Population screening for the common G985 mutation causing medium-chain acyl-CoA dehydrogenase deficiency with Eu-labeled oligonucleotides and the DELFIA system

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We have screened 10 171 neonatal blood spots from the Trent and West Midlands regions of the UK for the common G985 mutation to more accurately establish the incidence of medium-chain acyl coenzyme (Co)A dehydrogenase (MCAD) deficiency. We have used a technique involving PCR and Eu-labeled allele-specific oligonucleotides detected by using time-resolved fluorometry on the dissociation-enhanced fluorescence immunoassay (DELFIA) system for the detection of the G985 mutation. We have also evaluated the feasibility of neonatal screening with this technique. We identified 158 G985 heterozygotes and no G985 homozygotes. The calculated incidence of MCAD deficiency in the population studied (all mutations, assuming 90% of MCAD mutations are G985) is 1 in 13 426 (95% confidence limits 1 in 10 070–1 in 18 791). At the optimum cutoff criteria, the technique has a sensitivity of 97.5%, specificity of 99.6%, and positive predictive value of 80.2%. We conclude that this study confirms that MCAD deficiency is a common inherited metabolic disease and is a candidate for neonatal screening. The methodology used is robust and suitable for large-scale population studies such as this. The technique is also potentially suitable for screening.

INDEXING TERMS: time-resolved fluorescence • disease frequency • neonatal screening

Medium chain acyl coenzyme (Co)A dehydrogenase (MCAD) deficiency is a potentially fatal inherited disorder of mitochondrial fatty acid oxidation.4 Presentation is often in early infancy with a sudden life-threatening episode of hypoglycemia and encephalopathy following a period of fasting and stress [1]. Symptoms may occur in the immediate postnatal period [2], and MCAD deficiency may go undiagnosed. In one large study the first acute episode was fatal for 29% of subjects [3]. Survivors may go on to have further severe hypoglycemic episodes with consequent morbidity before the diagnosis is made [4]. Long-term prophylactic measures to prevent the onset of symptoms in known individuals are simple and inexpensive [5].

One point mutation (G985) accounts for ~90% of all disease alleles causing MCAD deficiency [6]. Data from many countries have shown that MCAD deficiency due to the G985 mutation is most common in western Europe and that there is a founder effect [7]. Two studies from the West Midlands and Trent regions of the UK have estimated the frequency of G985 homozygote individuals to be 1 in 6400 (from 410 specimens) and 1 in 13 400 (from 479 specimens) respectively [8, 9]. However, the small numbers of specimens used in these surveys means that these estimates are very imprecise.

Neonatal screening for MCAD deficiency has been suggested in view of the possible high incidence, potentially fatal outcome, and simple treatment of the disorder [10]. Potential methods include the detection of octanoyl carnitine in blood spots by tandem mass spectrometry [11], cis-4-decenolate in blood spots by gas chromatography–mass spectrometry (GC-MS) [12], and the detection of urine hexanoylglucose by GC-MS and selective ion monitoring [13].

Metabolite screening by GC-MS and tandem mass

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4 Nonstandard abbreviations: CoA, coenzyme A; MCAD, medium-chain acyl-CoA dehydrogenase; GC-MS, gas chromatography–mass spectrometry; and DELFIA, dissociation-enhanced fluorescence immunoassay.
spectrum would be specific for MCAD deficiency but manpower requirements and the initial cost of equipment would be high. One advantage of tandem mass spectrometry is that a broad spectrum of diseases may be detected from one specimen with minimal cost for additional diseases. The technique may also replace current methods of screening for phenylketonuria. The sensitivity of these methods has not been proven and it is possible that metabolites for some disorders may not be detected in the immediate neonatal period. An alternative may be a DNA-based screening program, which would be specific and sensitive for a particular disease but would be limited to the detection of only those individuals with the specific mutation(s) being used. The setup costs would be expected to be cheaper than GC-MS or mass spectrometry, although reagent costs may be higher.

