DNA-based carrier screening in primary healthcare: screening for aspartylglucosaminuria 
mutations in maternity health offices

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Large-scale genetic screening programs are complex enterprises in which ethical, technical, medical, and socioeconomical aspects have to be handled with professional expertise. Establishment of automated, relatively robust, and inexpensive laboratory techniques is one step of this path. Here a pilot carrier-screening program for the mutations causing aspartylglucosaminuria was carried out for pregnant women in primary care maternity health offices. Women (1975) were tested before their 12th week of pregnancy, and 31 heterozygotes were detected. The sampling was based on dried blood strips, facilitating convenient handling and inexpensive mailing to the laboratory. The mutation detection technique, solid-phase minisequencing simplified by the use of scintillation microplates and automated equipment, proved to be rapid, simple, inexpensive, and reliable, with a low repeat rate (2.5%). In conclusion, we found that good collaboration between the primary healthcare unit, the laboratory, and counseling experts, combined with modern laboratory technology, facilitate reliable low-cost genetic testing.

INDEXING TERMS: hereditary disorders • polymerase chain reaction • mutation detection • genetic testing

The recent technical development in molecular genetics has facilitated large-scale detection of inherited gene defects. Accurate diagnosis of affected individuals, both post- and prenatally, as well as detection of healthy heterozygous carriers of recessively inherited diseases is possible by means of DNA tests. For common genetic diseases such as cystic fibrosis (CF), prevention programs based on community-wide carrier screening have become feasible. However, genetic screening presents many problematic issues: technical, educational, financial, and ethical. Recommendations by the World Health Organization on community carrier screening have defined qualifications for the testing method: The gene test must be simple, reliable, and inexpensive, providing clear and indisputable information on the presence or absence of the gene defect in question (see 1).

We have developed a mutation detection methodology, the solid-phase minisequencing technique, which meets the criteria of a large-scale testing system. The method is based on amplification of the DNA fragment spanning the mutation under study by using a 5' -biotinylated PCR primer, binding of the amplification product on a streptavidin-coated matrix, and primer-guided single-nucleotide incorporation at the site of the mutation [2]. The result of the test is obtained as a clear-cut numeric value unequivocally defining the genotype. Known point mutations as well as deletions or insertions in any gene can be identified by this technique. The method is robust, quantitative, reliable, and easily automated, and can be adapted for use with nonradioactive labels [3]. A double-labeling system for the simultaneous detection of two different mutations has also been developed [2]. The minisequencing method is at present routinely applied for detection of >15 inherited or somatic mutations [4–9]. It has also been utilized in the detection of mutations causing CF [10], in detection of minor malignant cell populations [11], and in forensic identification of individuals [12]. The procedure has recently been further simplified by utilizing an automated microplate washer and a scintillating microplate material in combination with a microplate scintillation counter [13].

The prospects for screening for single gene defects in Finland are characterized by the unique features of the population. Isolation and founder effects have led to gains and losses of some disease-causing genes. Consequently, ~30 mainly autosomal recessive diseases are unusually frequent in the Finnish
population, comprising “the Finnish disease heritage” [14], whereas, for example, CF and phenylketonuria are rare or totally absent. A common origin and subsequent spreading of the mutation have also resulted in the prevalence of one major disease-causing mutation in the population—a very favorable situation in genetic screening [4–6, 15–17].

Aspartylglucosaminuria (AGU), a typical representative of the Finnish disease heritage, is a recessively inherited lysosomal storage disease caused by deficient activity of aspartylglucosaminidase [18]. The disease is clinically characterized by severe mental retardation, susceptibility to infections, and various mild connective tissue manifestations [19]. The carrier frequency of the disease is 1:50–1:70, with regional differences [20]. The predominant mutation (AGU \(_{\text{Fin}}\) major), which is responsible for 98% of the disease alleles, is a point mutation (G\(_{488} \rightarrow C\)) causing a substitution of cysteine to serine (Cys\(_{163} \rightarrow \text{Ser}\)) [4, 21]. In addition, another rare mutation (AGU \(_{\text{Fin}}\) minor) has recently been identified in seven Finnish AGU patients. This 2-bp deletion in the second exon of the AGA gene results in a frameshift and premature termination of translation [22]. All of these patients are compound heterozygotes with the AGU \(_{\text{Fin}}\) major mutation in one allele and the AGU \(_{\text{Fin}}\) minor mutation in the other. Over 99% of the Finnish AGU alleles can be detected with a search for these two mutations.

