Mutagenically separated PCR (MS-PCR): a highly specific one step procedure for easy mutation detection

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ABSTRACT

With increasing knowledge about the causal role of genetic defects in clinical diseases the necessity is apparent to have procedures for rapid diagnosis of point mutations. We developed a PCR-based technique, whereby both normal and mutant alleles can be amplifed in the same reaction tube, using different length allele-specific primers. Furthermore the allele-specific primers introduce additional deliberate differences into the allelic PCR-products that drastically reduce crossreactions in subsequent cycles. This mutagenesis separates the amplification reactions of the alleles performed in the same tube. Subsequent identification of the PCR-products is done by gel electrophoresis and shows at least one of the two allelic products. Therefore, in addition to simple handling, MS-PCR provides a within-assay quality control for the exclusion of false negative results. The feasibility of this technique has been tested using six different mutations. The high sensitivity of MS-PCR also allows screening for mutation carriers in pooled DNA samples.

INTRODUCTION

In the fast growing field of molecular genetics many new mutations are discovered every day. To delineate common characteristics for carriers of functional mutations it is essential to identify additional affected individuals. For the repeated detection of genetic defects very simple diagnostic tools are required. Most current methods for the detection of point mutations are based on the polymerase chain reaction (PCR) (1–3) but others work on different principles (4, 5). We developed a new PCR-based technique for the detection of a point mutation in the human apolipoprotein B gene (R3500Q) underlying familial defective apoB 100 [FDB, (6)], a disease in which heterozygosity leads to hypercholesterolemia (7) and thus may constitute a risk factor for coronary heart disease (8). We also show that this technique can be used in general for the identification of allelic DNA. The new technique was designed to be easier in handling and to give safer results than current techniques, as will be discussed below.

MATERIALS AND METHODS

DNA preparation

EDTA-blood samples were obtained from a normal homozygote, a heterozygote, and from a mutant homozygous patient. The status of these samples in respect of the analysed mutations was confirmed by independent methods as described (1, 9). DNA was obtained essentially as described by R.Higuchi (10). 500 µl EDTA-blood samples were mixed (1:1) with lysis buffer (0.32 M sucrose, 10 mM Tris HC1 pH 7.5, 5 mM MgCl₂, 1 % Triton X-100) to destroy red blood cells. After centrifugation (up to 1 min at 10,000 g) supernatant was removed. Lysis buffer treatment was repeated until yellow-white pellets were obtained. The pellet was finally resuspended in a mix of 500 µl nonionic detergent buffer (50 mM KCl, 10 mM Tris HCl pH 8.3, 2.5 mM MgCl₂, 0.1 mg/ml gelatine, 0.45% Nonidet P40, 0.45% Tween 20) with 30 µg of proteinase K. After incubation at 55°C for 2 hours, proteinase K was destroyed and DNA denatured by 10 min incubation in a boiling water bath.

Primers

In each MS-PCR-setup three primers are used, two allel-specific 'MS-PCR'-primers with both sequences derived from the same DNA-strand and one primer with a sequence derived from the complementary strand. The MS-PCR-primers are described in Table 1. The complementary strand primers are (5' – 3'): CP1 = GGATCCAGGAGTCCAGATCCCCAGC, CP2 = GGAC-AGACAGACAGACAGGTGGCAGG, CP3 = CAGGACGAAACTGAGCCCCATGCGG, CP4 = TCATCCGCAGAGACACTCACGGCGGCAGCC, and CP5 = CAGCAGGAAATAGAGCAGAATGATGCAGAC. The sequences of primers P1, P2, P3, and P4, used in the first example of MS-PCR, are given in the legend to Figure 1.

