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Log-PCR: A New Tool for Immediate and Cost-Effective Diagnosis of up to 85% of Dystrophin Gene Mutations

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BACKGROUND: Duchenne (DMD) and Becker (BMD) muscular dystrophies are caused by mutations in the dystrophin gene. Despite the progress in the technologies of mutation detection, the disease of one third of patients escapes molecular definition because the labor and expense involved has precluded analyzing the entire gene. Novel techniques with higher detection rates, such as multiplex ligation-dependent probe amplification and multiplex amplifiable probe hybridization, have been introduced.

METHODS: We approached the challenge of multiplexing by modifying the PCR chemistry. We set up a rapid protocol that analyzes all dystrophin exons and flanking introns (57.5 kb). We grouped exons according to their effect on the reading frame and ran 2 PCR reactions for DMD mutations and 2 reactions for BMD mutations under the same conditions. The PCR products are evenly spaced logarithmically on the gel (Log-PCR) in an order that reproduces their chromosomal locations. This strategy enables both simultaneous mapping of all the mutation borders and distinguishing between DMD and BMD. As a proof of principle, we reexamined samples from 506 patients who had received a DMD or BMD diagnosis.

RESULTS: We observed gross rearrangements in 428 of the patients (84.6%; 74.5% deletions and 10.1% duplications). We also recognized a much broader spectrum of mutations and identified 14.6% additional cases.

CONCLUSIONS: This study is the first exhaustive investigation of this subject and has made possible the development of a cost-effective test for diagnosing a larger proportion of cases. The benefit of this approach may allow more focused efforts for discovering small or deep-intronic mutations among the few remaining undiagnosed cases. The same protocol can be extended to set up Log-PCRs for other high-throughput applications.

Duchenne muscular dystrophy (DMD)[5] (MIM 310200) and Becker muscular dystrophy (BMD) (MIM 300376) are common inherited disorders of muscle. DMD was originally described by Conte in 1831, Marion in 1840, and Duchenne in 1850 *(1)*. The disease is characterized by wasting of skeletal and cardiac muscle and progresses to immobility and death. It is transmitted as an X-linked recessive trait that affects males in >99% of the cases. The prevalence of DMD at birth is about 1 in 3500 males, whereas the BMD prevalence (1 in 35 000) has probably been underestimated *(2, 3)*.

Because DMD is a lethal X-linked disorder, one third of all mutated alleles are removed each generation because they are carried by affected males, who rarely have children. According to Haldane's rule *(4)*, the rate of the appearance of mutated alleles must equal the rate of their removal *(5)*. Thus, the frequency of new mutations has to be very high (up to 10^{-4}), and the mutation spectrum is extremely heterogeneous. Both DMD and BMD are caused by mutations in the dystrophin gene locus [*DMD*,[6] dystrophin (muscular dystrophy, Duchenne and Becker types)] *(6)*, which consists of 79 exons and 8 tissue-specific promoters. Dystrophin is not detectable in DMD patients, whereas in BMD patients some dystrophin—albeit abnor-

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[5] Nonstandard abbreviations: DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; Log-PCR, multiplex PCR design in which PCR products are evenly spaced logarithmically on a gel; MAPH, multiplex amplification and probe hybridization; MLPA, multiplex ligation-dependent probe amplification.

[6] Human genes: *DMD*, dystrophin (muscular dystrophy, Duchenne and Becker types); *AMELX*, amelogenin (amelogenesis imperfecta 1, X-linked); *AMELY*, amelogenin, Y-linked.

