

QUANTITATIVE ANALYSIS OF THE DYSTROPHIN GENE BY REAL-TIME PCR

NELA MAKSIMOVIC¹, ANA ANDJELKOVIC¹, VEDRANA MILIC RASIC²,
VIDOSAVA RAKOCEVIC STOJANOVIC³, BILJANA KASTRATOVIC KOTLICA⁴, S. BRANKOVIC⁵,
TATJANA DAMNJANOVIC¹, BILJANA JEKIC¹, VERA BUNJEVACKI¹, LJILJANA LUKOVIC¹,
DIJANA PEROVIC¹, SUZANA CVJETICANIN¹ and IVANA NOVAKOVIC¹

¹ Institute of Human Genetics, Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia

² Child and Adolescent Neurology and Psychiatry Clinic, Faculty of Medicine, University of Belgrade,
11000 Belgrade, Serbia

³ Clinic of Neurology CCS, Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia

⁴ Clinic of Obstetrics and Gynecology, Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia

⁵ Faculty of Science and Mathematics, University of Prishtina (Kosovska Mitrovica), 28000 Kosovska Mitrovica, Serbia

Abstract - Duchenne and Becker muscular dystrophy (DMD/BMD) are severe X-linked neuromuscular disorders caused by mutations in the dystrophin gene. Our aim was to optimize a quantitative real-time PCR method based on SYBR® Green I chemistry for routine diagnostics of DMD/BMD deletion carriers. Twenty female relatives of DMD/BMD patients with previously detected partial gene deletions were studied. The relative quantity of the target exons was calculated by a comparative threshold cycle method ($\Delta\Delta Ct$). The carrier status of all subjects was successfully determined. The gene dosage ratio for non-carriers was 1.07 ± 0.20 , and for carriers 0.56 ± 0.11 . This assay proved to be simple, rapid, reliable and cost-effective.

Key words: Duchenne/Becker muscular dystrophy, Real-time PCR, SYBR® Green, $\Delta\Delta Ct$ method, carrier detection

INTRODUCTION

Duchenne and Becker muscular dystrophies (DMD and BMD) are X-linked neuromuscular disorders with incidences of 1:3500 and 1:30000 male births, respectively (Emery, 1991). DMD and BMD are caused by mutations in the dystrophin gene (DMD gene) located at the Xp21.2 region. Approximately 65-70% of DMD and 85% of BMD patients show intragenic deletions of one or several exons of the gene. Most of the mutations are located at the proximal (exon 3-7) and central (exon 45-52) regions of the gene (Prior, 2005; Hu, 1990; <http://www.umd.be>).

These regions are known as “deletional hotspots”. A small proportion of the mutations (6%) involve duplications and in the remaining DMD/BMD cases, the disease emerges as a result of small mutations, including point mutations or microdeletions/insertions (Hu et al., 1988; Nishino, 2002). Mutations either are inherited from asymptomatic female carriers (70%), occur *de novo* or arise from germline mosaicism (30%) (Worton, 1988).

Due to the X-linked nature of the disorders, males carrying the mutated gene are affected, and females are the carriers of the disease. The absence

of an efficient treatment for these progressive disorders makes the determination of carrier status very important for genetic counseling and prevention of the disease. Multiplex PCR technique has been widely used for the detection of common deletions in affected males (Beggs et al., 1990; Chamberlain et al., 1988). However, carrier identification in asymptomatic female relatives of deletional DMD/BMD probands is still difficult due to the presence of both normal and mutant copies of the gene. During the past decades, several biochemical and molecular methods have been developed in order to overcome this problem. Such methods include the measurement of the serum creatinine phosphokinase (CPK) level, immunohistochemical methods (Panigarhi, 2001), DNA-based linkage analysis (Clemmens et al., 1991), Southern blotting (Den Dunnen et al., 1989), fluorescent *in situ* hybridization (FISH) (Ligon et al., 2000) and reverse transcriptase-polymerase chain reaction (RT-PCR) (Roberts et al., 1991). All these approaches are time-consuming and may suffer from limited sensitivity. Recently, multiplex ligation-dependent probe amplification assay (MLPA) (Gatta et al., 2005), multiplex amplifiable probe hybridization assay (MAPH) (White et al., 2002) and quantitative real-time PCR have been suggested as alternative methods to the current diagnostic approaches.

