Rapid Detection of Human Dystrophin Gene Mutations by Multiplex Semi-Quantitative PCR (MSQ PCR)

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Abstract

A PCR-based protocol is described for the simultaneous detection of deletions, duplications and some point mutations. The method was applied to 334 DNAs of male subjects affected with Duchenne or Becker muscular dystrophy, screened for mutations of the Dystrophin gene. The PCR amplifications involved the enhancer sequence, the muscular promoter sequence and 30 different exons. In total, 119 intragenic deletions, 21 intragenic duplications and 3 point mutations were detected.

The present method of multiplex semi-quantitative PCR amplification of selected sequences is as reliable as cDNA probing, but more sensitive and much faster. Therefore, it is the method of choice for single-step screening for deletions, duplications, insertions and for those base substitutions which affect the double strand conformation in solution.

Key words: Duchenne, Becker, Dystrophin, mutation, PCR.

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Mutations of the dystrophin gene produce clinical phenotypes corresponding to the classical Duchenne (DMD) or Becker (BMD) muscular dystrophy. Less frequently, the clinical affection may be very mild (myoglobinuria, cramps, elevated serum CK) [4] or expressed more at the cardiac level (dilative cardiomyopathy) than in the skeletal muscles [8, 11].

The detection of an alteration of the DNA sequence in the dystrophin gene is a very important diagnostic element when the clinical presentation is ambiguous. Moreover, the clear definition of the genetic status of a given proband is essential for providing genetic counselling and eventually prenatal diagnosis in at-risk pregnancies of his female relatives.

The dystrophin gene is more than 2.5 Mb long and the coding sequence is 14 Kb, subdivided in 82 exons. About 60% of the mutations are intragenic deletions, 8% are duplications and 32% are base substitutions, deletions or insertions [3].

The advent of PCR technology introduced the method of multiplex amplification of exonic sequences for detection of intragenic deletions [1, 2].

We present here a PCR-based protocol for the simultaneous detection of deletions, duplications and some point mutations.

Material and Methods

DNA was extracted by salting out procedure, as described by Miller [7]. The PCR reactions were performed by a MJ Res.(USA) Thermocycler. Each reaction was carried out in a total volume of 10 ul containing 32 ng of genomic DNA, 3.1 pM of each primer, 120 µM of each dNTP, 1x reaction buffer (67mM Tris HCl, pH 8.8, 16.6 mM (NH4)2SO4, 1.5 mM MgCl2, 0.01% Tween-20) and 0.08 unit of RTB DNA polymerase (AMED, Italy).

The cycling parameters were : a first denaturation step at 94°C for 3 min, followed by 21 cycles (94°C for 30 sec, 65 C for 2 min). The total number of cycles was never exceeding 21, in order to obtain a semi-quantitative PCR.

Primers were provided by GenSet (France) and by MedProbe (USA), while the dNTPs mixture was from Pharmacia.

Multiplex reactions were set up by selecting amplification products which could be clearly separated by electrophoresis without reciprocal interference.

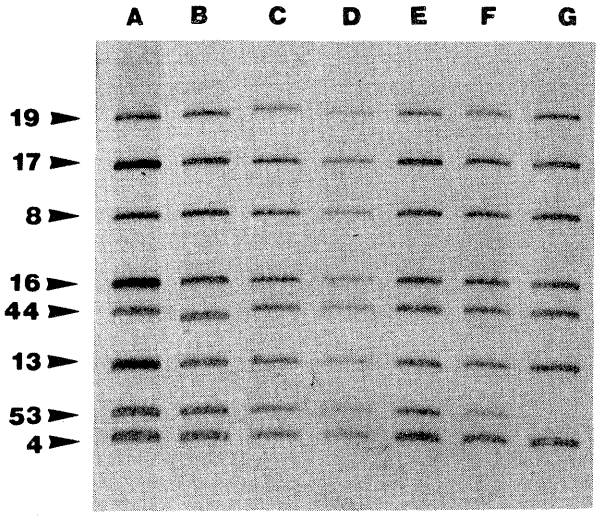

Figure 1. Screening of mutations of the dystrophin gene by high-resolution polyacrylamide gel electrophoresis: intragenic duplication involving exons 13, 16, 17, 19 (lane A), intra-exonic deletion (exon 44, lane B), A → G substitution (exon 19, lane C), positive control (male subject, lane D), positive control (female subject, lane E),heterozygosity for a deletion involving exon 53 (lane F), intragenic deletion involving exon 53 (lane G).

