ABSTRACT

Mutation detection in the DMD gene defective in Duchenne (DMD) and Becker muscular dystrophies (BMD) is complicated by the presence of 79 exons. The majority of recognized mutations are, however, copy number changes of individual exons, which traditionally have been identified by three common multiplex polymerase chain reaction (PCR) assays and/or Southern blotting. Here we report the use of the newly developed quantitative assay multiplex ligation-dependent probe amplification (MLPA) to determine the copy number of each of the 79 DMD exons in 182 males and 14 carrier females referred to our diagnostic facility on the clinical suspicion of DMD or BMD. The MLPA method confirmed all previously recognized mutations and identified an additional 28, including four point mutations. Also, the assay reliably identified 7 carrier females, which are usually not easily recognized. In our hands the method is highly reproducible, easy to handle, and has increased our mutation pick-up rate by a total of 33%.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is one of the most common inherited neuromuscular diseases, affecting 1 in 3500 males. It is an X-linked disorder caused by mutations in the DMD gene (Worton and Thompson, 1988). Covering 2.4 Mb, DMD is one of the largest known human genes (Den Dunnen et al., 1989). The gene contains 79 exons encoding a 14-kb mRNA (Koenig et al., 1987). Mutations leading to a truncated protein cause the severe phenotype of DMD, whereas mutations retaining the mRNA reading frame cause the more benign phenotype of Becker muscular dystrophy (BMD) (OMIM: 300376) (Monaco et al., 1988; Koenig et al., 1989). Knowing the exact mutation in a patient is therefore prognostic and an important part of the diagnostic tool of these diseases.

Large rearrangements in the gene are found in about two thirds of DMD patients, with approximately 60% carrying deletions, 5%–10% carrying duplications and the remaining caused by point mutations and small insertion/deletions (Koenig et al., 1987; Den Dunnen et al., 1989; Gillard et al., 1989). Several approaches for detection of deletions/duplications have been published, where the three multiplex polymerase chain reactions developed by Beggs, Chamberlain and Kunkel, are the most commonly used for a clinical diagnostic set up (Chamberlain et al., 1988; Beggs et al., 1990; Kunkel et al., 1991). These methods allow a detection of 90%–95% of the deletions in male patients. Until recently, quantitative Southern blotting was the only reliable method for detecting duplications and identifying carrier females within the families (Den Dunnen et al., 1989; Yamagishi et al., 1996). Quantitative Southern blotting is, however, time-consuming and requires several hybridization steps. Quantitative PCR analysis is a relatively recent alternative in which the primers of a multiplex PCR analysis are fluorescent labeled, allowing for the PCR products to be accurately sized and quantified by capillary electrophoresis (Yau et al., 1996). These methods also allow detection of duplications, and identification of carriers of deletions and duplications. However, the incomplete coverage of the exons will leave duplications/deletions outside the hotspots undetected. In 2002 the quantitative PCR-based method, multiplex amplifiable probe hybridization (MAPH; Armour et al., 2000) was published. This method allows scanning of all 79 exons of the DMD gene for their copy number (White et al., 2002), and thus can detect deletions and duplication, as well as being suitable for carrier diagnosis. Although the method seems
to be highly reliable, we find it difficult and laborious to use. It requires spotting of the individual DNA samples on small pieces of nylon filter, prehybridization, hybridization, several washing steps of the filters, transfer of the individual filters to PCR tubes, followed by two steps of multiplex PCR, and finally analysis of the PCR products.

Recently, the multiplex ligation-dependent probe amplification (MLPA) method was introduced (Schouten et al., 2002), which allows the relative quantification of up to 40 different nucleic acid sequences in a single reaction tube. The method has proven to be accurate and reliable for identifying deletions and duplications in several genes (Erlandson et al., 2003; Hogervorst et al., 2003; Montagna et al., 2003; Taylor et al., 2003; Rooms et al., 2004).

Here we describe our results and experience with the MLPA DMD kits, P034 and P035, commercially available from MRC Holland, Amsterdam, The Netherlands. These two sets of probes allow a scanning for deletions and duplications of all 79 DMD exons in just two PCR reactions. Testing this method on 196 DMD samples we have been able to detect and define several deletions, duplications, and rearrangements that were not identified by the three commonly used multiplex PCR approaches. Furthermore, we have been able to identify carriers within the families. The power of the technique is further illustrated by the identification of four intra-exonic mutations that otherwise require extensive sequencing to detect. This rather simple technique can easily be implemented in diagnostic laboratories and will improve the diagnostic service for the families.

