These factors are variably associated with the first process, and they work together to effect the amniochorionic system that is the main source for production not only of IL-8 (13) but also of various prostanoids (14, 15). Therefore, the highly variable concentrations of IL-8 in the CVS may be attributable to the extent of and/or the stages in which these factors contribute to cervical-decidual-amniochorionic activation. However, the precise mechanism is to be investigated further. Moreover, the kind of signals involved in increasing active Cp in CVS need to be better understood.

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PCR-Oligonucleotide Ligation Assay from Dried Blood Spots, Eva-Liisa Romppanen and Ilkka Mononen* (Kuopio University Hospital, Department of Clinical Chemistry, P.O. Box 1777, FIN-70211 KUOPIO, Finland; * author for correspondence: fax 358-17-173186, e-mail ilkka.mononen@messi.uku.fi)

PCR followed by oligonucleotide ligation assay (PCR-OLA) is a molecular method that can be used for the detection of nucleotide sequence variants. During the past 10 years it has been used successfully for numerous diagnostic applications (1–15). PCR-OLA is based on the enzymatic ligation of two oligonucleotides that anneal next to each other onto the PCR-amplified target DNA. Even a single-nucleotide mismatch between the oligonucleotides and the template precludes the ligation. Automated PCR-OLA on a microplate format in combination with ELISA detection technology is a powerful method for testing numerous samples (2). One bottleneck for the effective use of PCR-OLA in population screening programs has been the time-consuming and tedious preparation of DNA samples that is necessary to eliminate PCR inhibitors (16).

Dried blood spot (DBS) specimens on filter blotters are widely used for collection, storage, and shipping of blood samples in screening programs for newborns (17), and they have been used for genotypic confirmation of positive screening tests (18). PCR inhibitors can be eliminated from DBS specimens by eluting the spots (19, 20), by fixing the spots to the disk with methanol (21, 22), or by extracting DNA from the disk (23, 24). To increase the throughput of PCR-OLA, we treated DBS specimens with methanol and used the methanol-fixed samples for the PCR-OLA analysis of the major mutation (AGUFin) (25), which is responsible for Finnish-type aspartylglycosaminuria (AGU; McKusick 208400). We tested the methanol treatment on DBS specimens collected on five different types of glass fiber or filter paper blotters. The results show that PCR-OLA from methanol-treated DBSs enables rapid and reliable detection of genetic variances.

We collected EDTA blood samples from 115 Finnish individuals with unknown genotype and 20 carriers of AGUFin, making these anonymous after collection. The genotype of the AGUFin carriers was confirmed by restriction fragment length polymorphism (25). DBS specimens were prepared by dropping 10–20 μL of fresh or previously frozen (–20 °C) blood onto Merckocuant® blank strips (Merck), GF/C glass microfiber filters (Whatman), BFC 180 specimen collection paper (Whatman), Schleicher & Schuell (S&S) fiber paper no. 2992, or S&S fiber paper no. 903. The membranes were air-dried for 2 h, followed by application of one drop of methanol (~50 μL) to the DBS area. The methanol was allowed to evaporate in a fume hood for 1 h, and the membranes were stored desiccated at room temperature until analysis. In addition, for each membrane type, 10 DBS specimens that had not been treated with methanol were analyzed by PCR-OLA as controls.

A disk 1.5 mm in diameter, representing ~0.5–1 μL of
whole blood, was punched out of the membrane with a handheld punch and placed directly into a PCR tube. The punch was first rinsed in 2 mol/L HCl and then in distilled water after each sample to prevent cross-contamination between specimens (26). A 429-bp segment of the glycosylasparaginase gene containing the AGUfin mutation site (25) was amplified using primers 5'-TGTAGCT-CCTTAAGATG-3' and 5'-CCAGTAGCTTCCAT-GCT-3' (27) in a final volume of 30 μL. Amplification reactions contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 6 μmol of each dNTP, 7.5 pmol of primers, and 0.24 U of DynaZyme DNA polymerase (Finzymes). The cycling program in a PTC-100 Thermal Cycler (MJ Research) consisted of a first cycle of 96 °C for 10 min, 84 °C for the time needed to add DNA polymerase, 51 °C for 45 s, and 72 °C for 1 min, followed by 30 cycles of 1 min at 94 °C, 45 s at 51 °C, and 1 min at 72 °C, with a final extension for 9 min at 72 °C. The oligonucleotide ligation assay for the detection of the AGUFin mutation was performed as described by Delahunty et al. (6). The absorbance readings of the mutant and the wild-type (WT) ELISA reactions after a constant color development time of 15 min were measured at 510 nm with a Tecan Spectrafluor spectrophotometer. The ratio of the absorbance of the WT allele-specific reaction to the mutant allele-specific reaction was calculated to determine the genotype of the sample. A homozygote for the WT allele was identified by a ratio >5, and a homozygote for the mutant allele was identified by a ratio <0.2. The ratio of the two absorbances for a heterozygous individual was close to 1. In each PCR-OLA series, we used one negative control in which no DBS was added, and as a positive control we used DNA from a known carrier of the AGUFin mutation. For confirmation of the genotype obtained in PCR-OLA analysis of the DBS specimens, DNA from each EDTA blood sample was isolated with a Split Second® DNA Preparation kit (Roche) and 0.5 μL of the DNA preparation was used for PCR amplification as described above. PCR-OLA has been shown to accurately detect both the WT genotype and the heterozygous AGUFin genotype (6).