In the present study, we have optimized the quantitative real-time PCR assay based on SYBR® Green chemistry for determining the dosage of DMD gene exons 6, 47, 52 for direct DMD/BMD carrier detection. Quantitative real-time PCR is a high precision approach for gene dosage studies that displays digital data corresponding to the changes at gene level (Wilhelm, 2003; Nosaeid, 2009; Ruiz-Ponte et al., 2006).

MATERIALS AND METHODS

Patients

Blood samples were obtained from 2 unrelated obligate female carriers (characterized by two affected DMD sons in one case and by affected BMD father in the other) and 18 possible carriers from 9 fami-

lies with sporadic cases (characterized by only one affected son or brother). Affected males had partial dystrophin gene deletions involving either exon 6, 47 or 52, previously detected by multiplex PCR. In parallel, normal female controls were used as wild-type reference. Informed written consents were obtained from all individuals participating in this study.

DNA extraction

Genomic DNA was extracted from 5 ml peripheral blood by standard salting-out (Miller et al., 1988). The concentration and purity of the isolated DNA were determined by measuring the absorbance at 260 and 280 nm. DNAs were diluted in distilled water to acquire a concentration of 6 ng/ μ l.

Real-time quantitative PCR

The sequences of primers used for amplification of exons 6, 47 and 52 were as published on the Leiden muscular dystrophy pages (<http://dmd.nl>). The real-time PCR amplifications were carried out in optical grade 96-well plates (Microamp, Applied Biosystems, USA) in a 15 μ l reaction volume including 13.8 μ l SYBR® Green Master mix (Fermentas, Germany), 0.6 μ l of each primer and 6 ng of DNA. All samples were analyzed in duplicate for both reference and test exon. For each sample, one of the unaffected dystrophin gene exons was used as an endogenous control. In each assay normal control and no-template control were included. PCR reactions were performed on the 7500 real-time PCR system (Applied Biosystems, USA) using the following conditions: preincubation 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing and elongation step at 64°C for 1 min. Each complete amplification stage was followed by a dissociation stage at 95°C for 15 sec, 60°C for 1 min, and then the temperature was ramped up to 95°C. Fluorescence intensity was measured once per cycle at the end of the elongation phase. Amplification was followed by melting curve analysis performed according to the dissociation stage data (Liu et al., 2006). Reactions with a single peak at expected Tm were considered for further analysis.

Data analysis

Data evaluation was performed using the 7500 data analysis software (version 3.5) and Microsoft Excel. Quantitative analysis was performed by comparative Ct. The Ct parameter is defined as the cycle number at which the amplification plot passes a fixed threshold. Mean Ct was the mean Ct value of duplicate amplification. ΔCt was calculated as the difference between the Ct values of the DMD test exon (exonA) and reference exon (exonB). $\Delta\Delta Ct$ values for each tested individual were calculated using ΔCts of the healthy female as a calibrator following the equation: $\Delta\Delta Ct_{\text{exonA-exonB}} = (\Delta Ct_{\text{exonA-exonB}})_{\text{targetsample}} - (\Delta Ct_{\text{exonA-exonB}})_{\text{calibrator}}$. The relative fold increase (R) in exon quantity in the tested person was calculated using the equation: $R_{\text{exonA/exonB}} = 2^{-(\Delta\Delta Ct_{\text{exonA-exonB}})}$ (Livak, 2001). Using this method, a $\Delta\Delta Ct$ ratio is expected to be about 1 in normal controls, about 0.5 in the females that are carriers of the deletion and 1.5 in females that are carriers of the duplication.

Optimization and validation of real-time PCR assay

In order to validate the $\Delta\Delta Ct$ method for the relative quantification of exons 6, 47 and 52, genomic DNA (gDNA) was isolated from the blood of a healthy female and 2-fold serial dilutions ranging from 50 to 3.125 ng/ μ l were generated (50, 25, 12.5, 6.25, 3.125 ng/ μ l). For each dilution, three different PCR reactions were set up. Each reaction contained a pair of primers specific for one of the three exons of interest, and was run in duplicate. The ΔCt values for each exon pair were calculated ($Ct_{\text{exon6}} - Ct_{\text{exon47}}$, $Ct_{\text{exon6}} - Ct_{\text{exon52}}$, $Ct_{\text{exon47}} - Ct_{\text{exon52}}$) and plotted against the logarithm of the amount of gDNA in each dilution. Afterwards, trend lines were drawn using Excel software (Microsoft).