PCR reaction conditions

Individual analysis for the FDB mutation. All reactions were performed in a Gene ATAQ Controller (Pharmacia). For individual diagnosis 2 µl DNA-solution were used with 6 µl 10 X PCR-buffer (500 mM KCI, 100 mM Tris HCl pH 8.3, 0.01% gelatine), 1 mM MgCl₂, 10 µM each of the four dNTPs, 0.1 µM primer P3 (primer sequences see Figure 1), and 1.5 units Taq-polymerase (Beckman) in a 60 µl reaction volume.
P1 was used at 0.1 μM, primer P2 at 0.075 μM. A mineral oil layer was used as vapour barrier. Cycling was 45×[1 min 95°C, 1 min 65°C, 1 min 72°C], 4×[2 min 65°C, 2 min 72°C], 5 min 72°C. 10 μl of the reaction products were analysed by agarose gel electrophoresis.

Reaction conditions for all other examples are detailed in the results section.

**Hot start PCR.** Hot start reactions were performed essentially as described (15). 20 μl aqueous solution including 2 μl 10×PCR-buffer, 1 mM MgCl₂, primers and dNTPs were covered with an AmpliWax™ PCR Gem barrier (Perkin Elmer Cetus). 40 μl of a solution containing 4 μl 10×buffer, 1 mM MgCl₂, 1.5 units Taq-polymerase and the sample DNA were put on top of the barrier. Final concentrations of dNTPs were 20 μM each.

**Analysis of pooled samples for the FDB mutation.** To increase the sensitivity of PCR-analysis for the mutant allele in a mixture with a multifold excess of normal alleles, we developed a three-step procedure. The rationale for the following protocol is given in the results section. 2 μl DNA solution were mixed with 10 μl of a reagent mix to obtain final concentrations (neglecting contributions from the sample) of 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 0.001% (w/v) gelatine, 1.5 mM MgCl₂, 11.67 μM for each of the dNTPs, 0.124 μM primer P2 and 1.17 units Taq-polymerase per reaction. Without mineral oil overlay four cycles of annealing and extension were performed [4×(2 min 60°C, 2 min 72°C), 5 min 72°C], to obtain double stranded DNA from all denatured single stranded normal template. Concentration of KCl, Tris–HCl and gelatine were kept constant in subsequent amplifications. 58 μl of a second reagent mix were added to obtain final (accumulated, theoretical) concentrations of 0.5 mM MgCl₂, 2 μM for each of the dNTPs, 0.0213 μM primer P2, 0.1 μM P1 (molar primer ratio P1/P2 = 4.7), 0.1 μM P4 and 1.75 units Taq-polymerase in 70 μl reaction volume. The DNA was amplified with mineral oil overlay [20×(1 min 95°C, 1 min 60°C, 1 min 72°C), 4×(2 min 60°C, 2 min 72°C), 5 min 72°C]. 7 μl of this reaction were transferred to a new tube and mixed with 43 μl of a third reagent mix to obtain final (accumulated, theoretical) concentrations of 0.5 mM MgCl₂, 5.3 μM for each of the dNTPs, 0.0243 μM primer P2, 0.144 μM P1 (molar primer ratio P1/P2 = 5.93), 0.014 μM P4, 0.1 μM P3 and 1.425 units Taq-polymerase in 50 μl. Amplification was [40×(1 min 95°C, 1 min 65°C, 1 min 72°C), 4×(2 min 65°C, 2 min 72°C), 5 min 72°C]. 15 μl of the reaction product were analysed.

**Agarose gel electrophoresis**

Agarose gel electrophoresis was performed using 3.5% NuSieve GTG Agarose (FMC)/1% Agarose NA (Pharmacia) composite gels, 1×TAE-buffer [50×TAE = (242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0)/liter], 0.5 μg ethidiumbromide/ml; samples were mixed with 0.2×the volume of loading buffer (40% sucrose, 0.1 M EDTA, 0.25% bromphenol blue (Sigma)); electrophoresis was for 1-2 hours at 5 V/cm. Gels were photographed using a Reprostar UV-transilluminator (300 nm, CAMAG) and Polaroid type 667 instant film.
Cycle Hybridisation of alleles with P1

