filter discs at room temperature for 7, 14, 21, 28, and 35 days also did not significantly alter the mean cholesterol values, as is apparent from the \( F \) statistic (0.97) and the \( P \) value (0.45; Table 1). The mean triglyceride concentrations given in Table 1 indicate that dried serum is stable for triglyceride measurements and that storage for different time periods at room temperature does not alter the mean values significantly \( (F = 0.89; P = 0.50) \). An excellent correlation was observed between triglyceride concentrations in fresh samples and samples dried on filter paper and analyzed on day 0 (Fig. 1B).

Dried blood on filter paper may be useful for cholesterol and triglycerides. It has been used extensively for mass screening programs \( (1, 2, 6–8) \) and for measurement of glucose, insulin, glycosylated hemoglobin \( (9, 10) \), and steroids \( (11) \).

The adaptation of lipid assays to dried blood is ideal for pediatric applications and in multicenter studies where the costs and safety of sample transportation to a distant laboratory are limiting considerations. However, because serum was used in the present study, this approach needs to be evaluated in whole blood, and the use of filter paper matrix as a means of sample transportation would also need to be validated in the context of a multicenter study.

We conclude that cholesterol and triglycerides are highly stable in dried serum and are readily transferable to a liquid phase. The good agreement between values in dried serum and fresh samples supports the validity of the measurement of these analytes in dried serum.

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References


Mutation Screening of the Entire Coding Region of the Protoporphyrin Oxidase Gene Using Denaturing Gradient Gel Electrophoresis and Denaturing HPLC, Lene Christiansen, Anette Bygum, Marianne Kæhne, Alice Jensen, Magens Harder, and Niels Erik Petersen (1) Department of Clinical Biochemistry and Clinical Genetics, Odense University Hospital, DK-5000 Odense C, Denmark; (2) Department of Dermatology, Marselisborg Hospital, 8000 Aarhus, Denmark; * author for correspondence: fax 45-6541-1911, e-mail lene.christiansen@ouh.fyns-amt.dk

Variegate porphyria (VP) is characterized by decreased activity of protoporphyrinogen oxidase (PPOX), the penultimate enzyme in the heme biosynthetic pathway. VP belongs to the group of mixed porphyrias and presents with both cutaneous manifestations and acute neurovisceral attacks. Genetically, VP is associated with mutations in the gene encoding PPOX and is usually inherited as an autosomal dominant trait with low clinical penetrance \( (1, 2) \). Although a few founder mutations predominate in certain populations, VP generally is a heteroallelic disease, with a variety of mutations in the PPOX gene being responsible for the decreased activity of PPOX \( (3, 4) \). The 5.5-kb PPOX gene is located on chromosome 1q22-23 and contains 1 noncoding and 12 coding exons \( (5) \). To date, \( >80 \) different mutations and polymorphisms have been identified in the PPOX gene \( (3–12) \).

Characterization by mutational analysis of suspected cases of VP should be considered an important clinical diagnostic tool because of the similarities in clinical presentation of VP, porphyria cutanea tarda (PCT), and hereditary coproporphyria. Furthermore, the use of DNA diagnostic methods enables detection of latent or asymptomatic mutation carriers at risk of developing VP.

Here we describe assays for easy and reliable screening of the entire coding region of the PPOX gene as well as 74 bp of the noncoding exon 1 and 8–41 bp of the flanking intron sequences, using denaturing gradient gel electrophoresis (DGGE) and denaturing HPLC (dHPLC). Because VP frequently is confused with the more common PCT, we screened for PPOX mutations in DNA from 40 sporadic PCT patients, using established assays to detect possible misclassified VP cases.

The study protocol was approved by the relevant Regional Ethical Committees. All participants received written information and gave signed consent to participate in the study.

The 40 patients included in the study were previously diagnosed as having PCT based on the clinical presentation and the urine porphyrin excretion patterns. Feces porphyrin measurements were not available. Genetic analysis for disease-related mutations in the uroporphyrinogen decarboxylase gene were negative \( (13) \).

DNA was isolated from 10 mL of EDTA-stabilized blood by standard procedures \( (14) \).

The primers used for PCR amplification of 13 DNA fragments covering the 3' part of the noncoding exon 1
and the coding exons 2–13 are listed in the on-line supplement (http://www.clinchem.org/content/vol47/issue6/). We used MELT87 (15) to construct the PCR primers in accordance with the theoretical melting profiles of each DNA fragment to keep the genomic part of each PCR product in one or two low-melting regions. In addition, all primers were designed to facilitate usage of the same PCR conditions for amplification of all DNA fragments.

Each DNA fragment was amplified in a total reaction volume of 50 μL containing PCR buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl; Boehringer Mannheim), 200 μM each dNTP (Boehringer Mannheim), 0.8 μM of one of each pair of sense and antisense primers (Amersham Pharmacia Biotech), 100 ng of genomic DNA, and 1 U of Taq DNA polymerase (Boehringer Mannheim). For optimal PCR amplification of the fragment covering exon 13, the concentration of MgCl₂ was decreased to 1 mM; for amplification of the fragments covering exons 2 and 4, only 50 ng of template DNA was used.

