c.650_7309del	1	Mother	Non-Carrier
		2	Index Case	c.6763_7098del
2	del47_48 c.6763_7098del	3	Mother	c.6763_7098del (Carrier)
		4	Sister	c.6763_7098del (Carrier)
		5	Female Cousin	Non-Carrier
3	del3_6 c.94_530del	6	Index Case	c.94_530del c.1483_1602del (del13)
		7	Mother	Non-Carrier
4	del50 c.7201_7309del	8	Index Case	c.7099_7309del (del49_50)
		9	Mother	c.7099_7309del (Carrier)
		10	Sister	c.7099_7309del (Carrier)
5	del45_50 c.6439_7309del	11	Index Case	c.6439_7309del
		12	Mother	c.6439_7309del (Carrier)
6	delDp427m c.-244_31+?del	13	Index Case	c.-244_31+?del
		14	Mother	c.-244_31+?del (Carrier)
		15	Female Cousin	c.-244_31+?del (Carrier)
7	del3_19 c.94_2380del	16	Index Case	c.94_2380del
		17	Mother	c.94_2380 (Carrier)
8	del45_47 c.6439_6912del	18	Index Case	.6439_6912del
		19	Mother	.6439_6912del (Carrier)
9	del45_50 c.6439_7309del	20	Index Case	c.6439_7309del
		21	Mother	c.6439_7309del (Carrier)
10	del47_52 c.6763_7660de	22	Index Case	c.6913_7660del (del48_52)
		23	Mother	c.6913_7660del (Carrier)
11	del45 c.6439_6613 del	24	Index Case	c.6439_6613del
		25	Mother	Non-Carrier
12	del50 c.7201_7309del	26	Index Case	c.7099_7309del (del49_50)
		27	Mother	Non-Carrier
13	del45 c.6439_6613 del	28	Index Case	c.6439_6613 del
		29	Mother	c.6439_6613 del (Carrier)
14	del4_8 c.187_831del	30	Index Case	c.187_831del
		31	Mother	Non-Carrier
15	del47_52 c.6763_7660del	32	Index Case	c.6763_7660del
		33	Mother	c.6763_7660del (Carrier)
16	del50 c.7201_7309del	34	Index Case	c.7201_7309del
		35	Mother	c.7201_7309del (Carrier)
17	del45_50 c.6439_7309de	36	Index Case	c.6439_7309del
		37	Mother	c.6439_7309del (Carrier)
18	del49_50 c.7099_7309del	38	Index Case	c.7099_7309del
		39	Mother	c.7099_7309del (Carrier)
19	del50 c.7201_7309del	40	Index Case	c.7201_7309del
		41	Mother	c.7201_7309del (Carrier)
20	del8_12 c.650_1482del	42	Index Case	c.650_1482del
		43	Mother	c.650_1482del (Carrier)
21	del45_50 c.6439_7309del	44	Index Case	c.6439_7309del
		45	Mother	c.6439_7309del (Carrier)
22	del8_12 c.650_1482del	46	Index Case	c.650_1482del
		47	Mother	c.650_1482del (Carrier)
23	del13 c.1482_1602del	48	Index Case	c.1482_1602del
		49	Mother	c.1482_1602del (Carrier)
24	del48 c.6913_7098del	50	Index Case	c.1482_1602del (Carrier)
		51	Mother	c.6913_7098del