There is no established technology for the routine screening of large numbers of specimens for specific DNA mutations. However, the previously developed time-resolved fluorescence assays for the detection of PCR products are highly sensitive, rapid, and allow automation. Pilot screening studies for the Z mutation in alpha1-antitrypsin deficiency [14] and the AF508 mutation in cystic fibrosis [15] with time-resolved fluorescence and the dissociation-enhanced fluorescence immunoassay (DELFIA) system (Wallac, Turku, Finland) have been reported. A method for the detection of the G985 mutation in MCAD deficiency with Eu-labeled allele-specific oligonucleotides has also recently been described [16].

We have screened 10 171 neonatal blood spots from the West Midlands and Trent regions of the UK for the G985 mutation by using the PCR and time-resolved fluorescence assay [16]. We report here the sensitivity and specificity of this method for population surveys of the G985 mutation frequency and the feasibility of the method for population screening.

**Materials and Methods**

**SPECIMENS**

Dried blood-spot specimens left over after completion of neonatal screening tests from the Herefordshire, Shropshire, and North and South Worcestershire districts of the West Midlands Health Region and 11 of the 13 districts of the Trent Health Region were used. All procedures were accepted by the local ethical committees of these districts. Data from the 1991 national census shows that the districts included within the Trent Health Region had an estimated white population of 88.9–97.1% [17]. The estimated white population was 98.4% in Shropshire and 98.7% in Herefordshire and Worcestershire [18].

In all cases the routine screening tests were completed before any spare sample was used for DNA analysis. The spare specimens were subsequently collected anonymously and assigned a code number. The code was to be broken only in the event of a G985 homozygote being detected. Blood spots from the West Midlands (5014 specimens) had been fixed by steaming for 12 min, whereas the Trent blood spots (5157 specimens) had not been fixed. Blood spots from both regions were subsequently treated identically.

**SAMPLE PREPARATION**

One 3-mm blood-spot disc from each card was punched into a clean microtiter well and treated with 100 µL of methanol. The spots were allowed to dry thoroughly before liberating DNA by boiling in 60 µL of sterile water (Parkfields Sterile Supply Unit, Wolverhampton, UK) [19]. The spot and supernatant were stored at 4 °C for up to 14 days until analysis was complete.

**DNA AMPLIFICATION**

PCR was carried out in singlicate in Microamp thin-walled tubes on a Geneamp 9600 thermal cycler (Perkin-Elmer, Norwalk, CT). Specimens (96) were amplified in each run, including known G985 homozygote, G985 heterozygote, and A985 homozygote (i.e., the normal gene) specimens as controls, and water blanks at the beginning and end of the plate to check for contamination. The primer sequences were 5'-Bio GAG CAC CAA GCA ATA TCA TT-3' and 5'-Bio GCA ATA GAA GCA TAA TAG GTA-3' as described by Iitiä et al. [16]. Each reaction mixture contained 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl2, 50 mmol/L KCl, 0.1 g/L gelatin, 1 µmol/L each primer, 0.02 µmol/L dNTPs, 0.75 U of Taq DNA polymerase, and 20 µL of blood-spot supernatant in a final volume of 50 µL. Thirty cycles of the following program were carried out: 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C. Finished PCR products were stored at 4 °C for up to 14 days before detection of the genotype with allele-specific hybridization.

