Génotyping: a new application for the spent dialysate in peritoneal dialysis

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**Abstract**

**Background.** The dialysate of patients on peritoneal dialysis (PD) is used to determine the concentration of growth factors and cytokines, and as a source of resident peritoneal cells for subsequent culture experiments. We hypothesized that the cells contained in spent dialysate samples obtained at the time of the peritoneal equilibration test (PET) and subsequently stored may represent a source of DNA from a given PD patient.

**Methods.** We characterized a protocol of DNA extraction from dialysates obtained in PD patients after a long dwell during the initial PET and kept frozen up to several years. After amplification of the source DNA by strand displacement using the bacteriophage φ29 DNA polymerase, we performed polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis for the Glu298Asp polymorphism of ENOS to demonstrate the suitability of the extracted DNA for genotyping.

**Results.** A significant amount of DNA (mean yield 12 μg/ml dialysate) was extracted from frozen dialysate samples. The extraction yield was not influenced by the duration of storage at −20°C. Following amplification, the DNA extracted from the dialysate was used successfully for genotyping the Glu298Asp polymorphism of ENOS, as demonstrated by parallel analyses using DNA extracted from the peripheral blood and sequencing.

**Conclusions.** These results demonstrate that the dialysis effluent obtained at the time of the initial PET and stored at −20°C is a reliable source of DNA that can be used subsequently for PCR amplification, RFLP analysis and sequencing.

**Keywords:** DNA; peritoneal dialysis; PET; PCR; polymorphism

**Introduction**

The peritoneal equilibration test (PET) is an established procedure consisting of a 4h dialysis exchange to characterize the peritoneal membrane (PM) solute transport characteristics in patients treated by peritoneal dialysis (PD) [1]. The PET is usually performed within the first month of PD initiation and once a year thereafter. The test has important clinical applications, since the permeability characteristics of the PM influence the dialysis prescription as well as patient and technique survival [2].

In addition to permeability assessment, the effluent taken from PD patients is used to determine the concentration of cytokines and growth factors, as well as other important molecules such as the cancer antigen 125 (CA125) reflecting mesothelial cells mass [3–6]. The effluent dialysate is also enriched in resident peritoneal cells, including mesothelial cells, macrophages and lymphocytes, with a total cell count of up to 50 cells/μl [7]. These cells, and particularly mesothelial cells, can easily be harvested from spent dialysate, in order to develop primary cell cultures that can be used for various cell and molecular biology techniques [6,8].

Several lines of evidence suggest that growth factors and cytokines, together with nitric oxide synthase (NOS) isoforms, play a central role in the regulation of vascular density and permeability in the PM [9,10]. Polymorphisms within the genes that encode these molecules [11–14] may lead to subtle differences in their transcription, which could in turn contribute to the large interpatient variability observed in baseline PM transport characteristics [2,15]. Studies to evaluate the potential influence of genetic variants on the PM permeability will require a large number of patients, ideally from a restricted geographic area and with a similar genetic background. Such studies will certainly be facilitated by the retrospective inclusion of well-characterized PD patients, although obvious limitations are the difficulty of obtaining DNA samples from PD patients who died before inclusion or were lost to
follow-up, as well as the ethical concerns linked to genetic research on archived material [16].

In our centre, the surplus dialysate samples obtained in PD patients undergoing a PET are stored systematically at −20°C for biochemical studies relevant to the PM. In order to assess the potential usefulness of the peritoneal cells contained in these samples for genotyping, we have characterized a protocol of DNA extraction and amplification from the frozen dialysate. Our results demonstrate that the dialysis effluent is a reliable source of DNA that can be used for polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and sequencing.