Aliquots of the PCR amplification products were loaded on 10% acrylamide gels (29:1 acrylamide:bis-acrylamide) containing 5% glycerol and run on vertical electrophoresis at 40 mA for 2 hrs.

The polyacrylamide gel slabs were silver stained according to Herring et al. [6]. The gels were placed for 10 min in a 10% ethanol, 0.5% acetic acid aqueous solution and transferred into a 0.17% silver nitrate solution, where they remained for 25 min. After a rinse in distilled water (3 min) the gels were transferred to the developing solution (30 g/l NaOH, 0.027% formaldehyde). When the bands become evident, the reaction was stopped by tranferring the gels in a 5% acetic acid solution. After staining, the gels were dried by a Biorad Gel Dryer (mod. 583) at 60°C for 1 hr.

Results

A typical picture of an electrophoretic separation of multiplex PCR products corresponding to different exons of the Dystrophin gene is shown in Figure 1.

Multiplex amplification enables to detect the presence of intragenic deletions through the failure of amplification of some exons (lane G). Moreover, the semi-quantitative PCR, coupled with the silver staining, gives the opportunity to detect differences in the intensity of single bands which reveal the presence of intragenic duplications (lane A) or dosage effects in subjects heterozygotes for deletions (lane F). The deletion of a limited number of nucleotides in an amplification product causes the alteration of its electrophoretic mobility and may detect the presence of small intra-exonic deletions (lane B).

Electrophoretic mobility shifts may be be produced also by single base substitutions which affects the double strand conformation in solution [9]. Therefore, some point mutations may be directely detected on 10% polyacrylamide gels (lane C).

In our experience, a total of 382 DNAs of male subjects were screened for mutations of the Dystrophin gene. Among them, 344 were affected with Duchenne or Becker muscular dystrophy, while 38 were affected with limb-girdle muscular dystrophy or showed ambiguous clinical phenotypes.

The PCR amplifications involved the enchancer sequence, the muscular promoter sequence and the following exons : 2, 3, 4, 5, 6, 7, 8, 12, 13, 16, 17, 19, 21, 26, 32, 34, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53 and 60. In total, 119 intragenic deletions, 21 intragenic duplications and 3 point mutations were detected.

Discussion

The PCR protocol for screening of mutations of the dystrophin gene requires a considerable number of primers and a minimum of 9 multiplex PCR reactions in order to screen all the exons.

However, this apparent disadvantage is fully compensated by the fact that the procedure is incomparably simpler and faster than the cDNA probing. On the other hand, it was reported that most of intragenic deletions [10] or duplications [5] may be detected by the amplification of 18 exons only.

In the commom laboratory practice it is usual to perform first a screening for intragenic deletions running PCR products on agarose gel and then to screen again for duplication or point mutations with more sensitive methods. The protocol presented here, involving semi-quantitative PCR coupled with silver staining allow to perform all the screenings in a single run, which is particularly useful when dealing with prenatal diagnosis.

The time required is about 7 hrs : 30 min for the preliminary PCR work (handling of the samples, dilutions, preparation of the PCR mix), 100 min for the amplification by the Thermocycler, a minimum of 2.5 hrs for the electrophoretic separation, 1 hr for silver staining and 1 additional hr for the gel drying.

By comparison, the time needed to screen the same samples by a single cDNA probe would be : 1-3 hrs for digestion with the restriction enzyme, 1 hr for the control of the digestion by minigel, 12 hrs for the Southern blot, 16-24 hrs for the hybridization and a minimum of 48 hrs for autoradiography. Therefore, in order to screen all the gene sequence by using sequentially the 8 cDNA probes on the same filter, a minimum of 24-25 days would be required.

cDNA probing is time consuming, tecnically demanding and it involves the use of hazardous radiochemicals, but

it is generally unable to detect microdeletions or microduplications and never could detect point mutations.

On the contrary, PCR method is reliable, fast and if ethidium bromide is substituted by silver staining, is completely safe.

In conclusion, as shown here for the dystrophin gene, the multiplex semi-quantitative PCR amplification of selected sequences is presently the most suitable method for single-step screening for deletions, duplications, insertions and for those base substitutions which affect the double strand conformation in solution.

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