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DNA extraction and quantification from touch and scrape preparations obtained from autopsy liver cells

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Abstract

The objective of the present study was to develop a simplified low cost method for the collection and fixation of pediatric autopsy cells and to determine the quantitative and qualitative adequacy of extracted DNA. Touch and scrape preparations of pediatric liver cells were obtained from 15 cadavers at autopsy and fixed in 95% ethanol or 3:1 methanol:acetic acid. Material prepared by each fixation procedure was submitted to DNA extraction with the Wizard® genomic DNA purification kit for DNA quantification and five of the preparations were amplified by multiplex PCR (azoospermia factor genes). The amount of DNA extracted varied from 20 to 8,640 µg, with significant differences between fixation methods. Scrape preparation fixed in 95% ethanol provided larger amount of extracted DNA. However, the mean for all groups was higher than the quantity needed for PCR (50 ng) or Southern blot (500 ng). There were no qualitative differences among the different material and fixatives. The same results were also obtained for glass slides stored at room temperature for 6, 12, 18 and 24 months. We conclude that touch and scrape preparations fixed in 95% ethanol are a good source of DNA and present fewer limitations than cell culture, tissue paraffin embedding or freezing that require sterile material, culture medium, laboratory equipment and trained technicians. In addition, they are more practical and less labor intensive and can be obtained and stored for a long time at low cost.

Key words
- Autopsy
- Congenital anomalies
- DNA
- Touch preparative
- Scrape preparative
- Polymerase chain reaction

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Introduction

Congenital anomalies are structural, functional, biochemical or behavioral alterations observable at birth. They involve about 3% of liveborns in the general population (1) and represent the major cause of post-neonatal death in the city of Ribeirão Preto (2), corresponding to about 25% of the cases of pediatric autopsy at the University Hospital of the Faculty of Medicine of Ribeirão Preto, University of São Paulo (3).

Although many of the anomalies observed at autopsy can be diagnosed by dysmorphologic examination to establish the syndromic and etiological diagnosis, other procedures are often necessary, ranging from cytogenetics to biochemical and/or molecular genetic techniques. Cytogenetics is of great value for the study of congenital anomalies, especially

those presenting multiple defects, but requires the presence of viable cells and an appropriately equipped laboratory. Its disadvantages are a high cost, time-consuming procedures (4) and limitation by lack of growth or by contamination with bacteria and fungi (5). In addition, non-numerical chromosome alterations or alterations below the level of optical resolution (microdeletions) cannot be identified by this method but require molecular techniques.

DNA can be extracted from different tissues, fixed or not. The choice of a method should consider not only the quality and quantity of extracted DNA, but also the costs of collection, preservation and storage. Touch and scrape preparatives are easy to obtain at autopsy and can be obtained from different tissues, providing a large number of samples (6-8). They do not require complicated technical training and require only new and clean glass slides (9,10) and a fixative fluid, materials of low cost. Fixation can be performed with ethanol, acetone, methanol or by air drying, among other techniques, with care taken to always preserve DNA integrity (11,12). The slides thus obtained can be stored for long periods of time at room temperature (13) and also provide a large number of whole cells with intact nuclei, which can be recovered for DNA extraction for different purposes.

Touch and scrape preparatives are useful means of obtaining material for molecular studies and can be easily obtained from patients, for example, by washes or scrape preparatives of the oral cavity (14-16). However, this cell source is not adequate at autopsy since the oral cavity presents marked contamination with cells and food debris and biological agents such as bacteria and fungi that may impair certain analyses. On the other hand, at autopsy it is possible to obtain better preserved cells, with a lower risk of tissue contamination with foreign elements. Thus, liver touch and scrape preparatives represent an excellent cell source.

The objective of the present study was to define and validate a method for the collection and fixation of liver cells obtained by touch and scrape preparatives at autopsy for DNA extraction and amplification for the purpose of diagnosing congenital anomalies and other genetic diseases.

Material and Methods

The touch and scrape preparatives used in the present study were obtained from 15 children of both sexes ranging in age from 25 min to four years who presented some type of congenital anomaly, and submitted to autopsy at the Pathology Service of the University Hospital, Faculty of Medicine of Ribeirão Preto (HCFMRP-USP), from July to December 2001. The project was approved by the Research Ethics Committee of HCFMRP-USP.

A fragment of the left liver lobe measuring approximately 4 x 3 x 0.5 cm was removed from each liver and its surface was used for the collection of touch (group TP) and scrape preparatives (group SP). The fragments were first washed in running water to remove excess blood and 4 touch preparatives were then performed by simply pressing new and clean microscope slides against the surface of the fragment. Scrape preparatives were then obtained with a new and clean surgical knife blade and deposited on three new and clean microscope slides.