When PCR amplification of the AGUFin mutation site was applied to untreated DBS specimens collected on various membranes, the PCR mixtures became deep brown because of the elution of heme and its degradation products from the filter blotters, and the OLA reaction was successful for only 30 of 50 of these samples. In the other 20 samples, no amplified target DNA was detected after PCR in ethidium bromide-stained agarose gel electrophoresis, indicating that a failure in the PCR step was the cause for unsuccessful OLA assays. When the corresponding DBS specimens were amplified after the methanol treatment, the reaction mixtures were colorless and clear after PCR, and OLA was successfully accomplished for every specimen, verifying the importance of the methanol treatment of the blotters. We also found that prolongation of the denaturation step at 96 °C to 10 min before addition of DNA polymerase improved the PCR amplification of the target DNA. The PCR procedure was optimized for DBS disks of 1.5 mm in diameter in a reaction volume of 30 μL. The amplification of the target DNA was often lower when disks with 2 mm in diameter were used in the same conditions, and disks larger than 2 mm did not properly fit into the V-bottomed PCR tubes or plates (28).

We tested the applicability of five different filter blotters for collection of blood for PCR-OLA by genotyping 115 Finnish blood samples with unknown AGUFin genotype and 20 blood samples from known carriers of the AGUFin mutation. PCR-OLA was successful after the methanol treatment on each DBS collected on various filter blotters (Table 1). The constant color development time of 15 min in the ELISA led to absorbance readings higher than the measuring range of the spectrophotometer in many samples. These absorbances are marked as >3.0 in Table 1. In 114 of 115 samples collected on five different membranes (Table 1), the absorbance ratio of the WT reaction to the mutant reaction was 5.3–45.3 (median, 26.0), indicating the normal genotype. In 1 of the 115 samples, the ratio was 0.8, which was in the same range as in DBS prepared from frozen blood of known carriers of AGUFin mutation (range, 0.8–1.8; median, 1.0; n = 60; Table 1), indicating a carrier phenotype. All of the genotyping results of PCR-OLA from DBS were verified with PCR-OLA from Split Second DNA samples, and they were in full conformity. The 2.5th, 50th, and 97.5th percentile absorbances for the WT and the mutant PCR-OLA reaction, and the absorbance ratio of the WT and the mutant reactions for DBS specimens collected on five different filter blotters and for Split Second DNA samples are shown in Table 1. With each filter blower type that was studied, the absorbance ratio of the WT reaction to the mutant reaction in normal samples was significantly higher (Mann–Whitney U-test, P <0.001) than the corresponding ratio in samples with the heterozygous genotype. Although the absorbances were lower for the DBS than for the Split Second DNA samples, the genotype was determined correctly in each DBS sample. DBS specimens prepared from blood samples that had been stored frozen at −20 °C for several years were also analyzed successfully by PCR-OLA. The results show that all of the five tested blotters can be used for reliable DNA diagnostics by PCR-OLA according to the present procedure. The membrane type seemed to affect the assay to some extent because a slight variation in absorbance readings was seen (Table 1). However, this variation did not prevent the interpretation of the genotype in any of the samples.

We believe that this novel methodology can easily be adapted to various PCR-OLA applications for research or diagnostic purposes. The use of DBS specimens in the detection of nucleotide sequence variations by PCR-OLA simplifies the collection, storage, and shipment of blood samples. Rapid handling of DBS specimens would be facilitated by use of an automated punching equipment, which is already commercially available. The simplified sample preparation reduces risk of human error and increases the usefulness of PCR-OLA, e.g., for use in large-scale population screening programs.
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Genes were also examined for sequence variations, and subsequent sequencing revealed the presence of the C282Y, H63D, and S65C mutations. The frequencies of the HFE mutations in both patient groups are presented in Table 1. In addition, a T-to-C transition in intron 2 (IVS2+4T→C) was found in 70% of the patients and was always present in either the heterozygous or homozygous state in H63D and S65C mutants, giving rise to multiple band patterns in exon 2 (Fig. 1). The IVS2+4T→C mutation previously had been published by Douabin et al. (9). Reverse transcription-PCR analysis demonstrated that this mutation had no effect on the splicing of the HFE gene.