**FIG. 1.** Average dosage quotient (DQ) for the individual Duchenne muscular dystrophy (DMD) exons and internal reference peaks depicted with one SD. The order of the exons corresponds to the actual order on the electropherogram. A: P034 samples. B: P035 samples.
Patients

Since 1985 our laboratory has been one of two diagnostic laboratories for DMD in Denmark. We have received blood or purified DNA samples from patients and their families during this period. DNA from blood samples was isolated by a standard salting-out method. The DNA samples have been repeatedly reanalyzed using the currently available methods. All referrals came either from a paediatric or neurologic department, or through a clinical genetic department. The reason for referral was either for diagnostic purpose (i.e., confirmation of a diagnosis), for carrier diagnosis of female relatives or for prenatal diagnosis.

Multiplex PCR

Our multiplex assays for deletion detection were slight modifications of the Chamberlain et al. (1988), Beggs et al. (1990), and Kunkel et al. (1991) sets, using the condition recommended at the Leiden Muscular Dystrophy pages (www.dmd.nl/). Three separate PCR reactions allow screening for deletions in 23 exons.

MLPA reaction and fragment analysis

The DMD-MLPA test is developed and manufactured by MRC Holland. It will screen the copy number of all 79 exons in two reactions, using the kit P034 and P035. In short, 5 μL (50–500 ng) target DNA was denatured for 5 min at 98°C after which 3 μL of the probe mix were added. The mixture was heated at 95°C for 1 min and incubated at 60°C overnight (16 hrs). Ligation was performed with the temperature-stable Ligase-65 enzyme for 15 min at 54°C. After inactivation of the ligase, the ligated products were amplified by PCR according to the manufacturer’s protocol using one primer labeled with 6-FAM. PCR was carried out for 33 cycles (30 sec at 95°C, 30 sec at 60°C, and 60 sec at 72°C) and the resulting fragments were analyzed on an ABI model 310 capillary sequencer using the Genescan software (Applied Biosystems, Foster City, CA). The sizes of exon-specific peaks were identified according to their migration relative to the GS-500 size standards. Data were exported to an Excel spreadsheet and the relative peak area for each probe was calculated as a fraction of the total sum of peak areas in each sample. Subsequently, the fraction of each peak was divided by the average peak fractions of the corresponding probe in control samples to give the dosage quotient (DQ, Taylor et al., 2003). Expected DQs in men for single copy fragments are 1, for deleted fragments 0, and 2 for duplicated fragments. For females the expected DQs for noncarriers are 1, and for carriers of a deletion/duplication 0.5 and 1.5, respectively.

Confirmation of deletion and duplication of single exons

The MLPA results from male patients were initially assessed visually for the detection of deletions, as the absence of DMD-specific peaks. Absence of DMD peaks corresponding to two or more contiguous exons was taken to represent a genuine deletion and no further investigations were performed. The absence of only one DMD peak in males, corresponding to a single exon, was investigated further using PCR primers flanking the exon.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Gender</th>
<th>Deletions</th>
<th>Duplications</th>
<th>Point mutations</th>
<th>Negative</th>
<th>LGMD2I</th>
</tr>
</thead>
<tbody>
<tr>
<td>196</td>
<td>182 males</td>
<td>87 (3)</td>
<td>14 (14)</td>
<td>4 (4)</td>
<td>66</td>
<td>11 (11)</td>
</tr>
<tr>
<td>14 females</td>
<td>7 (7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses are new mutations.
in question as given in the Leiden database (www.dmd.nl/) or by Southern blot. In females, the absence of a single peak was investigated by Southern blot or quantitative PCR. If a deletion could not be confirmed by PCR, the absence of the MLPA peak could be due to a point mutation within the exon, destroying the annealing of the MLPA probes. Potential PCR products of these exons were sequenced. Duplicated DMD peaks corresponding to two or more contiguous exons were taken to represent a true duplication and no further investigations were performed. Duplication of a single exon was confirmed by real-time PCR using the SDS7000 (Applied Biosystems), with the GAPDH locus as a genomic control target or by using fluorochromelabeled primers from the multiplex PCR reactions and calculating the ratio of the peak area between the exon in question and one or two control areas from nonduplicated exons.