Immediately after collection, the slides were placed in appropriate plastic tubes containing 95% ethanol for 10 min (groups TP95 and SP95) or 3:1 methanol:glacial acetic acid solution for 45 s (groups TPMA and SPMA). After fixation, the slides were air dried, wrapped in polypropylene film and stored in cardboard boxes at room temperature until the next stage of the experiment.

DNA was extracted using the Wizard® genomic DNA purification kit (Promega Corporation, Madison, WI, USA). Cells were carefully removed by scraping from a single touch

or scrape preparative slide with the aid of a new sterile surgical knife blade and the material obtained was placed in new clean 1.0-ml Eppendorf microtube to which 300 µl cell lysis solution, 100 µl nucleus lysis solution and 20 µl proteinase K (20 mg/ml, Promega) were added. The mixture was incubated overnight in a water bath at 50ºC, and 35 µl of the protein precipitation solution of the kit (not specified by the manufacturer) was then added and the sample kept in an ice bath for 5 min. Next, the mixture was centrifuged at 15,600 *g* for 4 min, the supernatant was transferred to a microtube containing 300 µl 100% isopropanol, mixed by inversion, and again centrifuged for 1 min and the supernatant was discarded. The pellet was resuspended in 300 µl 70% ethanol and centrifuged at 15,600 *g* for 1 min, and the supernatant was discarded. The pellet was air dried and then hydrated with 20 µl hydration solution. Finally, the extracted DNA was stored at -20ºC.

DNA was quantified at 1:100 dilution with a GeneQuant II RNA/DNA calculator spectrophotometer (Pharmacia Biotech, Cambridge, UK) at 260 nm wavelength for determination of double-stranded DNA.

DNA was amplified by PCR in five cases from each group in order to determine the quality of extracted DNA. A multiplex PCR system with a primer mix for the study of azoospermia factor - AZF (Yq11) was used (Table 1) and for this reason all subjects studied were males.

PCR was performed using 3 µl DNA, 0.8 µl Taq (*Thermus aquaticus*) DNA Polymerase (Gibco-BRL, Gaithersburg, MD, USA), 10.45 µl ddp water, 5 µl Taq DNA polymerase buffer (Gibco), 2.5 µl 2 mM deoxyribonucleotide 5' triphosphate, 1.25 µl dimethylsulfoxide, and 2 µl primer mix, placed in a PTC-100 thermocycler (MJ Research Inc., Watertown, MA, USA). The following cycles were used: 94ºC/5 min, 94ºC/30 s, 52ºC/45 s, and 65ºC/2 min, with 44 repetitions starting from the second step, and a final cycle at 65ºC/5 min.

After amplification, 5 µl of the material was stained with Orange G and submitted to 1.5% agarose gel electrophoresis (Gibco) in Tris borate EDTA with 5 µl ethidium bromide at 80 V for a maximum of 1 h using a 100-bp ladder as a molecular weight marker. The result was photographed with a Kodak digital camera.

To test if long-term storage affected the amount and quality of the extracted DNA, one slide each of touch and scrape preparatives with both fixatives, stored for 6, 12, 18 and 24 months on cardboard at room temperature, was submitted to the procedures described above.

Statistical analysis

Data were analyzed statistically using the GMC software. When testing for normality we observed that the experimental data were not normally distributed and therefore we opted for the Kruskal-Wallis test for analysis of variance of multiple independent samples.

Table 1. Primers used in multiplex PCR.

Name	Locus	Direct primer	Inverse primer	bp
Y6D14pr	DYS205	GGCTAGGTGCCAGCAAGTAGATCA	GTTCTCTTCCCCTGCATCAAG	134
Y6BaH34pr	DYS206	GCCCTTTGGTAAAAGCG	GAATTTGCAAGGGCTGC	910
Y6Hp35pr	DYS274	GGTACACACTCCATCCTGGAC	TAACGGCTACCTTTTAGGTGG	226
Y6Hp52pr	DYS239	GAACTGGCAGGATTAGCCTTC	GCTCAGAATCTGCGATCAG	258
Fr15-Hpr	ND	TAGCTTGGTTTTGCACCAGACGC	CACCCTCTGTATATGACCTGGC	313

ND = not determined.

Table 2. Concentration in ng/µl of DNA extracted from the different samples (total volume of each, 20 µl).

Groups			
TP95	TPMA	SP95	SPMA
90	420	460	50
1,165	1,950	7,500	3,680
1,160	20	8,550	30
4,170	20	8,640	50
560	885	450	3,980
340	2,580	2,960	1,210
1,585	335	4,670	6,540
455	265	1,430	2,980
90	1,195	130	50
775	395	7,500	500
1,725	1,095	8,360	90
985	775	2,090	1,040
520	215	2,070	2,970
1,200	250	3,400	1,900
1,250	355	3,700	1,540
Means			
1,071.33	717	4,127.33	1,774

TP = touch preparatives; SP = scrape preparatives; 95 = 95% ethanol; MA = methanol:glacial acetic acid (3:1).