RESULTS

Real-time PCR, based on SYBR® Green I chemistry was optimized for the detection of deletions of exons 6, 47 and 52 within the dystrophin gene. In order to assess the validity of this comparative threshold cycle method for relative quantification,

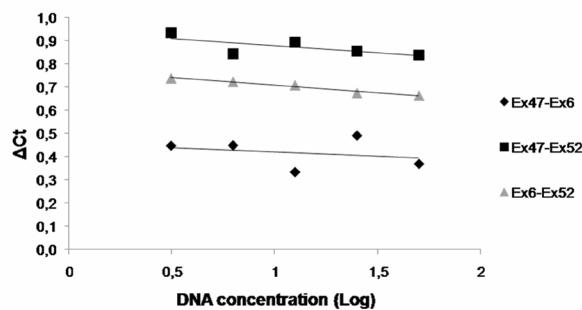


Fig. 1 Validation of the $\Delta\Delta Ct$ method for the relative quantification of exons 6, 47 and 52. The equations of the trend lines are: Ex6-52: $y=-0,038X+0,459$; Ex 47-52: $y= -0,066+0,774$; Ex47-6: $y= -0,060+0,937$.

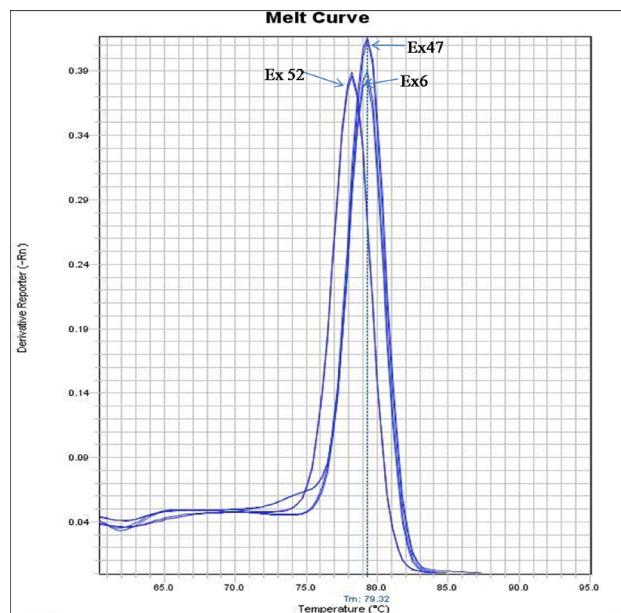


Fig. 2 Melting curve analysis of real-time PCR for DMD exons 6, 47 and 52.

standard curves for target and reference exons were prepared over serially diluted genomic DNA samples. The mean Ct values corresponding to each concentration were plotted against the log input of DNA. Trend line slopes were within an acceptable range ($-0.1 < \text{slope} < 0.1$), the PCR efficiencies of the target and the reference exons were approximately equal, which was a prerequisite for the accurate copy number assessment (Fig. 1). To observe the presence of any possible primer dimer or non-specifically amplified product formation, which

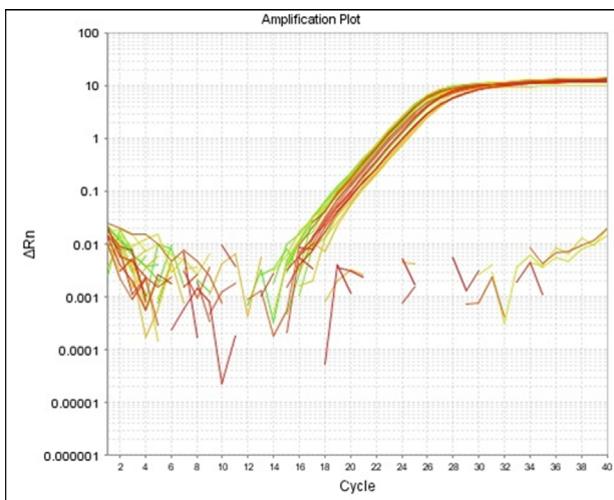


Fig. 3 Amplification curve analysis for DMD exons 6, 47 and 52,

usually creates additional peaks separate from the specific amplicon, dissociation curve analysis was performed for each amplification reaction. Results (single PCR product without any amplification of non-specific products is seen as one single sharp peak on the melting curve or one single band on agarose gels) showed that no detectable non-specific products were present in the reactions (Fig. 2). The duplicate runs of the exons of interest showed an almost complete overlap of parallel amplification plots (for all samples, the SD of Ct value was very low range) (Fig. 3). In total, samples from 20 individuals with known or unknown carrier status were tested using real-time PCR. The deletions of target exons were confirmed in 2 obligatory carriers and 8 out of 18 (44.44%) of suspected carriers. The gene dosage ratio (R) for non-carriers was 1.07 ± 0.20 and for carriers 0.56 ± 0.11 . The ratio ranges between carriers and non-carriers of the deletions did not overlap, allowing the accurate discrimination of deletion carriers and normal individuals.