The combination of the two phenomena, a well-designed mutation detection protocol and the existence of one major disease-causing mutation, made a health center-based carrier screening program tempting. In the present paper we report the applicability and accuracy of the minisequencing method in large-scale carrier screening of AGU in which 2000 pregnant women and the spouses of identified carriers were offered the screening test at maternity healthcare offices. The general outline of the project and the attitudes of the mothers and healthcare personnel will be reported separately.

**Materials and Methods**

**CARRIER SCREENING PROGRAM**

The gene test for AGU was offered to women at early pregnancy (mainly before 12 weeks of pregnancy) in two health centers in Helsinki, with a total of 13 maternity care units. Before the screening, training was organized for the personnel of maternity care units and laboratories of health centers, including basic knowledge in gene testing in general, AGU disease, and the whole screening program. Information on the gene test was mailed to the mothers after they had booked the first appointment at the maternity healthcare unit. At the first visit, the women who had decided to participate gave written consent, and blood samples were taken either during the first visit by nurses or after the visit in laboratories of the health centers. Only partners of the women identified as carriers were tested (stepwise screening). The test was conducted once a week, or when necessary in urgent cases. The test results were mailed to the noncarrier mothers and given personally by telephone by one of the authors (M.H.) to the carriers. The study protocol was approved by the Ethical Committees of the University of Turku, the National Public Health Institute, and the city of Helsinki.

**BLOOD SAMPLES**

Blood spots were collected on a dry blank strip (Merckquant 11860; Merck, Darmstadt, Germany; similar to urine test dipsticks), with a 0.6 × 0.6 cm\(^2\) area of blotting paper (Fig. 1). The samples were taken either by dropping capillary blood directly onto the strip from the fingertip, or by dipping the strip in a tube of EDTA-blood, if a venous blood sample was taken for other purposes. Two blood strips were collected from each participant. The blood spots were dried and mailed to the laboratory in sterile marked plastic bags. The samples were stable for at least 18 months at room temperature.

**DATA STORAGE**

During the course of the study the following data storage protocol was developed. When reaching the Helsinki University Central Hospital Laboratory Department, the samples and test requests were entered into the laboratory computer system (Multilab 1; Mylab, Tampere, Finland), which produced bar codes corresponding to each sample. The bar codes were used to prepare a worklist, which was utilized in all subsequent steps of the analysis. After the completion of the analysis, the Multilab program presented a list of the test results, which was faxed to the clinical geneticist of the screening team.

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**Fig. 1. Flow chart for the mutation detection procedure.**

The DNA of the blood spot is amplified with one biotinylated and one unbiotinylated primer spanning the mutation, and the amplification product is captured into streptavidin-coated scintillation microtiter plate wells and subsequently denatured. The nucleotides at the mutation site are identified by two separate reactions with primer-guided single nucleotide incorporation. The radioactivity of the wells is counted in a scintillation counter, and the result for each sample is obtained by using the ratio of mutation-specific nucleotide to normal nucleotide incorporation (R value).
DNA AMPLIFICATION AND PRIMERS
The oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) 381A DNA synthesizer. The primers for the amplification (PCR1 and PCR2) and detection (D1) of the AGU_Fin major mutation have been described previously [7]. The AGU Fin minor mutation was amplified with the primers PCR3 (5′-GTCAGTCACACTGCTCTCTC, nucleotide (nt) 129–nt 148 of the DNA sequence) and PCR4 (5′-CATCTTCTCATCTGCTTCAG, intron sequence, nt 59–nt 80 downstream from nt 281) [22], and detected by the primer D2 (5′-AGCCGTCACACTGCTCTCTC, complementary to nt 214–nt 195). The primers PCR1 and PCR3 were biotinylated at their 5′ ends.

Before the amplification, the pieces of blotting paper carrying the dried blood stains were incubated in 150 μL of PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.8), 1.5 mmol/L MgCl₂, and 1 mL/L Triton X-100) at room temperature for 1–2 h and boiled for 5 min. Of this sample, 42 μL was transferred (Finnpipette digital; Labsystems, Helsinki, Finland) to a well of a microplate (Falcon assay plates; Finnzymes, Espoo, Finland), and the PCR reagents were added (Biohit Proline electronic 8-channel pipettor; Biohit, Helsinki, Finland) to 0.2 mmol/L each of dATP, dGTP, dCTP, and dTTP; 0.2 μmol/L biotinylated primer; 0.5 μmol/L unbiotinylated primer; and 0.75 U of DyNaZyme DNA polymerase (Finnzymes) per reaction, in PCR buffer to a total volume of 60 μL. Thirty amplification cycles consisting of 92 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min were carried out in a thermal cycler (PTC-100 Programmable Thermal Controller; MJ Research, Watertown, MA).