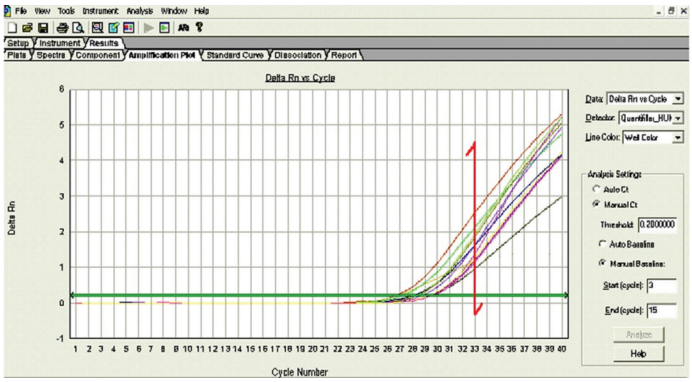

Figure 1. Pre-multiplex PCR validation and quantification of DNA samples

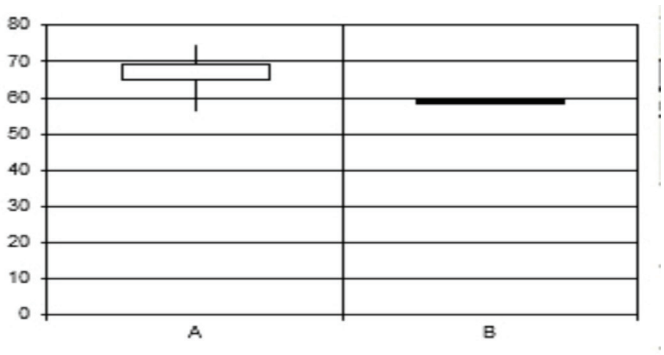

Figure 2. Tm differences of primers, A original sets B re-designed sets

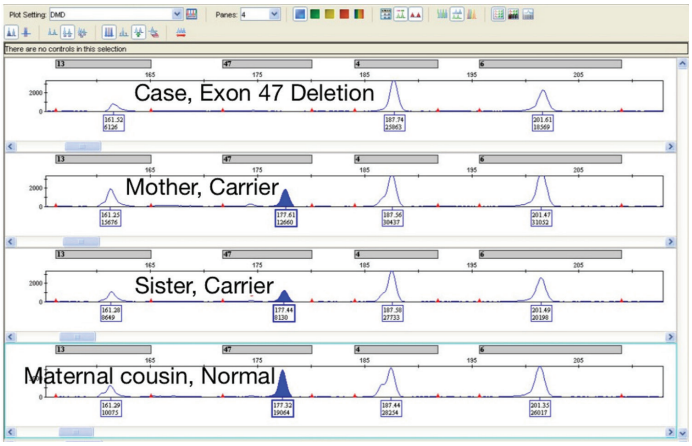

Figure 3. Electropherogram examples of samples

DISCUSSION

Herein we describe a novel, easier and faster multiplex quantitative PCR method to detect deletions or duplications for DMD carrier status. Furthermore, the developed method depends on capillary electrophoresis, which is convenient for analyzing large numbers of samples and their accompanied high sample throughputs. Compared with other methods, our method is a straightforward and inexpensive constant quantification method for measuring gene copy numbers. Even though our developed method does not cover the entire dystrophin gene, it is fast (multiplex PCR is amplified in 2.5 h, and the capillary gel electrophoresis takes about 30 min) and easy to perform for screening.

Duchenne muscular dystrophy in males is an often lethal neuromuscular genetic disorder without an efficient treatment. Up to now, therapy strategies have focused on identifying DMD carriers for early detection. Because DMD has an X-linked inheritance pattern, female relatives of DMD patients have a potential risk of being carriers. Although the detection of exon deletions in the dystrophin gene through conventional PCR methods can be used to analyze affected males, it can't be used to detect carrier status of females (8,10).

In the past, carrier status was frequently checked by haplotype analysis, fluorescence in situ hybridization, or separation of quantitative PCR products via gel electrophoresis (3,10,13-15).

Carrier status in family members could be assessed using different techniques, but a search of current literature indicates that the most promising technique is MLPA. When we compare MLPA and our QF-PCR test, the biggest advantages of MLPA are that it is commercially available and can screen all 79 exons. On the other hand, the MLPA technique requires more DNA, cannot detect maternal cell contamination, and is a more difficult and labor insensitive technique. Lai et al. compared the results from MLPA and conventional multiplex PCR assay, they found that two single deletion cases found by the multiplex PCR assay were detected as normal by the MLPA technique and they concluded that single exon deletion results from an MPLA should be confirmed by another technique (2,7,10,16,17). In this study, we have performed a new quantitative PCR-based detection method to identify deletion and duplication mutations in affected males and female carriers. Unlike other published studies, we combined the hot-spot regions in a single-tube amplification format, which could reduce laboratory handling time and cost for this technique. We also reduced the amount of DNA samples required for this test, which may be especially important in pre-natal diagnosis cases.

Multiplex PCR approaches may allow for the detection of different targets within a single tube. However, multiplexing procedures may include big challenges: the nature of PCR primers can lead to nonspecific or insufficient amplifications due to Tm differences (12,18). In our study, previously described primer sets had large Tm variations. After our adjustments, single tube multiplex amplifications are allowed.