mal—is visible on a western blot. The dystrophin gene spans 2.22 Mb, encoding the longest known primary transcript of the human genome. The most common mutations are large intragenic deletions or duplications that encompass one or more exons *(7)*. The effects on the transcript depend less on the extent of a deletion (or duplication) than on whether it disrupts the translational reading frame. In general, mutations that maintain the reading frame are associated with BMD, whereas deletions and duplications tend to disrupt the reading frame in DMD *(8, 9)*. A survey of 4700 mutations in the Leiden database (http://www.dmd.nl) indicates that the reading-frame rule holds for 91% of cases *(10)*. Deletion/duplication breakpoints may occur anywhere in the gene. There are, however, large differences in intron size, from 54 bp (intron 14) to 248 342 bp (intron 44). In addition, 20 consecutive exons (exons 23–42) are symmetric (0,0), and each one can be deleted without causing a frameshift. This feature accounts for the locations of 2 major disease-linked hot spots, one within exons 44–52 (710 kb) and the other within exons 2–19 (530 kb). The analysis of a limited number of exons has been thought to detect most of the deletions found in patients; thus, genetic testing for deletions has relied worldwide on different methods, which were originally based on the multiplex PCR technique. This technique initially was set up to analyze 18 *DMD* exons *(11, 12)*. Other PCR protocols were subsequently developed to cover some untested exons and to define deletion borders *(13, 14)*, and the descriptions of several additional multiplex primer sets have been published over the years. Possibly because of the different sensitivities and efficacies of all the multiplex PCR methods and/or the methods used for clinical diagnosis, deletions have been detected in different countries [Germany *(15)*, Greece *(16)*, Mexico *(17)*, Egypt *(18)*, Morocco *(19)*, Saudi Arabia *(20)*, India *(21)*, and China *(22)*] at frequencies that range from 50% to 65% of DMD cases. In the majority of laboratories, molecular diagnosis is performed via multiplex PCR alone. Other mutation types, such as atypical deletions, duplications, small mutations, deep-intronic deletions, and the insertion of repetitive sequences, remain undetected.

Additional diagnostic approaches, such as quantitative PCR *(23, 24)*, single-strand conformation polymorphism analysis *(25)*, denaturing HPLC *(26, 27)*, SCAIP ("single condition amplification/internal primer") sequencing *(28)*, multiplex amplifiable probe hybridization (MAPH) *(29)*, or multiplex ligation-dependent probe amplification (MLPA) *(30, 31)* are required to resolve these cases; however, the latest, more sophisticated techniques require specialized equipment and expertise that are very often unsuitable for a common diagnostic laboratory, especially in developing countries.

We have developed a novel tool, a multiplex PCR assay in which the PCR products are evenly spaced logarithmically on a gel (Log-PCR), that can detect deletions and duplications via analysis of all *DMD* exons in 4 multiplex PCRs that run under the same conditions.

We validated the method performing the pivotal analysis on a group of 506 DMD/BMD patients.

We found that this rapid, simple and inexpensive tool enabled a definitive molecular diagnosis in 85% of DMD/BMD patients.

Materials and Methods

PATIENTS

The unrelated Italian male patients (n = 506) received their diagnoses at the Servizio di Cardiomiologia e Genetica Medica of the Second University of Naples. The diagnosis was established from clinical features consistent with DMD or BMD, absent or altered dystrophin production *(1)* (as determined by immunofluorescence assay or western blot analysis), and/or a clear X-linked family history of the disease *(2)*. Informed consent was obtained from all of the patients in accordance with the guidelines of EuroBioBank or Telethon.

DNA from peripheral blood leukocytes was used in accordance with the standard operating procedures adopted by the EuroBioBank network and was stored at the Naples Human Mutation Gene Bank (Cardiomyology and Medical Genetics) or at the Telethon Institute of Genetics and Medicine.

PRIMER DESIGN

Dystrophin amplimers were divided into 2 groups. The first group consists of 40 amplicons of phase 1 or phase 2 asymmetric exons [(0,1), (0,2), (1,0), (1,2), (2,0), (2,1)] that produce a frameshift when absent or duplicated. This amplicon group is composed of 2 PCR sets, A and B. Set A includes exons 1, 3, 5, 7, 11, 17, 19, 21, 43, 45, 50, 52, 54, 56, 58, 61, 63, 66, 68, and 75; set B includes exons 2, 4, 6, 8, 12, 18, 20, 22, 44, 46, 51, 53, 55, 57, 59, 62, 65, 67, 70, and 76.

The second dystrophin amplimer group consists of 38 amplicons of symmetric exons [(0,0), (1,1), (2,2)]. Deletion or duplications involving these exons do not produce a frameshift. This group is also composed of 2 sets, C and D. Set C includes exons 9, 13, 16, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 48, 60, 69, 72, and 74, plus an amplicon for the *AMELX* gene [amelogenin (amelogenesis imperfecta 1, X-linked)] when required. Set D includes exons 10, 14–15, 23, 25, 27, 29, 31, 33, 37, 39, 41, 47, 49, 64, 71, 73, and 77, plus an amplicon for the *AMELY* gene (amelogenin, Y-linked) when required. The presence or absence of an amelogenin amplicon helps to distinguish female and male DNA and