SOLID-PHASE MINISEQUENCING
Three 10-μL aliquots of the PCR products and 40 μL of buffer [phosphate-buffered saline (137 mmol/L NaCl, 13.4 mmol/L KCl, 7.8 mmol/L Na₂HPO₄ · 2H₂O, 7.4 mmol/L KH₂PO₄), 1 mL/L Tween 20] were transferred (Biohit Proline electronic 8-channel pipettor) to the scintillation streptavidin-coated microplate wells (Streptavidin Covalent Scintistrips®; Wallac, Turku, Finland). For each sample, two wells for detecting the mutant nucleotide and one well for detecting the normal nucleotide were used, the capacity of one plate thus being 29 samples. The reaction for the normal nucleotide served as a verification of the success of the amplification. The microplates were incubated for 1.5 h at 37 °C with gentle shaking. The wells were washed six times with an automated microplate washer (DELFIA® Platewash, Wallac) at 20 °C with 350 μL of a buffer containing 1 mmol/L EDTA, 50 mmol/L NaCl, 1 mL/L Tween 20, and 40 mmol/L Tris-HCl, pH 8.8. Thereafter the wells were treated with 50 μL of 50 mmol/L NaOH (Biohit Proline electronic 8-channel pipettor) for 5 min at 20 °C. The washing steps were repeated. For the detection of the AGU_Fin major mutation, 100 μL (Biohit Proline electronic 1-channel pipettor) of PCR buffer containing 0.2 μmol/L detection primer D1, 0.5 U of DynaZyme DNA polymerase, and 0.02 μmol/L of [3H]dCTP [TRK 625, 2.37 TBq (64 Ci)/mmol; Amersham, Bucks, UK] in the normal-type reactions or [3H]dGTP [Amersham TRK 627; 1.11–1.3 TBq (30–35 Ci)/mmol] in the mutant-type reactions was added. The detection primer for the AGU_Fin minor mutation was D2, and the nucleoside triphosphates used were [3H]dTTP [Amersham TRK 576; 4.35–4.92 TBq (117–133 Ci)/mmol] and [3H]dATP [Amersham TRK 633; 1.59–3.11 TBq (73–84 Ci)/mmol]. The wells were incubated for 15 min at 50 °C and thereafter washed six times to remove the unincorporated label. The plates were air-dried for 5 min at 20 °C, sealed with a sticker, and counted directly without addition of scintillation fluid for 5 min in the microplate scintillation counter (MicroBeta™, Wallac). The results were obtained as counts per minute (cpm), and the R value defining the genotype of the sample was calculated by dividing the cpm value of the incorporated nucleotide corresponding to the mutation by the cpm value obtained with the normal one (R = cpm_mut/cpm_norm).

Our earlier studies have shown a clear distinction between normal homozygotes and carriers of the AGU_Fin major mutation by the R values [4, 13]. The mean R values were 0.01 for normal homozygous and 0.57 for obligate heterozygous samples when scintillation microplates were used.

INTERNAL CONTROLS
A sample from one volunteer heterozygote AGU_Fin major carrier and one normal homozygote were included in each microplate. These two control individuals remained the same throughout the study period. The control samples were treated identically to the patients’ samples. Control samples from one homozygous normal and one heterozygous individual were also used in the assays for the AGU_Fin minor mutation. In addition, negative controls (no sample) for PCR and the minisequencing reaction were included in each plate.

RESULTS
Altogether 1975 women of the 2077 who were offered the gene test (95%) chose to participate. Carriers (31) of the AGU_Fin major mutation were detected (carrier frequency 1:64, 95% confidence interval 1:98 to 1:47). All of the spouses of the carrier women were normal.

About 40% of the samples were collected directly from the fingertip in the maternity offices, and the rest of the samples were obtained by dipping the strip into EDTA-blood in the laboratories. Despite the fact that many blood spots were very small, only one of the fingertip samples was inadequate for PCR, and a new sample had to be requested.

