Detection of Mutation Δ F508 in the Cystic Fibrosis Gene Using Allele-specific PCR Primers and Time-resolved Fluorometry

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A method to detect the main cystic fibrosis (CF) mutation Δ F508 from dried blood spots, whole blood, or saliva using the polymerase chain reaction (PCR) and time-resolved fluorometry (TRF) is described. Samples are treated by boiling in mild alkaline solution, after which two allele-specific PCR reactions are performed. Allele-specific primers and a common biotinylated primer are used in the amplification reactions. To detect the PCR product, an europium-labeled oligonucleotide, complementary to the biotinylated strand of the PCR product, is used in a solution hybridization. Hybridization is done in streptavidin-coated microtitration wells, making the detection easy to perform. After a washing step, the bound label is detected using a time-resolved fluorometer. To analyze function of the assay, 20 dried blood spot samples were tested. PCR amplification of the deletion region combined with gel retardation assay was used as a control method. In the initial testing, 2 samples giving discrepant results in the two assays were found. In addition, 17 samples from known CF patients together with 6 normal control samples were analyzed. Among these patient samples, 10 homozygotes and 6 carriers for mutation Δ F508 were found.

In Caucasian populations, approximately one out of 2500 newborns is affected with cystic fibrosis (CF), making CF one of the most common autosomal diseases. CF causes defects in mucus secretion, which affect the respiratory system, pancreas, and sweat glands.⁽¹⁾ Today, diagnosis of CF is based mainly on the sweat chloride test and on the presence of elevated levels of immunoreactive trypsinogen (IRT).⁽²⁾

Recent cloning of the CF gene⁽³⁾ has increased our understanding of CF at the molecular level⁽⁴⁾ and has made possible exact diagnosis of the disease. It has also opened avenues of research for developing an effective treatment for CF.^(5,6) A 3-bp deletion (Δ F508) can be found in ~70% of CF chromosomes. The CF gene containing this mutation codes for a truncated protein lacking a phenylalanine residue at position 508. Because other mutations causing CF (30%) are heterogeneous, an approach that includes IRT measurement and determination of the deletion Δ F508 has been proposed for newborn screening of CF.⁽⁷⁾

DNA amplification methods,⁽⁸⁻¹⁰⁾ including the polymerase chain reaction (PCR),⁽¹⁰⁻¹²⁾ have made it possible to analyze mutations from minute amounts of sample DNA. PCR has been used to amplify the region around the main CF mutation Δ F508 before analysis using allele-specific oligonucleotides^(4,13) or to create an artificial restriction site in the deletion region, which can then be analyzed using restriction enzyme analysis.^(14,15) Also direct analysis of the Δ F508 deletion with PCR allele-specific primers can be used.⁽¹⁶⁾ In allele-specific PCR,⁽¹⁷⁻²⁰⁾ primers are designed to

match from the 3' end either with the normal or the deleted allele, making it possible to amplify only one of the alleles (Fig. 1).

In time-resolved fluorometry (TRF) rare-earth metal chelates are used as labels.^(21,22) The fluorescence coming from these chelates has a long decay time, which makes it possible to detect the specific signal after a time delay. During this delay time, the short-living background fluorescence coming from the sample has faded. The large shift between the excitation and emission wavelengths (Stokes shift) also contributes to the sensitive detection in time-resolved fluorometry.

We wanted to develop a simple and user-friendly method for detection of the CF deletion Δ F508. To achieve this, it is necessary to avoid laborious sample preparation methods and the electrophoresis procedures normally used. Boiling was tested in the sample preparation and solution hybridization^(23,24) was used in the detection of the PCR amplification product.^(25,26)

METHODS

Oligonucleotide Synthesis

All the oligonucleotides were synthesized using phosphoramidate chemistry and a Gene Assembler Plus (Pharmacia LKB Biotechnology, Uppsala, Sweden) oligonucleotide synthesizer. For biotinylation of the PCR primer, an aliphatic amino group was introduced in the 5' end of the oligonucleotide using 5'-O-dimethoxytrityl-N4-(trifluoroacetylaminodohexyl)-deoxycytidine-3'-O-(β -cyanoethyl-diisopropylamino)-phosphoramidate

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FIGURE 1 Principle of the assay.