to check for substantial maternal contamination in samples of DNA extracted from chorionic villi. Consecutive exons are never included in the same set. We excluded exons 78 and 79, which have never been found to be deleted in DMD and BMD *(32)*. For each exon of the *DMD* gene (NM_004006.1), we based our design of primer pairs so that we (*a*) produced a predetermined spacing of the PCR products on agarose gels after electrophoresis and (*b*) retained the chromosomal order of the exons. To establish the length of each fragment, we created constant spacing between electrophoretic bands by using the equation: $\log X_n = \log X_{(n-1)} + 0.047$, where X is amplicon length in bp. We empirically determined that this increment produced the optimal distance with conventional agarose gels. Fragment lengths are alternated between sets to retain the option of running sets A and B together and sets C and D together with higher-resolution electrophoresis methods. Moreover, we designed each primer to fit the following requirements: (*a*) 28–32 bp in length, (*b*) at least 13 C or G nucleotides, and (*c*) at least 3 C or G nucleotides at the 3′ end. We used BLASTn (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to check primer sequences to avoid matching with repeated human sequences and single-nucleotide polymorphisms. *AMELX* and *AMELY* sequences were obtained from GenBank (NM_182680 and NM_001143, respectively). We aligned and compared sequences with ClustalW software to select 2 pairs of primers, the first specific for *AMELX* alone and the second specific for *AMELY*. To avoid PCR artifacts due to polymorphic variation, we designed the primer sets to have 4 internal amplification controls: Exon 31 is present both in set D and within the amplimer of exon 32 in set C, exon 11 is present both in set A and within the amplimer of exon 10 in set D, exon 25 is present both in set D and within the amplimer of exon 24 in set C, and exon 34 is contained both in set C and in the amplimer of exon 35 in set D. These redundancies are very useful. For instance, we have found a polymorphic 84-bp deletion at the 5′ end of intron 30 (frequency, 0.015) that causes a failure to amplify exon 31, but the internal control helps us to distinguish between this polymorphism and true deletion of exon 31.

MULTIPLEX PCR CHEMISTRY

Each multiplex PCR was performed in a final volume of 15–20 μL containing 60–100 ng of DNA template, 10× buffer (final concentrations: 15 mmol/L Tris base, 20 mmol/L HEPES free acid, 25 mmol/L KCl, 10 mmol/L $MgCl_2$, pH 8.20), 2× polyol solution (final concentrations: 150 mmol/L maltitol, 350 mmol/L sorbitol), a primer mixture (total final concentration of primers, 4 μmol/L; each primer, 0.1 μmol/L), deoxynucleoside triphosphates (final concentration of each, 0.25 mmol/L), and 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems).

The cycling conditions of all 4 multiplex PCRs were identical, so we were able to perform all 4 reactions simultaneously with the same thermal cycler. The multiplex PCRs were carried out with a hot-start method on an MWG AG Biotech thermocycler. The cycling conditions consisted of a first step at 99 °C for 30 s, a pause at 85 °C (for *Taq* addition), and 21 cycles of 20 s at 97 °C and 7 min plus 20 s/cycle at 66 °C (total time, 4 h 30 min). Two microliters of each sample were run on a DNase- and RNase-free agarose gel (16 g/L SeaKem LE; Lonza), containing GelStar stain (10 000×; Cambrex) and 0.5× TTE buffer (final concentrations: 45 mmol/L Tris, 15 mmol/L taurine, and 0.3 mmol/L EDTA, pH 8.0). By using GelStar as the DNA-staining dye (10-fold more sensitive than ethidium bromide), we were able to reduce the number of PCR cycles to 21, thereby maintaining the PCR reaction within the logarithmic phase. Gel electrophoresis was performed for 50 min at 160 V (Fig. 1).

Results

The Log-PCR method allows the entire *DMD* coding sequence to be surveyed. The 4 multiplex PCR reactions represent a total of 57.5 kb. The evenly spaced Log-PCR amplicons range in size from 211 bp to 1 742 bp and provide a general overview of the gene. Large deletions and duplications appear on an agarose gel as contiguous bands that are absent (Fig. 2) or more intense (Fig. 3), respectively, in the 4 reactions.