Materials and methods

DNA purification from frozen dialysate and peripheral blood

DNA purification was performed using the Purgene Kit® (Genta, Minneapolis, MN). Anonymised, frozen samples (2 ml) of dialysis effluent were thawed on ice, and centrifuged at 2000 g for 10 min (Labofuge 6000, Heraeus-Christ, Osterrode). The cell pellet was mixed with 50 μl of supernatant and transferred to a sterile microfuge tube containing 250 μl of cell lysis solution and 1.5 μl of proteinase K solution (20 mg/ml). The cell lysate was mixed by inverting the tube 25 times and incubated at 37°C for 15 min. The lysate was mixed with 100 μl of protein precipitation solution, vortexed at high speed for 20 s, placed into an ice bath for 5 min, and centrifuged at 13 000 g for 3 min to obtain a tight protein pellet. The supernatant containing the DNA was transferred into a sterile microfuge tube containing 300 μl of isopropanol and 0.5 μl of glycogen solution (20 mg/ml). The sample was mixed by inverting the tube 50 times, incubated at room temperature for 5 min and then centrifuged at 13 000 g for 5 min. The supernatant was poured off and the tube was drained on absorbent paper for 5 min. Ethanol (70%) was added to wash the DNA pellet and the sample was centrifuged at 13 000 g for 1 min. After drainage on absorbent paper for 15 min, the DNA pellet was mixed with 10-20 μl of DNA hydration solution and incubated at 65°C for 1 h.

DNA purification from peripheral blood samples (3 ml) was performed with the Purgene Kit® (Genta) as recommended by the manufacturer.

DNA amplification

We used the strand displacement amplification with the bacteriophage φ29 DNA polymerase (GenomiPhi™ DNA Amplification Kit®, Amersham Biosciences, Piscataway, NJ) in order to increase the amount of DNA available. In this method, a limited amount of genomic DNA is combined with a sample buffer containing random hexamer primers and other reagents to produce high molecular weight fragment copies of the source DNA. Typically, each reaction of GenomiPhi™ DNA amplification generates 4–6.5 μg from 10 ng of purified human genomic DNA in 18 h. Briefly, 1 μl of solution containing a minimum of 1 ng of purified DNA was added to a 200 μl microfuge sterile tube containing 9 μl of sample buffer containing random hexamer primers. The tube was heated to 95°C for 3 min to denature the sample and subsequently cooled to 4°C on ice. For each amplification reaction, we combined 9 μl of reaction buffer containing deoxynucleotide triphosphates with 1 μl of enzyme mix (φ29 DNA polymerase) on ice, and added the entire 10 μl volume to the denatured sample with gentle mixing. The tube was then incubated at 30°C for 18 h. The sample was heated to 65°C for 10 min to inactivate the enzyme and then stored at 4°C.

**PCR and RFLP analyses, sequencing**

In order to demonstrate the suitability of the extracted DNA for genotyping, we used the PCR technique followed by restriction enzyme digestion to determine the G894T polymorphism of the exon 7 of ENOS, the gene that encodes the endothelial NOS. This polymorphism results in the conservative replacement of the glutamic acid at codon 298 by an aspartic acid (Glu298Asp) [12]. It has been shown to influence renal disease progression [17] as well as endothelial dysfunction in cardiovascular disease [18]. The PCR was carried out in a 20 μl volume with 1 μl (containing 10 ng) of genomic DNA from the dialysate or 1 μl (containing 500 ng) of genomic DNA from blood, 10 μM of each primer, 1.25 mM dNTP (Roche Diagnostics, Mannheim, Germany), 1 μl of Tag polymerase (Roche) and 2 μl of 10× buffer containing 15 mM MgCl2 (Roche). The PCR primers were generated to amplify the 248 bp fragment encompassing the G894T variant (forward primer: 5’AAGGCAGGAGACA GTGGATGGA3’; reverse primer: 5’CCCCGATACCTT TTTGTTGCTCA3’). Samples were first denatured for 5 min at 94°C and then incubated during 30 cycles: denaturation at 94°C for 1 min; annealing at 63°C for 1 min; elongation at 72°C for 2 min; and final extension at 72°C for 10 min.

The PCR products were digested by MboI (Life Technologies, Carlsbad, CA), which does not cut the G894 allele (corresponding to Glu298), whereas it produces two fragments of 158 and 90 bp in length from the T894 allele (corresponding to Glu298Asp) [12]. The PCR products resulting from MboI digestion were size-fractionated on 1.5% agarose, stained with ethidium bromide, purified by QIA Quick Gel Extraction Kit® (Qiagen Genomics In., Valencia, CA), and their identity verified by sequencing (Genome Express, Grenoble, France).