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Hybridisation of alleles with P1</th>
<th>Hybridisation of alleles with P2</th>
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<tbody>
<tr>
<td>1</td>
<td>(A) normal allele</td>
<td>(C) normal allele</td>
</tr>
<tr>
<td></td>
<td>no mismatch;</td>
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</tr>
<tr>
<td></td>
<td>no elongation</td>
<td>efficient elongation</td>
</tr>
<tr>
<td>2</td>
<td>(K) mutant allele</td>
<td>(O) mutant allele</td>
</tr>
<tr>
<td></td>
<td>3 mismatches;</td>
<td>3 mismatches;</td>
</tr>
<tr>
<td></td>
<td>efficient elongation</td>
<td>no elongation</td>
</tr>
<tr>
<td>3</td>
<td>(P)</td>
<td>(Q)</td>
</tr>
<tr>
<td></td>
<td>3 mismatches;</td>
<td>3 mismatches;</td>
</tr>
<tr>
<td></td>
<td>no mismatch;</td>
<td>no mismatch;</td>
</tr>
<tr>
<td></td>
<td>efficient elongation</td>
<td>efficient elongation</td>
</tr>
</tbody>
</table>

Figure 2. Principle of MS-PCR. In the schematic nucleotide sequence drawings base positions are symbolized by small circles lined up for all regular positions. The base at the position of the analyzed polymorphism is designed by 'M' (mutant) or 'N' (normal) in the original target and by 'm' or 'n' in the complementary strands, respectively. The large filled circles and squares symbolize additional mutagenic positions in the primers P1 and P2, where they differ from each other and from the original target. These substitutions reduce the probability of false priming. Corresponding complementary substitutions, that are introduced into the amplification products [(I), (K)] by PCR-mediated mutagenesis with these primers, are symbolized by empty circles and squares. Mismatches between primer and template are indicated by a larger distance of the—elsewhere parallel aligned—strands. The complete sequences of the primers used in MS-PCR and their relative positions are given in Figure 1. The regular amplification reactions are (B) —(F) —(I) —(M) —... for the mutant allele and (C) —(G)—(K)—(M) —... for the normal allele. Side reactions obtaining elongation products (E) and (H) are suppressed by competition in the MS-PCR (see text) and are put into brackets to indicate this fact.

performs a mutagenesis at the 2nd base position from its 3'-end. Thus, the two different amplification products (I), (K) which, after a few cycles of PCR, provide the vast majority of templates, differ from each other not only by the allelic mutation, but also at four other base positions, mutagenically changed by the primers (Figure 2 shows only the situation near to the 3'-ends of the mutagenic primers, with two of the mutagenically changed base positions). Thereby, crossreactions caused by annealing and elongation of the non-matching primer are drastically reduced (situations (L) and (O) in Figure 2) and the two reactions are practically separated from each other by selective mutagenesis. Thus, reaction specificity crucially depends on the specificity during the first cycle, where one deliberate mismatch increases the specificity of elongation on the original target (first cycle (A) —(H) Figure 2). This latter effect is similar to that used in the ARMS-method described by Newton et al. (3). If, however, deliberate mismatches between primers and templates are only used to increase specificity on the original template and placed at identical distances from the primers' 3'-ends and using identical nucleotide changes as in the ARMS-technique (3), they will no longer be present between PCR made templates and the non-matching primers. As a consequence, ARMS reactions had to be carried out in separate test tubes. In contrast, an essential effect in MS-PCR is the accumulation of mismatches between any one of the allelic primers and the PCR-product obtained with the other primer. A finding by Kwok et al. (16) who have shown that the differentiation of alleles is difficult if the mismatch at the 3'-end is C:A or involves a T was also observed in our experiments. This is especially true if separate reactions with primer P3 and only one of primers P1 and P2 are performed in a test tube with mismatching DNA. Lane 6 in Figure 3 shows an unspecific