We first developed reaction and cycling conditions for set A by testing various multiplex PCR protocols with different deoxynucleoside triphosphate, primer, and *Taq* concentrations and at various annealing/extension temperatures and times. We used 0.1 μmol/L of each primer (4 μmol/L total) and 60 ng of DNA template. Because we were initially unable to amplify all of the fragments homogeneously with one protocol, we investigated various modifications to the PCR chemistry. We found that all products were amplifiable with a buffer consisting of 15 mmol/L Tris base, 20 mmol/L HEPES free acid, and 25 mmol/L KCl. One or more products were not amplified with different salts (e.g., ammonium sulfate). We also investigated the addition of polyols or sugar alcohols to improve band homogeneity and consistency by testing different concentrations of 5 compounds, either alone or in pairs: D-sorbitol, D-mannitol, xylitol, maltitol, and *meso*-erythritol. A mixture of maltitol and sorbitol was the most effective combination. We found the optimal $MgCl_2$ concentration to be 10 mmol/L, a concentration that is at least 6-fold higher than typically used in PCR reactions. We then successfully applied these

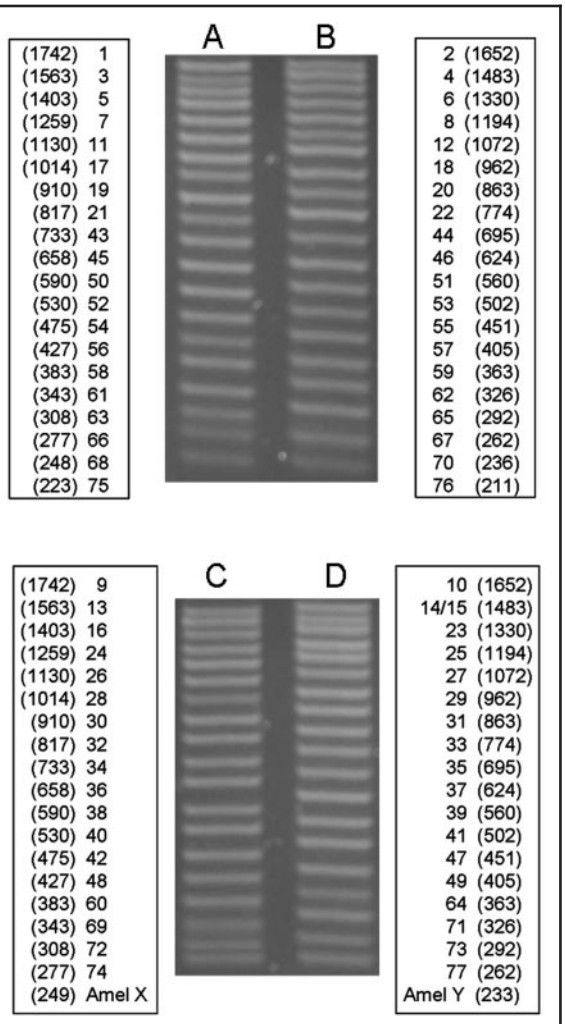

Fig. 1. Agarose gel electrophoresis of Log-PCR products from typical male DNA.

Patterns of equidistant bands are derived from the 4 multiplex reactions (A–D, see text). Two microliters of each PCR reaction were run for 50 min at 160 V in a gel containing 16 g/L agarose. Indicated next to each band are the exon number and amplicon size in bp (in parentheses). The migration of PCR products on the agarose gel follows their chromosomal order (5′ amplicons are larger than 3′ amplicons). Amel X, *AMELX*; Amel Y, *AMELY*.

conditions to the other 3 multiplex PCRs containing the B, C, and D primer sets.

We validated the Log-PCR method by retesting samples from a large cohort of DMD and BMD patients (n = 506). The patients were divided into 2 groups The first group consisted of 152 patients who had no apparent deletions according to the tests of Chamberlain and Gibbs *(11)* and Beggs et al. *(12)*. Our Log-PCR method revealed that 74 (48.7%) of the DMD and BMD patients in this group had mutations (23 deletions and 51 duplications) that were previously undetected. The second group consisted of 354 patients who had documented deletions. We verified the deletion endpoints in all of these patients and found larger deletions in 170 (48%) of the patients in this group. In brief, Log-PCR identified deletions in 74.5% of cases and duplications in 10.1% (see the figures in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue6). Our approach improved the detection rate of deletions and duplications by 14.6%. We identified 377 deletions with 127 different borders and 51 duplications with 43 different borders. Thus, each mutation is unique in almost all cases. We tested all deletions and duplications with the "reading frame checker" *(32)* and identified 21 exceptions to the reading-frame rule (see supplementary data in the online Data Supplement).