(modC).⁽²⁷⁾ Thirty modCs were introduced in the 5' end of the detection probe for labeling. Identical coupling conditions were used for the modC as for the other phosphoramidates. The oligonucleotides were purified using denaturing polyacrylamide gels according to standard methods.⁽²⁸⁾

Labeling of the Oligonucleotides

Biotinylation of the PCR primer was done using a biotin-amidocaproate *N*-hydroxysuccinimide ester (Sigma Chemical CO., St. Louis, Missouri) as described earlier.⁽²⁶⁾

The detection probe was labeled using Eu chelate W2014.⁽²⁷⁾ The chelate contains isothiocyanate as the reactive group. Labeling and purification were performed according to Dahlen et al.⁽²⁶⁾ The final probe contained 28 Eu chelates per oligonucleotide.

Sample Preparation

A disk 2 mm in diameter was cut from

the dried blood spot into 500- μ l Eppendorf tubes using a hand puncher. To prevent cross-contamination between the samples, the puncher was incubated in concentrated HCl for 2 min, rinsed with distilled water, and dried after each sample. A volume of 50 μ l of 10 mM NaOH, 1% Nonidet P-40 (Sigma, St. Louis, Missouri) was added and the sample was incubated at 100°C for 10 min. After incubation, the mixture was chilled on ice and briefly centrifuged to spin the solution to the bottom of the tube. Then 50 μ l of 10 mM CH₃COOH, 100 mM Tris HCl (pH 8.5) was added to neutralize the sample. Tubes were centrifuged for 5 min with an Eppendorf microcentrifuge 5415C (16000 RCF) before taking the sample into the PCR reaction. When saliva or whole blood was used, the sample was pipetted directly into 10 mM NaOH, 1% Nonidet P-40, and otherwise treated as above.

PCR Amplifications

PCR amplifications were performed in a

Perkin-Elmer Cetus Thermocycler using a program of 95°C for 50 sec, 53°C for 2 min, 73°C for 2 min, for 24 cycles unless otherwise stated. The total volume of each reaction was 50 μ l and the samples were added in volumes of 20 μ l. The final reaction contained 10 mM Tris HCl (pH 8.4), 0.2% Nonidet P-40, 5.0 mM MgCl₂, 200 μ M of each dNTP, 0.1 mg/ml bovine serum albumin, and 50 mM NaCl. PCR primers (Table 1) were used at 0.1 μ M concentrations.

Solution Hybridizations

To denature the amplification product, PCR reactions were boiled at 100°C for 5 min, cooled on ice for 5 min, and centrifuged for 5 min. Aliquots of 10 μ l were pipetted from each PCR reaction into two microtitration wells coated with streptavidin.⁽²⁶⁾ Hybridization solution (0.5 M NaCl, 25 mM Tris-HCl (pH 7.75), 0.005% Tween-40, 0.25 % BSA, 0.025% bovine globulin, 10 μ M DTPA, and 0.025% sodium azide), 200 μ l, contain-

TABLE 1 Oligonucleotides Used for the Allele-specific Amplifications and Detection of the PCR Product

Use of the oligonucleotide	Sequence from 5' to 3' end
5' Primer used in the gel retardation test	5'-GCCTGGCACCATTAAAGAA-3'
3' Primer used in the gel retardation test	5'-AGTTGGCATGCTTTGATGAC-3'
5' Primer in the amplification of the normal allele	5'-ACCATTAAAGAAAATATCATCTT-3'
5' Primer in the amplification of the deleted allele	5'-CCATTAAAGAAAATATCATTGG-3'
The common, biotinylated 3' PCR primer	5'-Bio-modC-CTCTTCTAGTTGGCATGCT-3'
Detection probe used in hybridization	5'-(EumodC) ₃₀ -GTTTCCTATGATGAATATAGATACAGAAGC-3'

ing 2 ng of the detection probe was pipetted above the samples. The solution hybridization and collection of the formed hybrids onto the microtitration wells were performed simultaneously at 47°C for 2 hr.

Unbound detection probe was washed away using an automated plate washer 1296-024 (Wallac Oy, Turku, Finland) and DELFIA washing solution (Wallac Oy). For detection of the bound hybrids, 200 µl of DELFIA Enhancement Solution (Wallac Oy) was added into the microtitration wells and shaken for 30 min at room temperature. During the incubation with enhancement solution, bound Eu is released from the detection probe into the enhancement solution and a highly fluorescent complex is formed. The fluorescence was then measured using a time-resolved fluorometer (ARCUS 1230 or a DELFIA Fluorometer 1232, Wallac Oy).