The result of the minisequencing-based DNA test is expressed in R values (cpm_mut/cpm_norm). This facilitates easy interpretation of test results and data storage. The mean R value for the 104 consecutive assays of the normal control was 0.026 (range 0.004–0.066), and for the study samples identified as noncarriers (1944 women, 31 spouses) the mean R value was 0.028 (range 0.001–0.084) (Table 1). For the heterozygote control sample the mean R value (103 assays) was 0.56 (range 0.28–0.99), and 0.53 (range 0.35–0.69) for the subjects identified as carriers (n = 31). Thus the R values fell into two clearly distinct categories with no overlap (Figs. 2 and 3), and the carriers could be distinguished from the normal homozygotes by a 19-fold increase in the average R value.

The reproducibility of the assay during the 1-year period was
Table 1. Variation of 53 assays performed.

<table>
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<tr>
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<th>[3H]dGTP, cpm</th>
<th>[3H]dCTP, cpm</th>
<th>RP/nc</th>
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<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
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<tr>
<td>Inter assay variation of controls</td>
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<tr>
<td>Normal control (n=103)</td>
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<td>30</td>
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<tr>
<td>Carrier control (n=104)</td>
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<td>1195</td>
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<td>Overall variation screened samples</td>
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<td>Homozygous normal (n=1975)</td>
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<td>2363</td>
</tr>
<tr>
<td>Heterozygous carriers (n=31)</td>
<td>685</td>
<td>206</td>
<td>1307</td>
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Calculated from all 53 assays performed and is presented in Table 1 and Fig. 2. The variation between assays was estimated on the basis of both the mean of R values of noncarriers in each run and of the R value of the carrier and normal controls, and the follow-up chart is presented in Fig. 3. The difference of the R value of the carrier and noncarrier controls ranged from 8- to 65-fold.

The spouses of the detected carriers were tested also for the AGUFH minor mutation with the minisequencing assay. None of them carried the mutation. The mean R value of the samples from spouses was 0.020 (range 0.003-0.061); for the assays of the normal control the R value was 0.014 (range 0.004-0.049); and for the assays of the heterozygous control, 0.56 (range 0.29-0.76). The difference in R value between the carriers and normal homozygotes was thus ~30-fold for the detection of the AGUFH minor mutation.

The complete testing procedure took ~9 h per series containing 39 samples on average. Sample coding took 1 h, preparation of the blood spots for PCR 2.5 h, the minisequencing assay 4 h, and the checking and reporting of the results 1.5 h. Thus the assay was in most cases performed during two working days. The first day included preparation, coding, and PCR amplification of the samples; the minisequencing took place during the second day. The cost of the reagents for one sample was ~$5 (US) and the cost of labor $7, thus giving a total cost of $12 per sample for the laboratory part of the screening.

All samples from the identified carriers were retested in the next analysis series to confirm the results. Because of analytical difficulties, the repeat rate for the test for the AGUFH major mutation was 2.5% (n = 44). Of these 44 samples, a failure of amplification was the reason for reanalysis in 24 samples, but reamplification of the same original blood sample from a second strip was successful in all these cases. Disproportion between the two parallel cpm values for the mutation-specific nucleotides required retesting of 20 samples. All of them proved to be normal homozygotes. The precise reason for this discrepancy remains unclear, but contamination of radioactivity from the neighboring well might be one cause. Also, moisture or static electricity on a plastic seal disturb the detection by altering the light path to the detectors of the scintillation counter.

**Discussion**

A laboratory method to be used in population-based screening for disease-causing gene defects has to meet stringent specifications. The DNA test must be reliable, simple, rapid, and inexpensive, and, preferentially, the data should be storable in a numeric format. Here we have demonstrated the application of a simple, reliable, and robust mutation detection system in a pilot population-screening program. The minisequencing procedure, combined with both efficient and easy sampling and efficient sample coding procedures available in large laboratories, proved to facilitate economical and reliable genetic screening.

A large-scale genetic screening program involves personnel from three different disciplines: primary healthcare, the test-performing laboratory, and clinical genetics. A good collaboration between these groups is an essential prerequisite for the success of the program. Here we have evaluated the role of the clinical laboratory and shown that not only the technical facilities and knowledge but also good contacts with healthcare workers and clinical geneticists must exist.