Steroids can be used as a palliative therapy for DMD progression, but there is currently no effective cure. In the last decade, different strategies have been studied, including antisense oligonucleotides, the molecules most used for RNA modulation. New technological developments are enhancing both diagnosis and therapeutic options for DMD patients. For the group with nonsense mutations, the European-approved molecule drug Translarna (ataluren) has been released. At the same time, genetic mutations may also be targeted with antisense-mediated exon skipping approaches (8,10).

CONCLUSION

In conclusion, this study showed an efficient, fast, and convenient semi-quantitative fluorescent PCR method and its clinical application for the molecular diagnosis of DMD patients and female carriers with deletion or duplication mutations. This method detects 75% of the mutations in DMD families and can be efficiently adapted to screen for female carriers even when DMD patient data are not available.

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Competing interests: The authors declare that they have no competing interest.

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Ethics Committee: This work has been approved by the Istanbul University, Cerrahpasa School of Medicine Ethics Committee.

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REFERENCES

1. Alcantara MA, Garcia-Cavazos R, Hernandez UE, et al. Carrier detection and prenatal molecular diagnosis in a Duchenne muscular dystrophy family without any affected relative available. Ann Genet 2001;44:149-53.
2. Birnkrant DJ, Bushby K, Bann CM, et al. Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and neuromuscular, rehabilitation, endocrine, and gastrointestinal and nutritional management. Lancet Neurol 2018;17:251-67.
3. Frisso G, Carsana A, Tinto N, et al. Direct detection of exon deletions/duplications in female carriers of and male patients with Duchenne/Becker muscular dystrophy. Clin Chem 2004;50:1435-8.
4. Hung CC, Chen CP, Lin SP, et al. Quantitative assay of deletion or duplication genotype by capillary electrophoresis system: Application in Prader-Willi syndrome and Duchenne muscular dystrophy. Clin Chem 2006;52:2203-10.
5. Grimm T, Muller B, Dreier M, et al. Hot spot of recombination within DXS164 in the Duchenne muscular dystrophy gene. Am J Hum Genet 1989;45:368-72.
6. Echigoya Y, Lim KRQ, Nakamura A, et al. Multiple exon skipping in the duchenne muscular dystrophy hot spots: Prospects and Challenges. J Pers Med 2018;8.
7. Gatta V, Scarciolla O, Gaspari AR, et al. Identification of deletions and duplications of the DMD gene in affected males and carrier females by multiple ligation probe amplification (MLPA). Hum Genet 2005;117:92-8.
8. Aartsma-Rus A, Ginjaar IB, Bushby K. The importance of genetic diagnosis for Duchenne muscular dystrophy. J Med Genet 2016;53:145-51.
9. Nakamura A. Mutation-Based Therapeutic Strategies for Duchenne Muscular Dystrophy: From Genetic Diagnosis to Therapy. J Pers Med 2019;9.
10. Falzarano MS, Scotton C, Passarelli C, et al. Duchenne Muscular Dystrophy: From Diagnosis to Therapy. Molecules 2015;20:18168-84.
11. Chamberlain JS, Gibbs RA, Ranier JE, et al. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 1988;16:11141-56.
12. Henegariu O, Heerema NA, Dlouhy SR, et al. Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques 1997;23:504-11.
13. Dolinsky LC, de Moura-Neto RS, Falcao-Conceicao DN. DGGE analysis as a tool to identify point mutations, de novo mutations and carriers of the dystrophin gene. Neuromuscul Disord 2002;12:845-8.
14. Ligon AH, Kashork CD, Richards CS, et al. Identification of female carriers for Duchenne and Becker muscular dystrophies using a FISH-based approach. Eur J Hum Genet.2000;8:293-8.
15. Chaturvedi LS, Srivastava S, Mukherjee M, et al. Carrier detection in non-deletional Duchenne/Becker muscular dystrophy families using polymorphic dinucleotide (CA) repeat loci of dystrophin gene. Indian J Med Res 2001;113:19-25.
16. Janssen B, Hartmann C, Scholz V, et al. MLPA analysis for the detection of deletions, duplications and complex rearrangements in the dystrophin gene: potential and pitfalls. Neurogenetics 2005;6:29-35.
17. Lai KK, Lo IF, Tong TM, et al. Detecting exon deletions and duplications of the DMD gene using Multiplex Ligation-dependent Probe Amplification (MLPA). Clin Biochem. 2006;39:367-72.
18. Elnifro EM, Ashshi AM, Cooper RJ, et al. Multiplex PCR: optimization and application in diagnostic virology. Clin Microbiol Rev 2000;13:559-70.