Figure 3. MS-PCR for individual diagnosis of FDB. For PCR-reaction conditions see methods. Different reaction series were set up to demonstrate the successful allele-specific analysis of the genotype at the FDB-mutation position by MS-PCR (lanes 2—4) using primers P1, P2, and P3, and to show the unspecific products that occur if PCR is performed in separate reactions containing P3 and either of primers P1 (lane 5—7) or primer P2 (lanes 8—10). Each series of three reactions comprised a heterozygote ('H', lanes 2, 5, 8), a normal homozygote ('N', lanes 3, 6, 9), and a mutant homozygote ('M', lanes 4, 7, 10). Analysis of the PCR-products was done by separation of the different sized allelic amplification-products by gel electrophoresis (cf. (4)). Lane 1 contains 2 μg of standard (1Kb-ladder, BRL). The reactions shown in lanes 2—4 did contain all three primers (P1, P2, P3) and gave the correct results: Lane 3, the 134 bp MS-PCR product from a normal homozygote, lane 4, the 114 bp product from a homozygous mutant, and lane 2, both products from the heterozygote. However, from all the samples a short amplification product was obtained when only primers P1 and P3 were used (lanes 5—7) and a long product was obtained with primers P2 and P3 (lanes 8—10). Lanes with unspecific amplification products are labeled at the bottom by triangles (lanes 6, 10). Specific amplification was only obtained in MS-PCR, that uses all three primers in a single reaction (lanes 2—4).
amplification product of the normal allele with the mutant allele-specific primer despite the fact that the two bases at the 3'-end did not match with the template (cf. Figure 2 (A) → (E) → (I) → (M)). Also, an unspecific amplification product of the mutant allele was obtained, when homozygous mutant DNA was amplified with the primer specific for the normal allele [(lane 10, Figure 3); Figure 2 (D) → (H) → (K) → (N)]. However, the same samples were correctly amplified if both allele-specific primers were brought into action in one tube (lanes 3, 4, Figure 3). This phenomenon may be understood as the result of competition for templates and other reaction components. A similar effect has also been observed by Ferrie et al. (17) who could demonstrate that single ARMS-reactions provide more specific results when an independent DNA fragment from another gene is coamplified. Thus, as demonstrated in lanes 2–4 of Figure 3, MS-PCR is a rapid and more simple tool for the analysis of homozygous and heterozygous states by performing one reaction per sample and subsequent electrophoretic analysis of the MS-PCR-products.

**General applicability**

Additional examples of MS-PCRs were developed to demonstrate that MS-PCR can be generally used for analysis of point mutations and to delineate a few general rules for the design of MS-PCR-primers and reaction conditions.