DELETIONS

We detected 66 deletions of single exons, 9 of which are undetected with standard tests. All single-exon deletions were confirmed via a single PCR with different primer pairs. Multiple deletions spanned 2 to 43 exons. We documented 41 deletions (11%) in the 5′ hot spot (exons 2–19), and 242 deletions (64%) in the 3′ hot spot (exons 43–52).

We refined the locations of the deletion endpoints in 44% of the cases with deletions in the 5′ hot spot and in 34% of the deletions in the 3′ hot spot. The largest deletions spanned exons 17–59 (1100 kb), exons 19–52 (820 kb), exons 10–44 (670 kb), exons 20–47 (615 kb), exons 20–45 (550 kb), and exons 8–42 (400 kb). We found 22 deletions outside the hot spots (see Fig. 4 in the online Data Supplement).

DUPLICATIONS

The Log-PCR also detects duplications when a small number of cycles is used. In the patient group with no previously documented deletions (n = 152), we detected 51 duplications, all of which were confirmed by real-time PCR analysis. Nine of these duplications involved a single exon. In the 5′ hot spot, the frequencies of deletions and duplications were 11% and 43%, respectively. We found the reverse situation in the 3′ hot spot, where the frequencies of deletions and duplications were 66% and <20%, respectively, suggesting different mutation susceptibilities in the 2 regions. The maximum duplication size was 1 Mb, spanning exons 33–60 (see Fig. 5 in the online Data Supplement).

NEGATIVE RESULTS

We found large deletions and duplications to be absent in 78 patients (15.4%). Such a finding is usually ex-

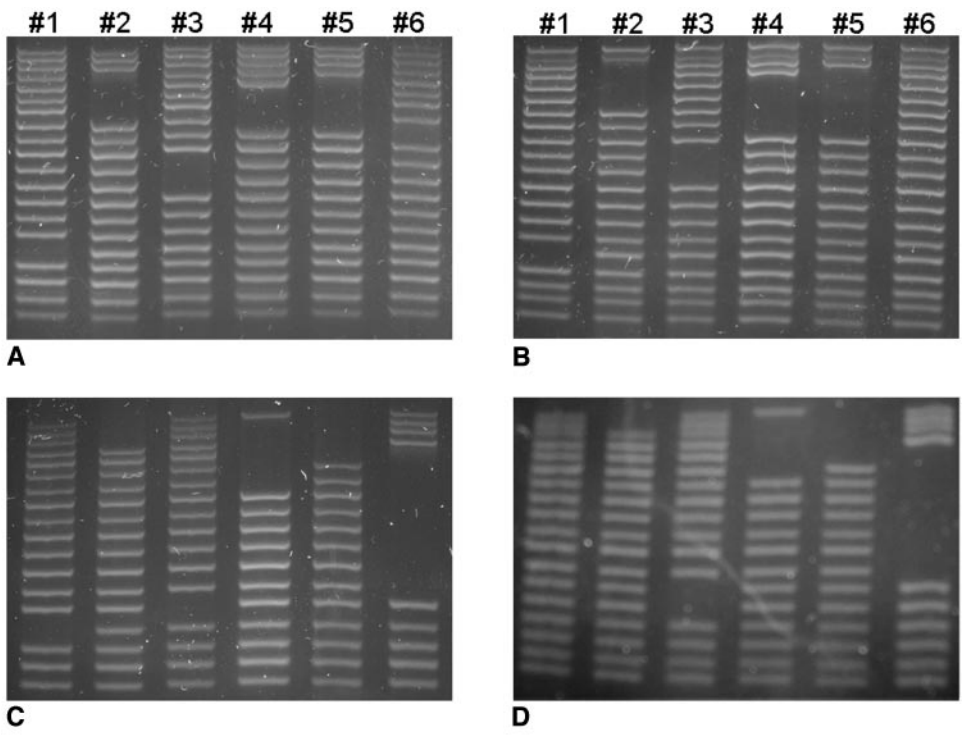

Fig. 2. Examples of deletions identified by Log-PCR testing.