**ALLELE-SPECIFIC HYBRIDIZATION**

The presence or absence of the G985 or A985 alleles was detected in separate wells in singlicate with Eu-labeled allele-specific oligonucleotides as described by Iitiä et al. [16]. All reagents and volumes were as previously described. The DELFIA system used comprised the 1296–024 platewash, plateshaker, and 1234 DELFIA research fluorometer. Briefly, the method involved addition of 20 µL of each PCR product to two consecutive wells on streptavidin-coated microtiter plates. Bound DNA was rendered single stranded after incubation with 50 mmol/L sodium hydroxide. The sequences of the allele-specific probes used were 3'-CCG TTA CCT TCA AC-Eu-5' (mutant allele) and 5'-Eu-GGC AAT GA-5' (normal allele) [16]. Hybridization buffer contained 100 µg of either normal or mutant probe in 10 mL of hybridization solution (DELFIA Assay Buffer with 0.8 mol/L sodium chloride). Hybridization buffer (100 µL) was added to each well and the plates were incubated at 37 °C. Unbound probe was removed by using a stringent wash temperature of 43 °C. DELFIA enhancement solution (200 µL) was added to each well and time-resolved fluorescence was measured in the DELFIA 1234 research flu-
orometer by using an excitation light band between 290 and 360 nm. Light emission was detected at 615 nm, and results of the normal and mutant counts (counts per second) and a ratio of mutant-to-normal counts were recorded.

ASSAY VALIDATION
The precision of the DELFIA system was investigated by pooling the PCR products of several reactions for each genotype to provide sufficient specimen for repeat analyses in the same and different runs (i.e., intra- and interrun precision for the DELFIA). The precision of the PCR stage was investigated by amplification of 20 different spots from the same specimen for each genotype in the same DELFIA assay (i.e., between-PCR precision). Overall method precision was investigated by amplification of 10 different spots from the same specimen for each genotype on separate occasions, followed by DELFIA analysis of the products in separate runs. Results are summarized in Table 1.

Validation of the method with eight authentic specimens for each genotype showed that G985 homozygotes and G985 heterozygotes had mutant counts >10 000 and a ratio of mutant-to-normal counts of >0.1. A985 homozygotes had mutant counts <10 000 and a ratio of <0.1 (Fig. 1).

STUDY DESIGN
To minimize the possibility of wrongly classifying any G985 heterozygotes or G985 homozygotes in the screening study, no absolute cutoff levels were set.

On the basis of the results with known genotypes, specimens giving mutant counts >10 000 and also a ratio of mutant-to-normal counts >0.1 were highlighted as probably from G985 heterozygotes or G985 homozygotes and referred to as group 1 (Fig. 2). These results were verified by a method based on that described by Greger et al. [20] by using the remaining supernatant of the original blood spot. The presence of the A to G mutation at position 985 together with a deliberate mismatch in the sense primer sequence creates an extra Styl restriction enzyme site. In the normal (A985), no restriction site is formed. For PCR the final primer concentration was 1 μmol/L in a 50-μL final volume. Digested products were separated on 12% polyacrylamide gels.

Approximately 5% of the remaining results from each run were chosen for verification (referred to as group 2, Fig. 2). These included those most likely to be false negatives, i.e., those with the highest mutant counts or ratios, and those with unsatisfactory PCR. A proportion of results with low mutant counts and ratios were also chosen to verify the A985 homozygote genotype. Samples in this group were further investigated before assignment of genotype, either by repeat DELFIA analysis alone or by verification with PCR amplification and Styl restriction enzyme digestion. All assignments of G985 heterozygosity were confirmed with the Styl method.

Specimens with the lowest mutant counts and ratios were assigned the A985 genotype without further investigation (referred to as group 3, Fig. 2).

Results
A total of 10 171 bloodspots were screened, 5157 from the Trent region and 5014 from the West Midlands region.

GROUP 1
Results (172) showed mutant counts >10 000 and a ratio of mutant-to-normal counts of >0.1 and were considered probable G985 heterozygotes or G985 homozygotes on the basis of results from known genotypes. After Styl digestion, 143 were proven G985 heterozygotes and 29 (17%) were proven A985 homozygotes (i.e., false positives). All 29 A985 homozygotes were reanalyzed by DELFIA. Their initial and repeat counts and ratios are shown in Fig. 3.