DISCUSSION

Due to the lack of efficient rehabilitation and treatment of progressive muscular dystrophy, counseling and prenatal diagnosis are the only available options nowadays. However, molecular diagnosis and car-

rier analysis of DMD/BMD is still difficult due to the size of the dystrophin gene, mutation diversity and the presence of a normal allele in female carriers. The aim of this study was to develop a rapid and reliable real-time assay for direct DMD/BMD carrier detection that in the future may be used for routine diagnostics. We selected exons 6, 47 and 52 of the dystrophin gene since they are located in two mutational hotspots of this gene. The assay is based on SYBR® Green I chemistry. The possibility that SYBR® Green I dye generated false positive signals due to its binding to nonspecific double stranded DNA was ruled out by performing melting curve analysis or gel electrophoresis of the PCR products. Using real-time PCR assay the carrier status of all subjects was successfully determined. The gene dosage ratio ranges did not overlap, allowing the accurate discrimination of carriers and non-carriers. However, we had access only to carriers of the deletions, so it has yet to be assessed whether the assay is able to determine the duplication carriers' status.

In our study, we did not choose an endogenous control outside the dystrophin gene. Instead, one of the unaffected dystrophin gene exons was used to quantify the other exon. This offered the possibility of internal quality control in diagnostics after the identification of a deletion and a different combination of test and reference exon for confirmation of the results (Joncourt et al., 2004).

Multiplex end-point PCR approaches such as MAPH, MLPA and EPFA, can be effectively used for the rapid screening of deletions and duplications in the entire DMD coding region in both affected males and female carriers (Traverso et al., 2006). Once the mutation is known, real-time assay allows rapid identification of carrier status in a single PCR within few hours. Furthermore, the results obtained by end-point PCR could be confirmed by using an independent set of primers. Thus, a combination of these different assays may provide secure and correct diagnosis of DMD/BMD.

SYBR® Green-based real-time PCR is highly sensitive, specific and reproducible, requires a minimal

quantity of DNA and is carried out in a single step (Traverso et al., 2006). The simplicity of the protocol allows its easy implementation in diagnostic laboratories. For routine application of this technique in the diagnosis of DMD/BMD it is necessary to develop additional assays for the analysis of other exons frequently involved in DMD rearrangements. Furthermore, it may be easily adapted for the screening of other genetic diseases that caused by deletions and duplications.