PCR was performed in a Perkin-Elmer GeneAmp® PCR system 9600 with the following conditions: denaturation at 95 °C for 5 min, followed by 40 cycles (for DGGE) or 35 cycles (for dHPLC) of denaturation at 94 °C for 1 min and annealing/extension at 66 °C for 5 min. PCR was terminated by extension at 72 °C for 10 min, followed by a denaturation/reannealing program of 99 °C for 7 min, ramping to 65 °C over 10 min, holding at 65 °C for 50 min, ramping to 37 °C over 10 min, holding at 37 °C for 50 min, and finally cooling to 4 °C.

The DNA fragments covering the 3' part of exon 1 and exons 3, 5, 6, and 8–13 were subjected to DGGE analysis as described elsewhere (16). For the 3' part of exon 1 and for exons 9, 10, and 11, 30–70% DGGE gels were used. For exons 3, 5, 6, and 13, 20–70% DGGE gels were used, and 20–60% gels were used for exons 8 and 12.

Because of the high GC content of the sequences surrounding exons 2, 4, and 7, we were unable to synthesize GC-clamped PCR products for DGGE analysis of these exons. Hence, these DNA fragments were analyzed using dHPLC. dHPLC analysis was performed using the Trans genomic Wave™ DNA Fragment Analysis System (Transgenomic). PCR product (5 μL) was injected into the column and eluted with a buffer gradient in which buffer A was 01 mol/L triethylammonium acetate (pH 7) containing 0.25 mL/L acetonitrile and buffer B was 0.1 mol/L triethylammonium acetate (pH 7) containing 250 mL/L acetonitrile. The PCR product covering exon 2 was eluted at column temperatures of 63 and 65 °C with the following buffer gradients at a flow rate of 0.9 mL/min. For 63 °C, the gradient was as follows: 0.0 min, 54% A–46% B; 0.1 min, 49% A–51% B; 4.6 min, 40% A–60% B. For 64 °C, the gradient was: 0.0 min, 57% A–43% B; 0.1 min, 52% A–48% B; 4.6 min, 43% A–57% B. For 65 °C, the gradient was: 0.0 min, 58% A–42% B; 0.1 min, 53% A–47% B; 4.6 min, 44% A–56% B.

The PCR product covering exon 7 was eluted at column temperatures of 60 and 64 °C with the following buffer gradients at a flow rate of 0.9 mL/min. For 60 °C, the following gradient was used: 0.0 min, 48% A–52% B; 0.1 min, 43% A–57% B; 4.6 min, 34% A–66% B. For 64 °C, the gradient was: 0.0 min, 54% A–46% B; 0.1 min, 49% A–51% B; 4.6 min, 40% A–60% B.

Using DGGE to screen for PPOX mutations in DNA samples collected from the 40 individuals, we found evidence of four different sequence variations. Using dHPLC to screen for PPOX mutation in exons 2, 4 and 7 in DNA from the individuals, we found one sequence variation in exon 7. Subsequent sequencing of the regions identified by DGGE and dHPLC revealed the underlying mutations, which are summarized in Table 1.

The DGGE and dHPLC assays established for the PPOX gene include mutation screening of 13 DNA fragments, which together cover the entire coding region of the PPOX gene as well as a 74-bp fragment of the noncoding exon 1 and 8–41 bp of the flanking intron sequences. This is the first report on screening of the entire coding region of the PPOX gene using only fast and high-sensitivity methods; previous investigations of this gene used either less sensitive or more labor-intensive methods for some segments of the gene (3, 11).

This mutation screening assay is suitable for diagnosis of VP, thereby facilitating differentiation between VP and PCT or hereditary coproporphyria cases as well as detection of asymptomatic carriers of VP. Five different PPOX gene sequence variations were detected in this study (Table 1). Three of these, P256R, R304H, and IVS10–22C→G, have previously been identified as common polymorphisms (3, 12, 17).

The novel intronic sequence variation detected, IVS6+7G→A, is not positioned in a conserved splice site. However, generation of a cryptic acceptor splice site in exon 11, L369P, is not positioned in a conserved splice site.

| Table 1. Sequence variation, position, and predicted impact of the five PPOX mutations identified. |
|---|---|---|---|
| No. of individuals | Sequence variation* | Position | Impact on protein |
| 1 | IVS8+7G→A | Intron 6 | ND b |
| 1 | 1044C→G | Exon 7 | P256R |
| 9 | 1188G→A | Exon 9 | R304H |
| 1 | IVS10-22C→G | Intron 10 | ND |
| 1 | 1383T→C | Exon 11 | L369P |

* Numbering is according to the cDNA sequence published by Nishimura et al. (18) (GenBank D38537), in which the first A of the translation initiation codon is assigned no. 278.

b ND, not determined.
synthesis of an abnormal mRNA in which 8 bp of the intron sequence are maintained in the mRNA (3). Thus, a similar effect of the IVS6+7G→A variation cannot be excluded, although the lack of generation of a new GT dinucleotide reduces the possibility of this consequence.

The L369P mutation identified in one patient was previously unknown and could be disease related because the introduction of a nonflexible proline residue is very likely to have a substantial structural impact on the protein. However, the fact that several missense mutations identified in the PPOX gene to date appear to be common polymorphic variations underscores that interpretations are to be made with caution. A more definite conclusion concerning the impact of detected mutations thus requires expression and enzymatic studies of the mutants in question.

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References