A deletion of one or more exons is seen as the absence of one or more consecutive bands on the gel. The electrophoretic results for all 4 multiplex PCR reactions (A–D) are indicated for 6 patients (#1–#6). Mix A (A): #1, del exon 61; #2, del exons 7, 11, 17, 19; #3, del exons 50, 52; #4, del exons 17, 19, 21; #5, del exons 11, 17, 19, 21; #6, del exon 43. Mix B (B): #1, del exon 62; #2, del exons 6, 8, 12, 18; #3, del exons 46, 51; #4, del exons 12, 18, 20, 22; #5, del exons 8, 12, 18, 20, 22; #6, no mutation. Mix C (C): #1, del exon 60; #2, del exons 9, 13, 16; #3, del exon 48; #4, del exons 13, 16, 24, 26, 28, 30; #5, del exons 9, 13, 16, 24, 26; #6, del exons 26, 28, 30, 32, 34, 36, 38, 40, 42. Mix D (D): #1, no mutation; #2, del exons 10, 14/15; #3, del exons 47, 49; #4, del exons 14/15, 23, 25, 27, 29; #5, del exons 10, 14/15, 23, 25, 27, 29; #6, del exons 27, 29, 31, 33, 35, 37, 39, 41. The deletion is from exon 60–62 in patient #1, from exon 6–19 in patient #2, from exon 46–52 in patient #3, from exon 12–30 in patient #4, from exon 8–29 in patient #5, and from exon 26–43 in patient #6. del, deletion.

plained by a failure to detect small or deep-intronic mutations. We have analyzed all of the exons and flanking intronic regions in these patients via high-throughput denaturing HPLC followed by direct sequencing, and we have identified a causative point mutation in 56 of these patients (unpublished data). This result improved the rate of detection of causative mutations to 95.7% (see Fig. 6 in the online Data Supplement), which is very close to the rate obtained by Zeng et al. *(35)*. In 4.3% of the cases, no mutations in the dystrophin gene could be identified after DNA analysis. We cannot exclude the presence of deep-intronic or atypical mutations. Alternatively, milder phenotypes may have been misdiagnosed as BMD, a situation that might occur especially when muscular dystrophy is diagnosed at a young age.

Discussion

The dystrophin gene spans a 2.2-Mb region at Xp21 that is exposed to intense deletion pressure in all populations. As a consequence, approximately 18 300 newborns with DMD are expected every year worldwide. No genetic background that influences this susceptibility to deletions has been recognized. Nevertheless, there should be an opposing mechanism that maintains the exceptional size of this gene. Resolving this issue requires reliable low-cost strategies for diagnosing the disease worldwide. The first tests were first designed by Chamberlain and Gibbs *(11)* and Beggs et al. *(12)*, and the 2 multiplex PCR assays (of 18 fragments) were immediately adopted as a quicker and less expensive alternative to Southern blotting with cDNA