For design of MS-PCR-primers, employed in the following experiments, we used a common scheme: One of the two allele-specific primers was 28 or 29 bases in size, the other primer was 49 bases long. The short primer does always contain one deliberate mismatch in one of the three bases that are adjacent to the 3'-terminal base. In the corresponding region also the longer primer has a deliberate mismatch but placed at a position different from that in the short primer. In addition, the long primer has two consecutive mismatches at positions corresponding to the 5'-end of the short primer to inhibit filling up heteroduplex molecules as explained above. The non-selective complementary strand primers were preferentially chosen at a distance to the MS-PCR-primers that yields PCR-products with a length between 100 and 300 basepairs. Such products are easily analysed on agarose gels with good separation of allelic variants due to the 20 bp difference introduced by the allelic MS-PCR-primers. To demonstrate that development of successful MS-PCRs needs no complicated optimization, we used a common reaction scheme for additional examples of MS-PCRs: All reactions were performed in final volumes of 20 μl including 2 μl 10×PCR-buffer (see methods), using 1 mM MgCl₂, 20 μM of each of the dNTPs and 0.5 units Taq-polymerase. Two MS-PCR-primers and one primer derived from the complementary strand were contained in each reaction, the complementary strand primers were always used at 0.1 μM, and the long MS-PCR-primers at 0.08 μM. For the short MS-PCR-primers different concentrations were tested to obtain two equally intense bands in heterozygotes. Different annealing temperatures were tested ranging from 55°C to 69°C. Generally, cycling was 40×[30 s 95°C, 45 s annealing temperature, 45 s 72°C], 3 min 72°C, 4°C hold, performed without oil overlay in a GeneAmp PCR System 9600 (Perkin Elmer). The number of cycles was sometimes different as indicated below. 10 μl of the PCR-products were analysed. Figure 4 shows results of these experiments. The sequences of the MS-PCR-primers ("CP...") are given in the materials section. In Figure 4A lanes 2–4 show the MS-PCR-results for a polymorphism in the 5'-noncoding region of the apolipoprotein E gene, a T→G exchange at position 638 [unpublished, numbering according to (12)], using primers MS1, MS2, and CP1. Annealing was at 60°C, 38 cycles were performed. The use of 43 cycles gave the same results (not shown). Lanes 5–10 present the products from two different MS-PCRs for a mutation in Codon 360 of the apolipoprotein A-IV gene [CAG→CAT: Gln→His, physiologic relevance discussed in (19)]. In one example MS-PCR-primer sequences (MS3, MS4) are derived from the upper, coding strand ("us") in Figure 4A, lanes 5–7), in the other example the primer sequences (MS6, MS7) were taken from the lower strand ("ls"), lanes 8–10. Complementary strand primers were CP2, and CP3, respectively. Figure 4B shows the analysis of two mutations in the lecithin cholesterol acyltransferase (LCAT) gene [relevance discussed in (20)]. The mutation in codon 123 ACA→ATA: Thr→Ile was analysed using primers MS8, MS9 and CP4 (lanes 2–4). The codon 158 mutation CCG→TGC: Arg→Cys was analysed using primers MS10, MS11 and CP5 (lanes 5–7). To meet the criterion of equal band intensity in heterozygote DNA we had to employ a molar primer ratio of 0.19:0.08 (MS10:MS11), which was the highest ratio of all examples shown (see Table 1). One might suspect that, by using this primer ratio, a difference of priming efficiency is exactly compensated by unsymmetric amplification...
a hot start amplification (see methods) prevents this artefact
dilutions performing 47 cycles again provided equally intense
assignments (lanes 2-4). However, also a primer artefact was
ACT—TCT: Thr—Ser [for relevance see (19)]. Initially primers
polymorphism in codon 347 of the apolipoprotein A-IV,
any essential unsymmetry in results over a range of two orders
bands for the heterozygote (not shown). Thus, we could not detect
were observed for the heterozygote DNA. Analysis of the same
samples after 100-fold dilution of the DNA
(amount of DNA, thus producing unsymmetry of products. Also,
amplified to a lower extent than other samples with a lower
the specific amount of sample DNA and number of cycles used.

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Ref.</th>
<th>Sequence (5'-&gt;3')</th>
<th>Conc. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo B Cod 3305</td>
<td>10586-10608</td>
<td>11</td>
<td>ggaa - (n&lt;sub&gt;2&lt;/sub&gt;) - aggGT</td>
<td>0.15</td>
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<tr>
<td>P1</td>
<td>10607-10628</td>
<td>11</td>
<td>ggaa - (n&lt;sub&gt;2&lt;/sub&gt;) - aggGT</td>
<td>0.08</td>
</tr>
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5'-Apo E:
| MS1          | 10586-10608 | 12 | ggaa - (n<sub>2</sub>) - aggGT | 0.15 |
| MS2          | 10586-10608 | 12 | ggaa - (n<sub>2</sub>) - aggGT | 0.08 |

Apo A-IV-Cod 360 bp:
| MS3          | 2398-2387 | 13 | ctga - (n<sub>2</sub>) - agcc | 0.12 |
| MS4          | 2398-2387 | 13 | ctga - (n<sub>2</sub>) - agcc | 0.08 |

Apo A-IV-Cod 360 bp:
| MS5          | 3414-2387 | 13 | ctga - (n<sub>2</sub>) - agcc | 0.15 |
| MS6          | 3435-2387 | 13 | ctga - (n<sub>2</sub>) - agcc | 0.08 |
| MS7*         | 3414-2387 | 13 | ctga - (n<sub>2</sub>) - agcc | 0.15 |

LCAT Cod 125:
| MS8          | 2314-2387 | 14 | gctg - (n<sub>2</sub>) - ctcGT | 0.16 |
| MS9          | 2314-2387 | 14 | gctg - (n<sub>2</sub>) - ctcGT | 0.08 |