Furthermore, it is essential that the coverage of the test, or multiple tests, is sufficient to yield a high detection rate of the gene defects causing the disease in the population to be screened. In this respect genetic heterogeneity, i.e., several gene defects causing the same disease, is a problem. Isolated populations with a relatively homogeneous gene pool are in a more favorable position in this respect than mixed populations showing a higher degree of genetic heterogeneity. The largest
experience on carrier screening has been obtained from Tay-Sachs disease in Jewish populations [23] and from beta-thalassemias in Mediterranean populations [24]. In both cases, however, the testing was based mainly on protein assays, namely hexosaminidase A activity and hemoglobin, respectively, thus alleviating the problems due to genetic heterogeneity. DNA tests detecting several mutations have recently been developed for these diseases as well, and are utilized in carrier screening with detection rates and costs comparable with the conventional tests [24, 25]. The interest towards large-scale carrier screening has recently been focused especially on CF, the most common single-gene disease among Caucasian populations. CF carrier screening in most populations is complicated by the diversity of the mutations, but kits capable of detecting several mutations have recently been developed [26–28], and are being utilized in pilot screening programs with detection frequencies ~90% [29–32].

There is a variety of PCR-based methods for detection of known mutations. These include hybridization with allele-specific oligonucleotide probes (ASO) [33]; restriction site variation analysis (RFLP), either based on mutation-specific RFLPs [34] or by creating restriction sites with allele-specific primers [35]; and the amplification refractory mutation system (ARMS) [36]. We have developed a solid-phase minisequencing method that offers several advantages as a screening test as exemplified in the present study. All reagents and equipment required are generally available. The system is easily automated with the help of basic laboratory equipment (pipettors, washer). The use of scintillation microplates minimizes the amount of manual labor. The minisequencing method is also applicable to nonradioactive detection [2, 3], but the use of tritium is cheaper, requires fewer steps, and handling of this weak beta emitter causes few safety problems. The minisequencing test can be applied to any known point mutation, deletions, and insertions. The same reaction conditions can be used for detection of several mutations, as exemplified by the detection of both the AGUFin major and minor mutations in the present study. The main drawback of the procedure is the inability to perform multiple mutation analyses in a single reaction. The development of the "DNA chip" technology [37] is a promising approach towards multimutation detection.

In the present study, the difference in R values between normal homozygotes and heterozygotes was on average 19-fold and the distinction between different genotypes, i.e., carriers and noncarriers, was unequivocal. The required repeat rate was low, being one sample per batch on average. For the first time the results of a DNA test can be treated statistically as is normally done in clinical chemistry.

The filter paper strips used in our study for sample collection enables convenient and inexpensive mailing of the samples and

Fig. 3. Week-to-week variation in R values. Mean R values of each series of samples calculated (A) from the normal and heterozygous (hz) control samples and (B) from the study samples identified as normal.
rapid preparation for PCR without need for additional reagents. The nurses participating in our study, usually without laboratory experience, found the method convenient both for themselves and for the women tested. No extra sampling was needed for the AGU carrier screening, because a capillary blood sample for a hemoglobin assay is regularly collected from the mothers at their first maternity clinic visit.

In evaluation of the cost effectiveness of a genetic screening program, many different aspects have to be taken into account. The structure, funding, and timing of the program all contribute to utilization and effectiveness. In the present study, a high participation rate was obtained while the AGU gene test was offered as a part of public maternity healthcare, entirely free for the participants. Counseling, reporting of the results, and overhead costs are major costs of screening programs, regardless of the organization of the healthcare system or the laboratory method used. Considering the analytical part only, the minisequencing test is of low cost.

The prospects of carrier screening programs in the Finnish population are promising both because of the high average education of the people, a well-organized healthcare system, and the homogeneity of the gene pool in the population. Enrichment of one predominant disease-causing mutation in the population is responsible for a group of ~30 autosomal recessive diseases known as the Finnish disease heritage. The recent description of a major mutation causing infantile neuronal ceroid lipofuscinosis (INCL), the INCL<sup>Fin</sup> [6], has created possibilities for carrier screening identical to those in AGU. In the near future, the disease-causing mutations in several other "Finnish" genetic diseases will be identified, thus increasing the interest in genetic testing. The high participation rate in the present study, and mainly positive attitudes among the general population toward genetic testing in Finland, as indicated in our earlier study [38], predict acceptance of future screening programs as well.

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