GROUP 2
A total of 595 specimens were in this group, and a further 15 G985 heterozygotes (2.5%) were identified. The results are discussed below according to the criteria for selection.

| Table 1. Assay validation: DELFIA, PCR, and overall method precision. |
|---------------|----------|----------|--------|----------|----------|----------|
|               |          |          |        |          |          |          |
|               | G985 Hom | G985 Het | A985 Hom |
|               | Mut       | Nor      | Mut      | Nor      | Mut       | Nor      |
| DELFIA        |           |          |          |          |           |          |
| Intrarun (n = 20) | 50 276 ± 3348 | 40 46 ± 2422 | 35 290 ± 7143 | 11 725 ± 12 566 | 4701 ± 1081 | 13 418 ± 12 332 |
| CV, %         | 7         | 60       | 20       | 11       | 23        | 9        |
| Interrun (n = 15) | 45 885 ± 9097 | 34 48 ± 1521 | 37 673 ± 8575 | 12 363 ± 16 560 | 34 70 ± 12 23 | 15 107 ± 20 403 |
| CV, %         | 20        | 44       | 23       | 14       | 35        | 14       |
| PCR (n = 20)  | 58 563 ± 8318 | 31 36 ± 1150 | 40 627 ± 5964 | 13 886 ± 16 121 | 6907 ± 2266 | 13 414 ± 28 186 |
| CV, %         | 14        | 37       | 15       | 12       | 33        | 21       |
| Overall (n = 10) | 40 880 ± 18 231 | 70 93 ± 3051 | 27 700 ± 11 672 | 11 359 ± 46 091 | 27 88 ± 1075 | 18 925 ± 84 104 |
| CV, %         | 45        | 43       | 42       | 39       | 39        | 44       |

Hom, homozygote; Het, heterozygote; Mut, mutant; Nor, normal.
Results with high mutant counts or ratios. A further 13 G985 heterozygotes were confirmed in this group of 329 samples (4%). Of these specimens, seven initially showed mutant counts >10,000 but a ratio of <0.1, and so were not included in group 1. A further four of these results had mutant counts >9000. The initial counts and ratios of all 13 G985 heterozygote specimens are shown in Table 2.

Results typical of homozygous a985 status. Results (235) with mutant counts <5000 and normal counts >60,000 were chosen for reanalysis to verify a proportion of the A985 homozygote specimens. No further G985 heterozygotes were identified from these specimens.

Results with low normal counts. A total of 31 results had <10,000 counts in both wells on the first DELFIA analysis, consistent with background amounts. These results were considered to represent inefficient PCR, and DELFIA analysis was repeated.

One of these specimens again gave <10,000 counts in both wells and the A985 homozygote genotype was confirmed by the StyI method. Two samples showed mutant counts >10,000 and a ratio >0.1 (i.e., fulfilled group 1 criteria), and were confirmed as G985 heterozygotes with the StyI method.

The remaining 28 specimens were assigned the A985 homozygote genotype on the basis of the second DELFIA result.

GROUP 3
In this group, 9404 samples were assigned the A985 homozygote genotype without further investigation.

The numbers of specimens assigned each genotype from the different groups are summarized in Fig. 2.

Although no G985 homozygotes were detected during the course of the study, one known G985 homozygote specimen was introduced blindly. This specimen was correctly identified with mutant counts of 27,039 and a ratio of mutant-to-normal counts of 5.81.

Discussion
After the discovery of a single prevalent mutation in MCAD deficiency (G985) [6], estimates of the incidence of MCAD deficiency in the Australian, North American, Japanese [8], northern European, Italian [9], Scottish [21], and French [22] populations have been based on analysis of the frequency of this mutation in small numbers of specimens (i.e., <1000). In contrast, this is a large screening study involving a method potentially suitable for large-scale population screening. This has enabled us to give a more accurate estimate of the incidence of MCAD deficiency in the Trent and West Midlands regions of the UK [23]. Since not all specimens had their genotype
confirmed by the StyI method, any bias in the data will be to underestimate the heterozygote frequency. However, only those specimens with the lowest mutant counts and ratios (mutant-to-normal counts) were assigned the A985 genotype without further investigation. Of these results, 235 (2.3% of the total) were verified with the StyI method and no G985 heterozygotes were detected, suggesting that misclassification was unlikely.