LCAT Cod 158:
| MS10         | 2343-2369 | 14 | gctg - (n<sub>2</sub>) - ctcGT | 0.19 |
| MS11         | 2343-2369 | 14 | gctg - (n<sub>2</sub>) - ctcGT | 0.08 |

Apo A-IV-Cod 347:
| MS12*        | 2349-2346 | 13 | gcgt - (n<sub>2</sub>) - ctcGT | 0.08 |
| MS13         | 2373-2346 | 13 | gcgt - (n<sub>2</sub>) - ctcGT | 0.13 |
| MS14*        | 2349-2346 | 13 | gcgt - (n<sub>2</sub>) - ctcGT | 0.08 |

Table I. MS-PCR-primer-pairs

Primers are listed as packages in pairs or as triplets if one primer was used in combination with two different primers in different samples (alternative primers are marked by *). Primer sequences are given in a shortened form, using n<sub>m</sub> as a substitute for m nucleotides corresponding to the published sequences (see 'Ref.'). Bases in upper case letters are at mutagenic or allelic positions. In addition, mutagenic positions are underlined. The third line of each package shows the complementary template region corresponding to the 3'-ends of the primers, the polymorphic position indicated by an 'x'. The column 'Cone.' provides the concentrations used in the final reaction volume.

to obtain equally intense bands, the ratio being valid only for
the specific amount of sample DNA and number of cycles used.
If some samples were overamplified they would actually be
amplified to a lower extent than other samples with a lower
amount of DNA, thus producing unsymmetry of products. Also,
use of different cycle numbers to amplify a low amount of DNA
would result in unsymmetric results. Therefore, we first analysed
the same samples after 100-fold dilution of the DNA (lanes
8-10). While total intensity of signals was reduced (compensated
in the photograph by longer exposure) two equally intense bands
were observed for the heterozygote DNA. Analysis of the same
dilutions performing 47 cycles again provided equally intense bands for the heterozygote (not shown). Thus, we could not detect
any essential unsymmetry in results over a range of two orders
of magnitude of DNA. Panel C shows the analysis of a polymorphism in codon 347 of the apolipoprotein A-IV, ACT—TCT: Thr—Ser [for relevance see (19)]. Initially primers
MS12, MS13 and CP3 were used. This provided correct zygosity assignments (lanes 2—4). However, also a primer artefact was
observed, marked by a 'x' left to the artefact in lane 2. Performing
a hot start amplification (see methods) prevents this artefact
formation (lanes 5—7). We also successfully tested the
substitution of MS12, which was involved in artefact formation,
by MS14 (lanes 8—10).

Pool-screening with MS-PCR
Carrier frequencies for most genetic diseases are so low that exact frequency estimations in unbiased populations samples are not available due to high costs. We therefore tested whether MS-PCR would allow us carrier screening in pooled samples. As an example we chose FDB for which previous estimates of carrier frequency ranged from 1/500 to 1/700 (21-23). To obtain a preferential amplification of the mutant allele, in which pooled samples is largely underrepresented, we used P1/P2 primer ratios of up to 6:1 (at molar basis). Some additional procedural modifications were made to enhance the sensitivity and specificity for pool analysis (details see methods). First, since the DNA was denatured at the end of the preparation procedure, unspecific priming of the normal allele by the mutant primer might occur as a consequence of the vast excess or the exclusive presence of normal alleles in the reaction mixture and because of the reaction start at nonstringent temperatures. This problem was eliminated by the preparation of double stranded DNA in precycles (annealing and extension) using only the primer specific for the normal allele. A second problem was primer artefacts (18) that occurred by increasing the PCR cycle number to obtain sufficient product from the underrepresented mutant allele. A two step protocol solved the problem: 20 cycles with primer P4 (located 5' to primer P3, see Figure 1) were performed, at a concentration of 2 µM for each of the four dNTPs to increase the selectivity of the allele-specific elongation (2). Further, the annealing temperature was reduced to 60°C to obtain a higher annealing efficiency of mutagenic primers with their templates. An aliquot of the reaction product was further amplified, now using primer P3, 5 µM dNTP (to obtain more product) and an annealing temperature of 65°C. To test the sensitivity of this