RESULTS

In order to get a reliable assessment of the MLPA method we decided to reanalyze all our DNA samples received for diagnostic purposes of Duchenne/Becker muscular dystrophy. Families, from whom a DNA sample from the proband or an obligate carrier was not available, were excluded. In total we investigated 196 DNA samples of which the majority, 182, were from male patients, 13 from obligate female carriers, and 1 sample from a young girl with a clinical picture strongly suspicious for DMD. Initially, we analyzed 10 control male samples and calculated the mean DQs and standard deviations for each peak. The mean DQ for all peaks was 1.01 (range, 0.94–1.17), standard deviation (SD) 0.04. The variations found for each peak are given in Figure 1. Typical results for deletions/duplications are shown in Figure 2. The MLPA screen of the 196 samples revealed a total of 94 deletions including a complex rearrangement with a noncontiguous deletion, 14 duplications, including a noncontiguous duplication, as well as 4 intra-exon mutations (Table 1).

Deletions

Out of the 182 male patients included in the screening we had previously identified a deletion in 84 using the common multiplex PCR approach. These deletions were only partly defined with respect to individual breakpoints. The MLPA screen confirmed all these deletions and identified in addition three; one patient with a deletion of exon 53, one with a deletion of exons 53–55, and one patient with a deletion encompassing exons 61–67. These deletions had escaped detection by the common multiplex assay due to the lack of primers covering the exons. In one case a deletion of exon 1 was found together with a deletion of exons 3–11. Exon 2 was normal, which was confirmed by PCR.

Of the 14 females sample, a deletion was found in seven, confirming their carrier status (Fig. 3).

Duplications

Fourteen duplications were identified, all occurring in male proband patients. In one patient a duplication of exon 44 was found together with a duplication of exons 51–55. This exon 44 duplication was confirmed using fluorescent-labeled primers and calculating the ratio between the peak area of exon 44 and exon 8. The exons between the duplications exhibited normal DQs.

Detection range

In order to assess the range of DQ for the different copy number aberrations, we analysed obligate carriers of both deletions and duplications. As illustrated in Table 2 the DQ range for both duplications and deletions is fairly narrow and nonoverlapping with the control samples.

Point mutations

In four cases MLPA indicated a deletion of a single exon, whereas PCR with exon flanking primers unexpectedly resulted

![FIG. 3. Distribution of the new deletions, duplications, and point mutations found in this study. Light-gray vertical bars indicate the exons included in our multiplex-polymerase chain reaction (PCR), used before the introduction of multiplex-ligation-dependent probe amplification (MLPA).](image-url)
in a product. Subsequent sequencing identified mutations within the individual exons (Table 3).

**Discrepancies between multiplex PCR and MLPA**

By the traditional multiplex PCR one of our male control DNA samples had unexpectedly previously been found to harbor a deletion of exons 16 and 17 predicted to give rise to an out-of-frame mRNA. The MLPA analysis of the patient indicated however only a deletion of exon 16 whereas exon 17 appeared normal. By changing the position of the multiplex primers for exon 17, we found that the entire exon 17 indeed was intact. Long-range PCR with an exon 15 forward primer and an exon 17 reverse primer, revealed a PCR product of approximately 7000 kb. Sequencing part of this product showed that the deletion covered part of intron 15, exon 16, and almost the entire intron 16 including the annealing sequence of the exon 17 forward primer used in the multiplex PCR. The deletion was also found in DNA isolated from a muscle biopsy.

**Manifesting female carrier**

A female patient with typical clinical symptoms of DMD was included in the MLPA screening. She was found to have a large deletion encompassing exons 33–43. There was no family history of DMD. Her mother did not have the deletion and her father was healthy. X-chromosome inactivation was biased in DNA from leukocytes, but normal in DNA from a muscle biopsy. She had a normal 46,XX karyotype.

**Alternative diagnoses**

Even in a comprehensive mutation screen for both deletions, duplications and point mutations, patients with no detectable mutations are always encountered. One of the obvious reasons is that the referral diagnosis in the first place is incorrect. According to the MLPA analysis, 78 of the male patients did not show a deletion or duplication. Fifty of these cases were sporadic. Limb-girdle muscular dystrophy (LGMD) is a differential diagnosis to Becker/Duchenne muscular dystrophy. The most common form of LGMD is LGMD2I (OMIM: 607155), caused by mutations in the FKHR gene (Brockington et al., 2001; Poppe et al., 2003). One mutation in this gene L276I (c.876C>A) is very common. The 50 sporadic patients were therefore tested for this mutation and 11 of these (22%) were found to have LGMD2I, being either homozygous for L276I or compound heterozygous for L276I and another mutation.