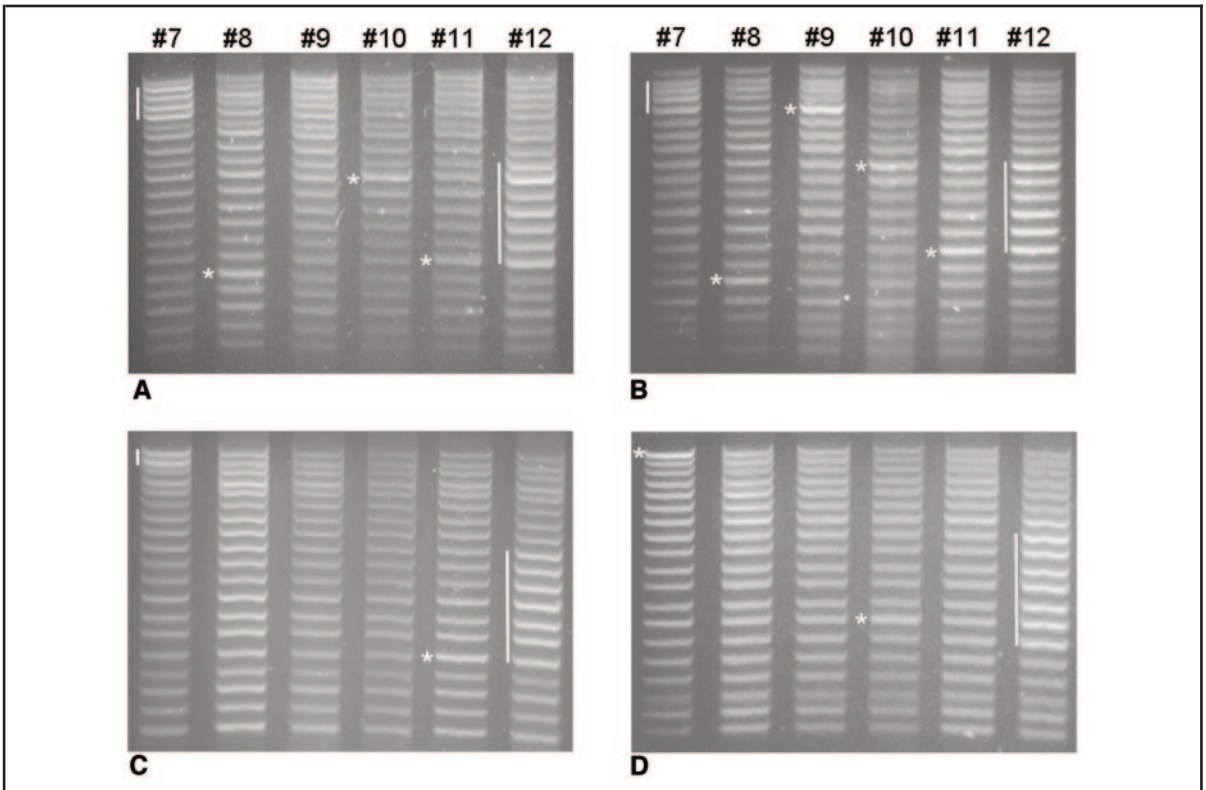

Fig. 3. Examples of duplications identified by Log-PCR testing.

PCR products with double the band intensity identify an exonic duplication. For detection, PCR products from the same mixture were run side by side to compare intensities. Note that identification of a duplication as a band with double the intensity is more reliable with a smaller number of PCR cycles [Mallikarjuna Rao et al. *(21)*] and staining with GelStar. We used Quantity One software (Bio-Rad Laboratories) to score electrophoresis bands. All duplications were confirmed by real-time PCR analyses. The electrophoretic results for all 4 multiplex PCR reactions (A–D) are shown for 6 patients (#7–#12). White asterisks and lines indicate duplicated exons. Mix A (A): #7, dupl exons 3, 5, 7, 11; #8, dupl exon 61; #9, no mutation; #10, dupl exon 45; #11, dupl exon 58; #12, dupl exons 43, 45, 50, 52, 54, 56, 58. Mix B (B): #7, dupl exons 4, 6, 8, 12; #8, dupl exon 62; #9, dupl exon 12; #10, dupl exons 44, 46; #11, dupl exons 57, 59; #12, dupl exons 44, 46, 51, 53, 55, 57, 59. Mix C (C): #7, dupl exons 9, 13; #8, no mutation; #9, no mutation; #10, no mutation; #11, dupl exon 60; #12, dupl exons 34, 36, 38, 40, 42, 48, 60. Mix D (D): #7, dupl exon 10; #8, no mutation; #9, no mutation; #10, dupl exon 47; #11, no mutation; #12, dupl exons 33, 35, 37, 39, 41, 47, 49. The duplication is from exon 3–13 in patient #7, involves exons 61 and 62 in patient #8, involves exon 12 alone in patient #9, is from exon 44–47 in patient #10, is from exon 57–60 in patient #11, and is from exon 33–60 in patient #12. dupl, duplication.

probes. These investigators claimed that these assays would identify 98% of deletions, but the true sensitivity is actually much lower. In addition, multiplex PCRs have 3 common drawbacks: (*a*) The occurrence of nucleotide variation or deletions in the sequences targeted by the primers can cause PCR failure (i.e., false signal for exon deletion); (*b*) a DNA sample containing salts or EDTA may reduce the PCR signal for exons amplified with A/T-rich primers (false signal for exon deletion); and (*c*) uncertainty may exist in the deletion/duplication endpoints, which are useful for predicting the reading frame.

An appreciable increase in the detection rate of deletions appears possible only if PCR tests are coupled with independent methods of analysis *(33–35)*.

MAPH *(29)* and MLPA *(30, 31)* are 2 new techniques that have been developed to detect deletions and are able to screen many target sequences simultaneously. MLPA is also commercially available (MRC-Holland) as 8 separate reactions for detection on aga-

rose gels. This method relies on hybridization of a sequence-specific probe to genomic DNA, subsequent amplification of the hybridized probes with a common primer set, and analysis of the resulting PCR products. As with conventional PCR, polymorphisms or single-base mutations in the probe-binding regions may affect results. The short length of the region identified by the specific probe (21 nucleotides) implies that mismatches at the binding site may prevent probe hybridization and hence prevent ligation and, ultimately, detection. Consequently, single-base changes may appear as exon deletions. In cases of exon rearrangements, such as translocations or the insertion of long interspersed elements, MLPA can miss mutations. Moreover, some true deletions randomly remain undetected with MLPA *(33)*. Thus, MLPA results should be confirmed with an independent method.