Table 3. Kruskal-Wallis test for the comparison of sample rank means.

Samples compared	Differences between means	Significance
TP95 x TPMA	7.3333	ns
TP95 x SP95	16.5000	0.01
TP95 x SPMA	1.7667	ns
TPMA x SP5	23.8333	0.001
TPMA x SPMA	9.1000	ns
SP95 x SPMA	14.7333	0.05

TP = touch preparatives; SP = scrape preparatives; 95 = 95% ethanol; MA = methanol:glacial acetic acid (3:1); ns = nonsignificant.

Table 4. DNA extracted from different samples stored for different times.

Samples	Storage time in months			
	6	12	18	24
TPMA	4,630	–	–	2,320
SP95	5,000	–	–	5,160
SPMA	–	4,670	2,390	–
TP95	–	6,540	2,330	–

TP = touch preparatives; SP = scrape preparatives; 95 = 95% ethanol; MA = methanol:glacial acetic acid (3:1).

Results

Touch or scrape preparatives were easily obtained in all cases, usually with good quality material. However, some of the slides showed large amounts of blood and in many cases, after fixation, they exhibited a turbid coloration of the fixative, indicating that part of the material had remained diluted in the latter. In other cases there was little material adhered to the slides even before they were placed in the fixative.

DNA extraction by the methodology used was efficient, as can be seen in Table 2, which shows the results of quantification for the 15 samples studied in each group. There was wide variation among touch and scrape preparative samples and between fixatives from 20 to 8,640 ng/µl, with a mean of 1,071.33 ng/µl for TP95, 717 ng/µl for TPMA, 4,127.33 ng/µl for SP95, and 1,774 ng/µl for SPMA.

Pair-wise comparison of the samples (Table 3) showed that there was a significant difference between TP95 and SP95, TPMA and SP95 and SP95 and SPMA, indicating that 95% ethanol is a better fixative than methanol:acetic acid and that scrape preparatives are better than touch preparatives. Comparison of TP95 and TPMA, TP95 and SPMA and TPMA and SPMA showed no significant differences at the 5% level.

With respect to the quality of the extracted DNA, PCR amplification with the primers used showed that there were no differences between fixatives and materials, as illustrated in Figure 1.

Table 4 shows the results of quantification of DNA extracted from long-term storage slides. The amount was similar to what was obtained with shorter storage time.

PCR amplification done on the DNA extracted from long-term storage at room temperature showed no difference when compared to short-term storage (data not shown).

Discussion

Congenital anomalies require attention and more in-depth studies since they are appearing at increasing frequency, not only in our region (2), but also in general in the Brazilian Southeast and in developed countries (17). This frequency tends to increase as avoidable causes of death such as prematurity, infections and malnutrition are better controlled, and also with better pre- and postnatal care. Thus, only causes of death of more difficult control persist; particularly important among them are congenital anomalies (3,17).

Paraffin-embedded tissue can be used as a source of cells for DNA extraction, although not all of them are susceptible to PCR and some fail to yield detectable products (18,19). The explanation for this failure is unknown, but may be due to differences in fixation. The use of 10% buffered formalin for more than 3 days reduces amplification (18). Shorter times of fixation can reduce the quantity of amplified material (19). The amount of DNA decreased progressively when non-fixed frozen sections, frozen sections fixed with 10% formalin for 10 min and paraffin-embedded sections were compared in terms of DNA extraction (19). The deleterious action of formalin is due to DNA cross-linking with proteins (20,21), which increases with time of fixation (11), as also observed for RNA (22). The quality of DNA extracted from paraffin-embedded tissue may also be inadequate due to the presence of phenols, salts, EDTA (23), paraffin, formalin (19,23), and substances used for staining (19). Thus, the degraded DNA generates an unknown amount of amplified products (23), usually smaller than that obtained with ethanol (24). Amplified products of up to 250 bp can be obtained from histological sections of tissues previously fixed in formalin and embedded in paraffin (25).

Cell culture, which would guarantee excellent material for molecular studies, is of limited application because it is usually employed only in specific cases such as multiple congenital anomalies. It is an expensive and time-consuming procedure which may also result in lack of growth and in contamination (1,3,5).

Another adequate source both in terms of quantity and quality is tissue fragments which are frozen in liquid nitrogen and stored frozen at very low temperatures (26-29), a procedure that involves relatively high costs.