Gel Retardation Test

DNA was liberated from the Guthrie disks by incubating the disks in 300 µl of reagent buffer (100 mM NaCl, 10 mM Tris, pH 7.6, 25 mM EDTA, 0.5% SDS, and 10 mg/ml proteinase K) overnight at 50°C on a shaker. After phenol/chloroform (1:1) extraction, the DNA was precipitated by addition of 150 µl of 7.5 M ammonium acetate (pH 7.5) and 750 µl of 96% ethanol. DNA was collected by centrifugation and resuspended in 10 µl of water. A 5-µl amount was used for the PCR amplification. After initial incubation at 94°C for 4 min, 35 PCR cycles (94°C, 1 min; 50°C, 1 min; 72°C, 2 min)

were run. The PCR amplifications were performed in a Hybaid Thermocycler.

In the gel retardation test, 10 µl of PCR product was mixed with the PCR product of known homozygous wild-type DNA, denatured (94°C for 5 min), and rehybridized (65°C, 5 min). The hybridization mixture was analyzed on a 12% polyacrylamide gel in parallel with the original PCR product.

Patient Samples

Known CF samples were from stored phenylketonuria cards from patients with verified CF. The other samples were collected randomly from daily routine phenylketonuria cards. Dried blood spot samples (Statens Seruminstitut, Copenhagen, Denmark) were first analyzed using a gel retardation test,^(29,30) as described above. The same blood spots were analyzed using allele-specific PCR and TRF for the detection of the PCR product. Samples giving discrepant re-

sults in the two assays were retested in both of the assays and the PCR products were sequenced directly and using PCR-derived cloned fragments. Dried blood spot samples (Oliver Latham Laboratory, N.S.W. Health Dept., Australia) from known CF patients were kindly provided by Dr. Bridget Wilcken.

RESULTS

Sample Preparation

Boiling has been described as a possible means of liberating the DNA from dried blood specimens for PCR amplification.⁽³¹⁾ We wanted to test if boiling in alkaline conditions could be used as the sample preparation process with different sample materials.

To test this, a blood disk (3 mm in diameter), 3 µl of EDTA blood, and 3 µl of saliva were prepared and used for amplification (Table 2). A clear positive signal in the normal specific amplification with all tested sample materials was achieved. The lowest ratio between positive and negative signals was achieved if blood was used as the sample material. Even with blood, over 30 times higher signal was achieved from the specific amplification than from the unspecific amplification reaction.

Optimization of the PCR Reaction

In the assay format, the concentration of the biotinylated PCR primer affects not only the amplification efficiency but also the collection of the formed hybrids during the detection of the PCR product. Primer concentrations (0.05, 0.1, 0.2, 0.4

TABLE 2 Different Sample Materials from a Homozygous Normal Person Were Tested in the TRF Assay

Sample	Normal specific PCR			Deletion-specific PCR		
	n ^a	mean ^b	CV% ^c	n	mean	CV%
Saliva	8	223,574	19	3	1,230	11
Blood	8	32,782	34	3	1,060	9
Blood spot	8	264,152	13	3	1,034	13

The 3-mm blood disk, 3 µl of saliva, or 3 µl of whole blood were prepared according to protocol described in the Methods.

^a n, number of replicate amplification reactions.

^b Mean signal obtained in the TRF assay as counts per second (cps).

^c CV, coefficient of variation.

μM) were studied and optimal results were achieved when 0.1 μM primers together with 10- μl samples in the hybridization reaction were used.

Annealing temperature was varied to get the optimal amplification efficiency and to see if the discrimination efficiency is affected by changes in the annealing temperature. (Temperature profiles between different PCR machines can vary considerably, and this might cause a problem when allele-specific primers are used.) Maximal hybridization signal was obtained using 56°C annealing temperature. Maximal discrimination between the normal and deleted sample DNA was also achieved at 56°C annealing temperature. The ratio between the specific and the unspecific reaction was 209 for the normal specific amplification and 321 for the deletion-specific amplification. There was very little indication of unspecific hybridization signal, even with the lowest annealing temperature tested (47°C) (Table 3).

The Hybridization Reaction

Kinetics of the hybridization reaction were studied by measuring separately the rate of hybrid collection and the rate of the hybridization reaction. Hybridization reaction reached maximal signal level after 2 hr of incubation. The collection of hybrids was complete after 1 hr. The combined hybridization/collection followed the kinetics of the hybridization, reaching maximal signal level after 2 hr (data not shown).

Probe concentration and temperature in the hybridization reaction were optimized using PCR product pools as target

DNA. Twenty-seven percent of the maximal signal level (obtained at 45–50°C) was reached if the hybridization was performed at 22°C and 46% of the maximal signal was obtained at 65°C. Concentration of the Eu probe was optimized following the signal-to-background ratio. The optimal ratio was achieved using 2.5–10 ng/ml Eu probe.