ASSAY PERFORMANCE

The data were analyzed to obtain the cutoff criteria that would maximize the sensitivity and specificity of the assay. For this analysis the 31 initial results in the portion of group 2 with low normal counts were replaced with their repeat values. Fig. 4 compares the constructed ROC plots for different cutoff amounts of mutant counts and ratio (mutant-to-normal counts). At a ratio cutoff of 0.09 the sensitivity was 97.5% and the specificity was 99.4%. At a mutant counts cutoff of 8000 the sensitivity was 100% and the specificity was 97.5%. However, the positive predictive values for these cutoffs are 73.3% and 38.3%, respectively. A better decision criterion would be a combination of a ratio $>0.09$ and mutant counts $>8000$. By using this criterion, the sensitivity was 97.5%, the specificity was 99.6%, and the positive predictive value was 80.2%. Only 38 false-positive and four false-negative results would have been generated with this criterion.

These four false-negative results were on specimens from the West Midlands: Three of them had mutant counts $>8000$ but a ratio of $<0.09$ due to high normal counts, possibly due to contamination of the PCR with normal DNA. Contamination control is a very important issue for PCR on this scale. We experienced a few problems with some contaminated runs showing as increased counts in the normal well of the water blanks.

Several of the false-positive results had mutant counts very much greater than the mutant counts for the true-positive G985 heterozygotes (Fig. 3). This was thought to have occurred because of splashing of normal probe into the mutant well during analysis, as probes were added to adjacent wells with a repeater pipette. Counts actually due to the presence of A985 DNA would then be detected in the mutant well. As the counts obtained from the normal probe were $\sim$10 times higher than those from the mutant probe, very high counts would therefore be observed in the mutant well. Addition of reagents with a fully automated system would reduce this possibility, as would the addition of mutant and normal probes to separate plates. In addition, the use of different labels for each mutation (e.g., Eu and Sm) would enable both mutations to be detected in one well [16].

Table 2. Counts and ratios (mutant-to-normal counts) for the 13 proven G985 heterozygotes that did not initially fulfill the criteria observed in the preparatory work.

<table>
<thead>
<tr>
<th>Region</th>
<th>Mutant counts (cps)</th>
<th>Normal counts (cps)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>West Midlands</td>
<td>19,007</td>
<td>229,685</td>
<td>0.083</td>
</tr>
<tr>
<td></td>
<td>11,649</td>
<td>130,810</td>
<td>0.089</td>
</tr>
<tr>
<td></td>
<td>29,873</td>
<td>467,107</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>19,228</td>
<td>202,752</td>
<td>0.095</td>
</tr>
<tr>
<td></td>
<td>21,328</td>
<td>228,508</td>
<td>0.093</td>
</tr>
<tr>
<td></td>
<td>8593</td>
<td>42,223</td>
<td>0.204</td>
</tr>
<tr>
<td></td>
<td>9472</td>
<td>51,387</td>
<td>0.184</td>
</tr>
<tr>
<td></td>
<td>9098</td>
<td>61,736</td>
<td>0.147</td>
</tr>
<tr>
<td></td>
<td>9816</td>
<td>57,209</td>
<td>0.172</td>
</tr>
<tr>
<td></td>
<td>1746$^a$</td>
<td>41,667$^a$</td>
<td>0.042$^a$</td>
</tr>
<tr>
<td></td>
<td>8066$^b$</td>
<td>89,489$^b$</td>
<td>0.090$^b$</td>
</tr>
<tr>
<td>Trent</td>
<td>12,726</td>
<td>130,276</td>
<td>0.098</td>
</tr>
<tr>
<td></td>
<td>12,565</td>
<td>352,115</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>9866</td>
<td>95,407</td>
<td>0.103</td>
</tr>
</tbody>
</table>

$^a$ Initial and $^b$ repeat values for one specimen.