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REFERENCES

- Beggs, A.H., Koenig, M., Boyce, F.M., and L.M. Kunkel (1990). Detection of 98% of DMD/BMD gene deletions by polymerase chain reaction. *Hum. Genet.* **86**, 45–48.
- Chamberlain, J.S., Gibbs, R.A., Ranier, J.E., Nguyen, P.N., and C.T. Caskey (1988). Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Res.* **16**, 141–156.
- Clemens, P.R., Fenwick, R.G., Chamberlain J.S., Gibbs R.A., de Andrade M., Chakrabarty R., and C.T. Caskey (1991). Carrier detection and prenatal diagnosis in Duchenne and Becker dystrophy families, using dinucleotide repeat polymorphisms. *Am. J. Hum. Genet.* **49**, 951–960.
- Den Dunnen, J.T., Grootscholten, P.M., Bakker, E., Blonden, L.A., Ginjaar, H.B., Wapenaar, M.C., van Paassen, H.M., van Broeckhoven, C., Pearson, P.L., and van G.J. Ommen (1989). Topography of the DMD gene: FIGE and cDNA analysis of 194 cases reveals 115 deletions and 13 duplications. *Am. J. Hum. Genet.* **45**, 835–847.
- Gatta, V., Scarciolla, O., Gaspari, A.R., Palka, C., De Angelis, M.V., Di Muzio, A., Guanciali-Franchi, P., Calabrese, G., Uncini, A., and L. Stuppia (2005). Identification of deletions and duplications of the DMD gene in affected males and carrier females by multiple ligation probe amplification (MLPA). *Hum. Genet.* **117**, 92–98.
- http://www.umd.be/DMD/W_DMD/index.html
- Hu, X.Y., Burghes A.H., Ray, P.N., Thompson, M.W., Murphy, E.G., and R.G. Worton (1988). Partial gene duplication in Duchenne and Becker muscular dystrophies. *J. Med. Genet.* **25**, 369–376.
- Hu, X.Y., Ray, P.N., Murphy, E.G., Thompson, M.W., and R.G. Worton, (1990). Duplication mutation at the Duchenne muscular dystrophy locus: its frequency, distribution, origin, and phenotypegenotype correlation. *Am. J. Hum. Genet.* **46**, 682–695.
- Joncourt, F., Neuhaus, B., Jostarndt-Foegen, K., Kleinle, S., Steiner, B., and S. Gallati (2004). Rapid identification of female carriers of DMD/BMD by quantitative real-time PCR. *Hum. Mutat.* **23**, 385–391.
- Leiden muscular dystrophy pages (<http://dmd.nl>)
- Ligon, A.H., Kashork C.D., Richards C.S., and L.G. Shaffer (2000). Identification of female carriers for Duchenne and Becker muscular dystrophies using a FISH-based approach. *Eur. J. Hum. Genet.* **8**, 293–298.
- Liu, J., Yan, M., Wang, Z., Wang, L., Zhou, Y., and B. Xiao (2006). Molecular diagnosis of alpha-thalassemia by combining real-time PCR with SYBR® GreenI and dissociation curve analysis. *Transl. Res.* **148**, 6–12.
- Livak, K. J., and T.D. Schmittgen (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. *Methods.* **25**, 402–408.
- Miller, S. A., Dykes, D. D., and H. F. Polesky (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* **16**, 1215.
- Nishino, I., and E. Ozawa (2002). Muscular dystrophies. *Curr. Opin. Neurol.* **15**, 539–544.
- Nosaeid, M.H., Mahdian, R., Jamali, S., Maryami, F., Babashah, S., Maryami, F., Karimipoor, M., and S. Zeinali (2009). Validation and comparison of two quantitative real-time PCR assays for direct detection of DMD/BMD carriers. *Clin. Biochem.* **42**, 1291–1299.
- Panigrahi, I., and B. Mittal (2001). Carrier detection and prenatal diagnosis in Duchenne/Becker muscular dystrophy. *Indian Pediatr.* **38**, 631–639.
- Prior, T.W., and S.J. Bridgeman (2005). Experience and strategy for the molecular testing of Duchenne muscular dystrophy. *J. Mol. Diagn.* **7**(3), 317–326.
- Roberts, R.G., Barby, T.F., Manners, E., Bobrow, M., and D.R. Bentley (1991). Direct detection of dystrophin gene rearrangements by analysis of dystrophin mRNA in peripheral blood lymphocytes. *Am. J. Hum. Genet.* **49**, 298–310.
- Ruiz-Ponte, C., Carracedo, A., and F. Barros (2006). Duplication and deletion analysis by fluorescent real-time PCR-based genotyping. *Clin. Chim. Acta.* **363**, 138–146.
- Traverso, M., Malnati, M., Minetti, C., Regis, S., Tedeschi, S., Pedemonte, M., Bruno, C., Biassoni, R., and F. Zara (2006). Multiplex real-time PCR for detection of deletions and duplications in dystrophin gene. *Biochem. Biophys. Res. Commun.* **339**, 145–150.
- White, S., Kalf, M., Liu, Q., Villerius, M., Engelsma, D., Kriek, M., Vollebregt, E., Bakker, B., van Ommen, G.J., Breuning,

- M.H., and J.T. den Dunnen* (2002). Comprehensive detection of genomic duplications and deletions in the DMD gene, by use of multiplex amplifiable probe hybridization. *Am. J. Hum. Genet.* **71**, 365–374.
- Wilhelm, J., and A. Pingoud* (2003). Real-time polymerase chain reaction. *Chembiochem.* **4**, 1120–1128.
- Worton, R.G., and M.W. Thompson* (1988). Genetics of Duchenne muscular dystrophy. *Annu. Rev. Genet.* **22**, 601–629.