This study protocol and the use of surplus dialysate samples for genotyping was approved by the Ethics Committee of the Université Catholique de Louvain.

Results

**DNA extraction from frozen dialysate**

The DNA concentrations obtained from 2 ml of frozen dialysate effluent and 3 ml of peripheral whole blood are shown in Table 1. The yield of DNA extraction from the dialysate was ~30-fold lower than that obtained from blood, which is expected in view of the small number of peritoneal cells present in the dialysate. However, the A260/A280 ratio was within the normal range (1.7–2), demonstrating DNA purity, and
subsequent DNA amplification using the GenomiPhi™ DNA Amplification Kit® allowed us to increase the amount of DNA available for testing. The duration of storage of the frozen dialysate had no influence on the yield of DNA extraction, since samples up to 7 years old gave similar results.

**Genotyping: PCR and RFLP analysis**

Despite the low yield of extraction, the DNA purified from dialysate samples was amplified successfully by PCR to a 248 bp fragment encompassing the G894T variant of ENOS (Figure 1A). The signal was similar to that obtained with DNA extracted from peripheral blood, the lower intensity reflecting the lower yield of extraction.

Figure 1B shows that genotyping by restriction analysis can be performed on the DNA extracted from dialysate. The 248 bp PCR fragment encompassing the G894T variant of ENOS (Figure 1A) was subjected to digestion with MboI. The wild-type G894 allele is not cut with MboI, whereas the T894 allele is cut into two fragments of 158 and 90 bp, respectively. Thus, patients homozygous for the wild-type G894 allele show a single band of 248 bp that is resistant to MboI, whereas patients homozygous for the T894 allele show two fragments of 158 and 90 bp, respectively. Accordingly, patients heterozygous for both alleles show a three-band pattern at 248, 158 and 90 bp (Figure 1B). The identity of all products of the MboI digestion was confirmed by sequence analysis.

**Discussion**

The PET is a semi-quantitative assessment of small solute transport that is performed routinely in PD patients. The dialysate obtained during this test offers a unique opportunity to collect biological information relevant for understanding peritoneal permeability. Thus far, the dialysate has been used to measure concentrations of different molecules and mediators, and to isolate resident peritoneal cells prior to culture. The fact that mesothelial cells, as well as other cells such as macrophages and lymphocytes, are present in the dialysate after a long dwell, combined with the storage of surplus dialysate samples from the initial PET was the rationale behind our attempt to demonstrate that these samples may represent a source of DNA from a given PD patient.

Our data demonstrate that: (i) DNA can be extracted from dialysis effluent drained after the night dwell from PD patients undergoing a PET, and stored at -20°C; (ii) the DNA extraction yield from the dialysate is lower than that of peripheral whole blood, although it is not influenced by the duration of the storage; and (iii) the extracted DNA can be amplified to perform unlimited DNA testing, including genotyping. The possibility of performing genetic analyses when the patient has died or moved away may be valuable for studies that will investigate the influence of common genetic variants in permeability variations observed among PD patients at baseline. It should be kept in mind, however, that the use of stored dialysate samples in this way may require a change in the ethical consent patients give for the use of their samples for research. In particular, patients should be informed at the time of sampling that the surplus material might be used for linked research; samples should be kept anonymized; and any potential harm to the donor or their surviving relatives should be carefully evaluated [16].

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**Table 1. Yield and absorbance of the DNA extracted from frozen dialysis effluent and peripheral whole blood**

<table>
<thead>
<tr>
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<th>Dialysis effluent (2 ml) n = 6</th>
<th>Whole blood (3 ml) n = 6</th>
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<tbody>
<tr>
<td>DNA concentration (µg/ml)</td>
<td>12 ± 2.7</td>
<td>531 ± 124</td>
</tr>
<tr>
<td>A260/A280 ratio</td>
<td>1.83 ± 0.04</td>
<td>1.81 ± 0.11</td>
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Data are expressed as mean ± SEM.
Conflict of interest statement. None declared.

References


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