Figure 5. Sensitivity of MS-PCR for pooled samples. Variable amounts of EDTA-blood from a heterozygous FDB-carrier and a homozygous normal proband were mixed to yield the leuocyte ratios indicated above the lanes. The homozygous normal control (N', lane 2, 134 bp) shows no mutant background band. In all lanes with samples containing any fraction of mutant alleles a mutant band (114 bp) is visible. The normal conditional control band is most efficiently suppressed in samples with the highest fraction of mutant alleles (lanes 3, 4). It becomes visible and more intense with decreasing amounts of mutant alleles in the sample. However, as the high primer ratio P1/P2 preferentially amplifies the mutant allele the normal allele is not as efficiently amplified as to suppress visible amplification of underrepresented mutant alleles at least down to ratios of 1 mutant allele in 6400 alleles.
reaction scheme, we analysed mixed samples that contained the DNA of a heterozygous carrier in an up to 3200 fold excess of DNA from a normal proband (allele ratio 1:6400, Figure 5). As demonstrated in lane 2, in case there was no mutant allele present in the mixture, no mutant amplification product (114 bp) was obtained but an amplification product of the normal allele (134 bp) was visible. If, however, there was a mutant allele present in the mixture, a mutant band appeared, while the normal product was suppressed with increasing amounts of the mutant allele (lanes 10—3). Thus, separated amplification of both alleles in a single tube with the use of different primer concentrations and accordingly different amplification rates allowed a highly specific and sensitive allele identification. In cases where a relative high initial concentration of mutant alleles is present in the sample pool there is only a silent amplification (product concentration too low for visualisation) of the normal allele (lanes 3, 4, Figure 5). Amplification of normal alleles to a visible product in the absence of a band specific for the mutant allele (Figure 5, lane 2), provides a conditional internal control signal of PCR-efficiency in pool-screening. In the presence of a mutant allele in the analyzed pool the mutation specific product demonstrates PCR-efficiency.

DISCUSSION

MS-PCR is a new PCR based method for the identification of previously known allelic mutations in nucleic acid sequences. The basic principle of the method is the introduction of mutations into the PCR-primer binding regions of the amplified DNA in an allele-specific manner, using allelic primers with mutagenic positions at different distances from their 3’-ends. In subsequent cycles these mutations prevent the formation of product by the erroneous elongation of the wrong primer.

The products formed during the MS-PCR process are specific for their respective alleles and can be directly identified by agarose gel electrophoresis with no need for additional manipulation. This is different in two other frequently used methods for the detection of point mutations. One is the ARMS-method (3), the other one uses restriction digestion of PCR-products (1). The latter technique requires the presence or creation of a polymorphic recognition site in the PCR-product and restriction enzyme digestion after PCR to obtain differently sized allelic products. In the ARMS-technique (3) two physically separated reactions for the two alleles are set up, thus doubling the number of PCRs to be performed. To overcome problems with false negative results, which can occur in single allele amplifying systems by events as simple as forgetting to pipet a reaction component, in these techniques the amplification of an independent fragment, e.g. from another gene, is used as an internal control. Misinterpretations due to errors in the individual setup should be impossible with MS-PCR since the use of a mastermix for the reagents and of positive and negative controls assures that the reaction components are correctly checked and any reaction not yielding at least one of the allele-specific bands would be easily recognized as incorrect. As a further advantage of MS-PCR we have shown that the competition and mutagenic separation of the allelic amplifications in a single tube may result in an essential increase of specificity (lanes 3, 4 Figure 3) as compared to two physically separated reactions (unspecific amplifications lanes 6, 10 Figure 3).