Denaturing Gradient Gel Electrophoresis Analysis of the Hemochromatosis (HFE) Gene: Impact of HFE Gene Mutations on the Manifestation of Porphyria Cutanea Tarda, Lene Christiansen,1 Anette Byggum,2 Kristian Thom-}

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Porphyria cutanea tarda (PCT) is the most common form of the porphyria disorders. PCT is caused by a decreased activity of the fifth enzyme in the heme biosynthetic pathway, uroporphyrinogen decarboxylase (UROD; EC 4.1.1.37). In familial PCT (fPCT), the disease is associated with mutations in the gene encoding UROD, but the majority of PCT cases are apparently sporadic (sPCT). Although clinical manifestations are predominated by cutaneous lesions, various degrees of liver damage are often associated with PCT. Clinically manifest PCT usually is provoked by exogenic factors, including alcohol, estrogen, viral hepatitis infections, HIV, and iron (1).

A mild to moderate iron overload is common in PCT, and several studies have revealed that the frequency of either of the two known HFE gene mutations associated with hemochromatosis, H63D and C282Y, is substantially higher in PCT patients than in the general population (2–8). This suggests that the inheritance of these mutations predisposes individuals to development of PCT.

Recently, another HFE gene mutation, S65C, was characterized, and analysis of a large group of hemochromatosis probands suggested that S65C may also be associated with hemochromatosis (9, 10). The purpose of the present study was to examine the HFE gene in Danish PCT patients for sequence variations, including the C282Y, H63D, and S65C mutations.

Using denaturing gradient gel electrophoresis (DGGE), we screened the entire coding region of the HFE gene in 57 unrelated PCT patients (15 with fPCT and 42 with sPCT). PCT diagnoses were based on the clinical picture and verified by biochemical findings. fPCT and sPCT cases were discriminated by mutation analysis of the UROD gene (Christianson et al., unpublished data).

Band patterns corresponding to the detected sequence variations in HFE are shown in Fig. 1. The DGGE analysis and subsequent sequencing revealed the presence of the C282Y, H63D, and S65C mutations. The frequencies of the HFE mutations in both patient groups are presented in Table 1. In addition, a T-to-C transition in intron 2 (IVS2+4T→C) was found in 70% of the patients and was always present in either the heterozygous or homozygous state in H63D and S65C mutants, giving rise to multiple band patterns in exon 2 (Fig. 1). The IVS2+4T→C mutation previously had been published by Douabin et al. (9). Reverse transcription-PCR analysis demonstrated that this mutation had no effect on the splicing of the HFE gene.

Fig. 1. DGGE gels showing the different band patterns in DNA fragments covering exons 2 and 4 of the HFE gene.

Lane 1, band pattern of a compound heterozygote for H63D and S65C, who is also homozygous for IVS2+4. Lane 2, band pattern of an H63D heterozygote/IVS2+4 homozygote. Lane 3, wild-type band pattern. Lane 4, band pattern of a compound heterozygote for S65C and IVS2+4. Lane 5, band pattern of an IVS2+4 homozygote. Lane 6, band pattern of an IVS2+4 heterozygote. Lane 7, wild-type band pattern. Lane 8, band pattern of a C282Y heterozygote. The 5’ part of exon 1 was amplified using the sense primer (GC) 50-5’ATAGGGGCAGAAGTGTGTTTCCACC-3’ and the antisense primer 5’ACGCGGATGGCCTCAGAAGT-3’. The 3’ part of exon 1 was amplified using the sense primer (GC) 5’-CTTGGACAGCAGAATAGCTGTAGGG-3’ and the antisense primer (AT) 5’-TTCGCCCGCAGCCCTCGGA-3’. Exon 2 was amplified using the sense primer 5’-ATGGTTAAGGCCTGTTGCTCTGCTG-3’ and the antisense primer (GC) 5’-ATGGTTAAGGCCTGTTGCTCTGCTG-3’ and the antisense primer (GC) 5’-ATGGTTAAGGCCTGTTGCTCTGCTG-3’.

Annealing of the PCR product was performed in a Perkin-Elmer GeneAmp PCR system 9600 using the following conditions: denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. PCR was terminated by extension at 72 °C for 10 min followed by a denaturation/ reannealing program of 99 °C for 5 min, ramping to 65 °C over 10 min, 65 °C for 10 min, ramping to 37 °C over 10 min, 37 °C for 10 min, and finally cooling to 4 °C. DGGE gels were prepared and run as described previously (12), with 20–60% gels used for the 5’ part of exon 1 and exon 5; 30–70% gels used for exons 2, 3, and 4; and 40–80% gels used for the 3’ part of exon 1. The presence of all four sequence variations was verified by restriction enzyme digestion using RsaI for C282Y and IVS2+4T→C, BstI for H63D, and HinfI for S65C.