The sensitivity was $97.5\%$ and the specificity was $99.4\%$. At a mutant counts cutoff of 8000 the sensitivity was 100% and the specificity was 97.5%. However, the positive predictive values for these cutoffs are 73.3% and 38.3%, respectively. A better decision criterion would be a combination of a ratio $>0.09$ and mutant counts $>8000$. By using this criterion, the sensitivity was 97.5%, the specificity was 99.6%, and the positive predictive value was 80.2%. Only 38 false-positive and four false-negative results would have been generated with this criterion.

Of the 173 specimens with normal counts $<60,000$ ($P <0.01$), 64% were from the West Midlands. The mean counts ($\pm$ SD) in the normal well for West Midlands and Trent specimens were 268,058 ($\pm$ 133,580) and 369,388 ($\pm$
The lower counts may therefore be explained by a lower yield of DNA from these spots after boiling, in comparison with the Trent specimens, which were not fixed. The use of unfixed specimens for screening would reduce the number of specimens to be repeated because of inefficient PCR.

Suitability of Assay for Population Screening

The availability of a suitable screening test is an important criterion for the establishment of a screening program [24]. Some desirable qualities of a screening method include acceptable sensitivity, specificity, and repeat rate. The method should also be easily automatable with adequate sample throughput and turnaround time and be cost beneficial.

The method used in this study demonstrated a sensitivity and specificity of 97.5% and 99.6%, respectively, at the optimum cutoff counts. However, G985 homozygotes have higher mutant counts and ratios than G985 heterozygotes and the assay would therefore be better at discriminating between G985 and A985 homozygotes than between G985 heterozygotes and A985 homozygotes. The method would therefore be expected to be more robust and yield even better sensitivity and specificity for the detection of G985 homozygotes in a true screening program rather than the detection of G985 heterozygotes as described here.

During the study period only one batch of Eu-labeled probes and two batches of streptavidin-coated plates and DELFIA reagents were used. There was no suggestion that there was a variation in absolute counts due to changes in reagent lots. However, the method may be susceptible to variation if used in a true screening program over a long period of time. It would therefore be important to validate the absolute counts with each new batch. A decision criterion based on ratios might therefore be more reliable. In this study, the sensitivity was 97.5%, the specificity was 99.4%, and the positive predictive value was 73.3% with a ratio cutoff of 0.09.

In our study only 31 specimens (0.3% of the total) were repeated because of inefficient PCR. The overall repeat rate will depend on the cutoff levels chosen. The reduction of the repeat rate for specimens with low counts has been mentioned above. The robustness of the assay for screening may also be improved by automation and more stringent contamination controls.

The suitability of screening for MCAD deficiency by using the G985 mutation will depend upon the prevalence of this mutation in the population being screened [25]. In the West Midlands and Trent regions 94% of mutations in 24 families diagnosed with MCAD deficiency over an 8-year period were G985 [23]. Some other rare mutations may be expected to interfere with the assay; for example a mutation occurring in the primer binding region would be expected to yield no PCR and hence background counts only. Combining a second-line test such as octanoyl carnitine or cis-4-decenoyl in blood spots [11, 12] on all G985 heterozygotes picked up would increase the number of MCAD-deficient individuals detected.

A nonradioactive method involving single-strand polymorphism minigels to detect the G985 mutation has also been described [26]. However, the technique was applied to only 48 people in the published report, and its potential for large-scale screening has not been proven.

MCAD deficiency appears to be a common inherited metabolic disorder in our population screened. Detection of the G985 mutation with PCR and Eu-labeled allele-specific oligonucleotides is suitable for large-scale studies of this kind. Neonatal screening could be undertaken by detecting the common mutation (G985) in blood spots by this method.

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References