The ease in the setup of new MS-PCRs and thus its general applicability was demonstrated by the use of a common scheme for primer design and reaction setup (see results section). The only optimisation necessary was the identification of the best annealing temperature. To increase the ease in data interpretation a subsequent optimisation step adjusted the relative concentrations of the two allele-specific primers in the reaction to yield equally intense fluorescence signals from the alternative allelic products upon ethidium bromid staining of the size separating agarose gels. From Table 1 it is apparent that in all cases the longer of the allele-specific primers was used at a lower concentration than the shorter one. This may be related to annealing advantages of longer primers at high temperatures. These differences in primer concentration do not respond to differences in the amount of DNA used in the MS-PCR as is demonstrated by the analysis of a 100-fold diluted sample (Figure 4B). It should be mentioned that the correct performance of MS-PCR is not restricted to the reaction condition frame we have used in the setup of the reactions shown in Figure 4. In fact, following a report on the improvement of specificity during amplification reactions by reducing the amount of dNTPs from 200 μM to 20 μM (2) we have found unambiguous results in the detection of the FDB mutation using dNTP concentrations ranging from 2—50 μM (data not shown).

Since all allele-specific amplifications with MS-PCR we have attempted to set up have been successful, we expect that the rules given in the results section for establishing MS-PCR assays are quite helpful. In one case, shown in Table 1, the initial design of the shorter primer for the ‘Apo A-IV-Cod 360’ analysis had to be changed (MS5 has been replaced by MS7). Possible explanations for the observed disadvantage of primer MS5 to form, in the presence of primer MS6, not sufficient PCR product for visualisation include inspecific interaction of MS5 with other primers, PCR product or genomic DNA as well as selfannealing or a too weak binding of its 3’-end to its target sequence. Assuming the latter would have the most prominent effect, the MS7 primer was designed, with the sequence similar to MS5, but modified to allow the formation of a G:C base pair near the 3’-end of the primer/template hybrid. The success of this measure suggests an important role of the stability with which the 3’-end of the primer anneals the template. This observation cannot currently be extended into a rule since in all cases primers that form the most 3’-located G:C base pair with their respective templates are also longer than their allelic counterparts. It appears to be important, though, to not create too big differences for allelic primers in their annealing efficiency, specifically at the 3’-ends. In this context the findings by Ferrie et al. (17) are quite interesting who have reported that deliberate purine/pyrimidine mismatches are less destabilizing than either purine/purine or pyrimidine/pyrimidine mismatches.

Since MS-PCR is dependent on reproducible annealing and extension kinetics, it might be argued that its use can be problematic in situations where only a few bases away from the allelic site of interest there is an additional, possibly frequent, polymorphism. If such a polymorphism exists outside the crucial 3’-end of the primer and results from a transition mutation (17), it may not have any effect at all. In case where polymorphisms affect MS-PCR it can be set up in a different orientation that is, in case the polymorphic site is located 3’ of the mutation to be detected the allele-specific primers will be upper strand primers and the amplification primer is lower strand and in case of a 5’ polymorphism it is the other way around. An example for this
strategy is shown in Figure 4A. A different strategy could be the synthesis of degenerated primers which contain the alternative bases at the second polymorphic site. The incorporation of an inosin into the primers at the second polymorphic site is yet another way to overcome these problems. Ehlen and Dubeau (2) have shown successful amplifications even if there are more than two such other polymorphisms and even if one such mutation occurs within the last four bases.

To unravel the phenotypic consequences of specific mutations it is often required to identify additional carriers of the same mutation. This is largely simplified by individual MS-PCR. A further advance in simplifying such a screening is achieved by the modified MS-PCR for analysis of pooled samples. The unique feature of MS-PCR to amplify both alleles in competition allows the amplification to visible product of the normal allele for use as conditional internal control of the PCR-efficiency. The formation of this control fragment is automatically suppressed if a mutant allele is present whose reaction product takes over the function as a control. Thus also in this modified procedure the safety inherent in MS-PCR is retained. From the mutations and applications for DNA amplification. Stockton Press, New York, pp. 31 – 38.

In conclusion we expect broad application of MS-PCR does also provide a measure for simplified its application in poolscreening for large epidemiologic projects as well as for specific problems in detection of allelic variation.

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REFERENCES