We propose Log-PCR as a new tool for complete screening of dystrophin exons and for sex testing. This method uses only 4 quantitative multiplex PCRs, which are run under the same reaction and cycling conditions. Protocols for quantitative PCR *(13)* and simultaneously amplifying all of the dystrophin exons *(28)* have already been described. The novelty of the Log-PCR approach is the achievement of both results in a single-step assay. It is applicable to genomic DNA extracted from blood or chorionic villi.

Multiplex PCRs are generally characterized by low product yield, product dropout, and nonspecific amplification. We carried out a long series of experiments to develop a robust quantitative multiplex PCR method that produces yields that are comparable for all amplicons and proportional to the initial amount of DNA template.

The idea of testing polyols and cosolvents originated from our review of early studies that found that the presence of sugars and polyols increased the thermal stability of proteins *(36)*. A later study demonstrated the use of trehalose for the thermostabilization of reverse transcriptase *(37)*. Including such compounds is crucial for the simultaneous and quantitative amplification of 57.5 kb of DNA fragments. The Log-PCR procedure substantially shortens reaction setup times and reduces reagent consumption, and these features support its use as a simple but universal test. We designed primers of 28–32 nucleotides in length with GC-rich 3′ ends that are never affected by latent polymorphic nucleotides. In addition, we used denaturing HPLC with 600 control samples to scan for rare variants in the annealing region. Thus, the Log-PCR was able to minimize the occurrence of false single-exon deletions, and we confirmed 66 of 66 single-exon deletions. Moreover, because most of the primers anneal to sequences far from exonic sequences, flanking intronic deletions can also be detected. For example, the Log-PCR method discovered a case of exon 22 deletion, which was not detectable with exonic primers because the missing nucleotides were in the flanking intron sequences and produced exon skipping.

To verify the reliability of the technique, we used the Log-PCR method with samples from a group of 506 patients with DMD or BMD that had been diagnosed at a single center according to homogeneous clinical criteria. We excluded samples from other sources to avoid introducing any bias. To our knowledge, this study is the most extensive single-center study of this kind ever done. Thus, the statistics for the dystrophin mutations we have described (506 cases) can be considered a very good approximation to the real frequency distribution of mutations. In fact, these data are not biased by the detection method because we used a homogeneous procedure to study all of the exons of every patient. In contrast, current databases include data produced with a heterogeneous group of methods and incomplete analyses. Such data sets are likely to overrepresent easy-to-discover mutations.

We hypothesize that geographic and ethnic differences are not important in X-linked lethal disorders, because all of the mutations have a short life in the pedigrees; however, because our data are derived from a Caucasian population, this distribution should be checked in other populations.

CLINICAL USE OF THE LOG-PCR

Complete visualization of all dystrophin exons allows mutation boundaries to be precisely defined. Such a comprehensive approach may be important when diagnostic studies are performed at a young age, when distinguishing between DMD and BMD is difficult. The identification of mutation endpoints may be important for future patient therapy via antisense oligonucleotide exon skipping *(38)*.

Log-PCR is a noninvasive, sensitive, and specific laboratory test for the diagnosis of DMD and BMD. The assay requires approximately 6 h and is cost-effective. It produces a report that is direct and easy to interpret, because all of the bands are ordered and evenly spaced. The assay requires the development of a reagent set that can be used in routine diagnostic laboratories. Compared with the MLPA reagents from MRC-Holland, the reagents should be 5 times less expensive, but labor requirements also should be addressed. The Log-PCR approach can also be used on microfluidics-based platforms (such as the Agilent 2100 Bioanalyzer) that reproducibly and completely quantify each fragment.

Log-PCR can also be useful for assessing carrier status (data not shown) when the mutation is known. We detected deletions in all of the carriers we tested.

We believe that the development of the Log-PCR method also provides a proof of principle for higher-throughput multiplex PCR methods. In the present study, we quantitatively amplified 57 kb of DNA sequence, and we expect this approach to be of use in resequencing large genomic regions (enrichment step). The method is therefore directly applicable to such resequencing without any further development.

Results obtained for a preliminary test series of samples sequenced on an ABI 3130XL DNA Sequencer (Applied Biosystems) have shown that direct sequencing of Log-PCR products is possible with common purification procedures and specific primers for sequencing.

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