**DISCUSSION**

We have applied the MLPA technique on a total of 196 DNA samples referred to our diagnostic facilities on the basis of DMD. In total 112 DMD mutations were identified (Table 1) of which 28 had not previously been discovered. Thus, the MLPA technique has increased our mutation pick-up rate by 33%. Moreover, the technique has enabled us to confidently identify carrier individuals.

Deletions in male patients are easily identified by the absence of the respective DQ value and as the majority of the identified deletions encompass more that one exon no additional analysis is necessary. On the other hand, duplications will only be detected if there is a clear difference between DQ of the normal and patient sample. In the present series the DQ found in the samples harbouring duplications did not overlap with the normal DQ range. However, when samples with a duplication of several exons were analysed, the calculated DQs for all peaks decreased, explaining the rather broad range of DQ found for duplicated peaks (Table 2). For carrier detection the same conclusion can be drawn. The DQ for either carriers of deletions or duplications did not overlap with DQ for normal samples making the analysis quite robust. We noted that the amount and especially the quality of the DNA are important for obtaining reproducible results. Purified DNA samples received from other laboratories without purification details often performed with variable quality, and occasionally exhibited DQ values outside the 95% confidence interval.

In any PCR-based technique, including MLPA, a false-negative signal (no PCR product) will occur if there is a polymorphism or rare variant at the target site for the primers/hybridization probes. We found a missing MLPA peak caused by intra-exonic mutations in four cases (Table 3) demonstrating that apart from changes in copy number, MLPA will also detect a subset of mutations within exons. All mutations were less...
than 6 bp from the ligation site of the respective MLPA probes. Three of these mutations lead to frame-shifts and one creates a premature stop codon, all predictive for a Duchenne-like phenotype. If the single deleted MLPA peak had been interpreted as the final result, we would have predicted an in-frame deletion for exons 3, 38, and 73, predictive of a Becker phenotype. These results underscore the importance of checking single duplications/deletions with alternative methods.

The person who was found to have deletion of exon 16 only is completely healthy, and has a histologic normal muscle biopsy and a normal creatine kinase level. The deletion was also present in DNA from a muscle biopsy, excluding mosaicism as an explanation for the phenotype. We conclude that a deletion of exon 16 only does not interfere with the essential function of dystrophin. In the Leiden database only one patient has previously been described with exon 16 only deletion (Adachi et al., 2002). No clinical information was however given.

Two of the 196 patients samples revealed an unexpected rearrangement of the DMD gene. One patient was found to have a noncontiguous duplication of exon 44 together with a duplication of exons 51–55. The patient has a typical DMD phenotype consistent with the predictive out-of-frame duplication of exons 51–55. The second patient presented with a deletion of the promoter Dp427c, exon 1, and exon 3–11, whereas exon 2 was present. Only a method like MLPA will allow the detection of such noncontiguous deletion/duplications, which could be of importance for the clinical prognosis and counseling.

A single manifesting carrier of DMD was identified. MLPA identified a deletion encompassing exons 33–43 consistent with an out-of-frame deletion. Western blot analysis revealed the presence of a truncated protein only. The patient’s karyotype was normal and X-chromosome inactivation in DNA from a muscle biopsy was likewise normal. There are three accepted explanations for manifesting carriers; disease-causing mutations present in both alleles, nonrandom X-inactivation and, as the final result, we would have predicted an in-frame deletion for exons 33–43 consistent with the predictive out-of-frame duplication of the DMD gene. The latter two explanations have been ruled out and it is therefore likely that the carrier harbors a deleterious mutation in her other DMD-allele.

We have found the disease-causing mutation in 57% of our analyzed samples, which is lower than usually quoted and there is still a considerable number of patients in whom no mutation has been found. Several patients will have point mutations, not detectable by MLPA. However, it is also likely that the clinical diagnosis is incorrect and that the clinical features overlap with that of DMD but are in fact a result of mutations located in other genes involved in correct muscular function. Indeed, we found that 11 of 50 (22%) sporadic patients had mutations in the FKRP gene resulting in LGMD 21. Thus, careful clinical evaluation is extremely important, and where no DMD deletions or duplications can be found LGMD should be considered and tested for.

In conclusion, the MLPA technique can significantly increase mutation detection in the DMD gene, and reliable identify carrier females harboring DMD copy number aberrations. The method is easy to handle, highly reproducible with good quality DNA, and because the assay only requires commonly used technology it can easily be implemented in a standard diagnostic laboratory. Identified copy number changes involving a single exon must however be interpreted with care and verified by alternative techniques.

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REFERENCES


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