Clinical Samples

Dried blood spot samples were first analyzed using PCR amplification of the deletion region combined with a gel retardation test. Among 20 selected samples, 10 normal homozygotes and 10 mutant homozygotes were found. Samples were coded and analyzed using allele-specific PCR combined with the TRF detection of the PCR product. Ten homozygous normal, 2 carriers, and 8 homozygous mutant samples were found (Fig. 2A). The two samples giving a different result in the two assays were retested in both systems and were found to be carriers according to both gel retardation and TRF assays. In addition, the deletion regions were sequenced and sequencing patterns indicating carrier samples were found.

To analyze function of the test further, 17 samples from known CF patients together with 6 control samples from normal patients were analyzed. A total of 10/17 patients were found to be homozygotes for mutation ΔF508 ; 6/17 were compound heterozygotes having mutation ΔF508 only in one of the chromosomes. One of the samples did not contain mutation ΔF508 (Fig. 2B).

The average ratio between the specific and nonspecific signal was 76 (from 32 to 181, 11 samples) for the normal sam-

ples and 105 (from 85 to 187, 8 samples) for the samples homozygous for mutation ΔF508 (Fig. 2A). When another set of samples was analyzed (Fig. 2B), average ratios of 198 (from 147 to 292, 5 samples) and 222 (from 180 to 284, 10 samples) were obtained, respectively.

DISCUSSION

To make DNA hybridization assay practical enough for routine clinical use, simple methods should be developed. Boiling in alkaline solution is an efficient way to prepare samples for PCR. The method is relatively easy to use and the amplification can be done from different sample materials.

Differences in the annealing temperature did not have a strong effect on the discrimination between the different alleles. On the other hand a 3-bp deletion makes it possible to have three mismatches at the 3' ends of the specific primers when compared to the cross-reacting allele. This makes the deletion ΔF508 an easy mutation to detect using allele-specific primers. Other mutations causing cystic fibrosis can be either deletions or point mutations.³² Even if the allele-specific amplification might not be the best method for simultaneous screening of multiple mutations, it could be used in combination with the IRT measurement in the neonatal screening of CF.⁽⁷⁾

In the initial testing of the clinical samples, two dried blood spots gave different results for the PCR amplification combined with the gel retardation test and for the allele-specific PCR combined with the TRF detection of the PCR product. When retested, these samples gave different results in the gel retardation assay and full agreement between the two assays was established. These results could be caused by the fact that occasionally the heteroduplex band found in heterozygotes is weak and not easily detected. By using hybridization as the detection method, it is possible to avoid misinterpretation of the result caused by artefactual PCR bands. A sensitive detection system^(33,34) also makes it possible to use a low number of PCR cycles, thus reducing the risk of PCR product contamination. Even if there are differences in the signals between different patient samples, high ratios between the specific and nonspecific signals make the interpretation of the results easy.

TABLE 3 The Effect of Annealing Temperature to the Specificity of the Method

Annealing Temperature (°C)	Normal specific PCR			Deletion-specific PCR		
	normal sample	mutant sample	ratio ^b	normal sample	mutant sample	ratio
47	158,962 ^a	885	180	1,618	205,678	127
50	169,400	1,181	143	1,400	254,283	182
53	198,725	975	204	1,207	257,313	213
56	225,979	1,081	209	1,069	343,306	321
59	8,856	1,002	9	796	116,562	146
62	902	1,013	1	1,685	2,284	1

Homozygous normal and mutant sample pools were analyzed.

^a Results are mean of three parallel amplification reactions analyzed using four parallel samples in the hybridization. Results are expressed in counts per second (cps).

^b Specific hybridization signal divided by the nonspecific hybridization signal.

FIGURE 2 Dried blood spot samples tested. The black bars indicate hybridization signal from the normal specific PCR reaction and open bars indicate hybridization signal from the deletion-specific PCRs. Herring sperm DNA instead of sample was used to control the PCR background (= Bg). (A) Twenty coded samples, analyzed using PCR amplification and a gel retardation test, were tested together with three control samples (normal = N, carrier = C, and mutant = M) in the TRF assay. Twenty-four cycles were used in the PCR amplification. (B) Seventeen samples from known CF patients were tested together with six samples from normal control patients. Twenty-six cycles were used in the PCR amplification.

Hybridization performed in the microtitration wells is a practical method, making it possible to analyze large numbers of samples. The procedure does not require preparation of gels or enzymatic manipulations of DNA, thus reducing the hands-on time considerably. In ELISA-based detection systems, an additional incubation with secondary labeling reagent is often needed. This can be avoided with label, which can be used directly in the hybridization reaction. In

addition Eu chelates are stable during storage, which is advantageous when economical and quality matters are important.

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