The present results show that the use of liver touch and scrape preparatives can be a good alternative because of the simple methodology involved, the amount and quality of the material obtained and the low cost of obtaining and storing the material, which is not impaired by long-term storage at room temperature. Touch and scrape preparatives vary widely in the amount of DNA extracted, possibly as a function of the presence of blood or even of inadequate adherence of the material to the slide (30), with the material being lost when the slide is placed in the fixative, with consequent turbidity of the fluid. To avoid this problem, the fragment could be washed in saline to remove most of the blood, and the surface to be used should

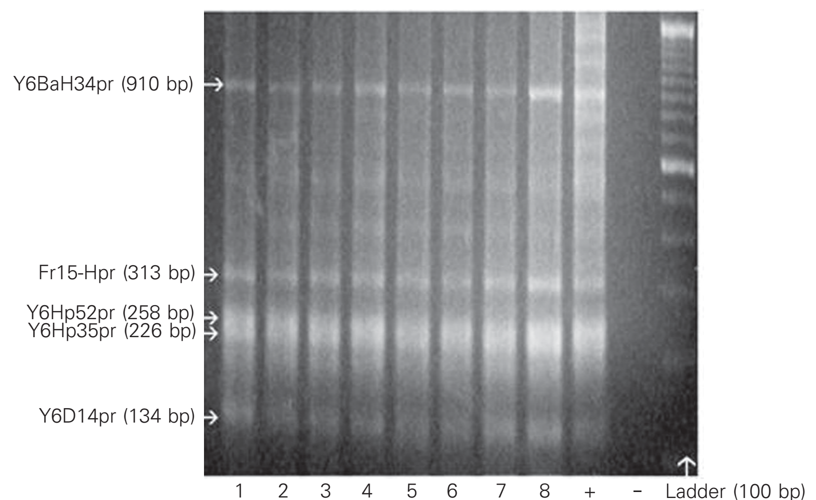

Figure 1. Amplification on 1.5% agarose gel of DNA extracted from touch and scrape liver preparatives at a concentration of 600 ng/µl. *Lanes*: *1*, SP95%; *2*, SPMA; *3*, SP95%; *4*, SPMA; *5*, TP95%; *6*, TPMA; *7*, TP95%; *8*, TPMA; +, positive control; – negative control; Ladder 100 bp. TP = touch preparatives; SP = scrape preparatives; 95% = 95% ethanol, MA = methanol:glacial acetic acid (3:1).

be dried before doing the touch or scrape preparative procedure. To guarantee cell adhesion to the glass, it is advisable to wait 30 s before placing the slides in the fixative. The preservation of the morphological and chemical characteristics of the tissue with this procedure would not be impaired since the material will not dry up. In any case, the quality of amplification is guaranteed, as shown in studies that demonstrated the use of material obtained from the skin of human patients with leishmaniasis that were first air dried and stained with Giemsa and then stored for up to 4 years without a coverslip (31). DNA extraction and amplification of Leishmania genes were possible even in cases in which cytologic analysis failed (31). Good results were also obtained with the amplification of *Mycobacterium tuberculosis* DNA obtained from air-dried sputum slides (13). The use of adhesive substances such as albumin and gelatin (30), silane, etc. is inadequate since the presence of any contaminating material should be avoided as much as possible when the objective is DNA extraction and amplification (23).

Logically, scrape preparatives would be expected to contain more cells and therefore to be more appropriate than touch preparatives, as actually indicated by our results. This agrees with literature data showing that fresh tissue scrape preparatives are more efficient than simple touch preparatives when evaluated by cytological methods for preoperative diagnosis (10,23). The amount of DNA which can be extracted must therefore be larger in this type of material. However, despite the difference, the mean quantity of DNA extracted from all groups was much more than needed for PCR, which usually requires 50 to 100 ng, with the possibility of using only 5 ng (32,33), or even than needed for Southern blotting, for which about 500 to 1000 ng is necessary (32). This means that any of these forms of obtaining tissue samples can be utilized. Literature data agree with these results since epidemiological studies involving DNA extraction, quantification and amplification using oral mucosa washes or scrape preparatives (16) have demonstrated that the amount of cells, and consequently of DNA, obtained permits the identification of different genes amplified by PCR. Tumor touch preparatives have also been utilized, with or without fixation to obtain DNA and to amplify specific genes such as p53 (34,35). As determined here, these cytologic methods are of low cost, easy to set up and execute, and can be utilized on a large-scale basis (16). These advantages are also taken into account when cells are to be obtained for cytologic analysis (10,36) or even for fluorescent *in situ* hybridization (FISH).

Not only the method for material collection, but also the fixative is of fundamental importance. The present results show that 95% ethanol is superior to methanol:acetic acid. Ethanol at 14% concentration, i.e., a concentration much lower than the one used here, has been used in studies of DNA extraction from oral mucosa cells obtained by oral brushing or washing and the results obtained have shown that DNA is of sufficient quality and quantity for the determination of gene sequences of different sizes (16). Ethanol is also the fixative used for cytologic analysis (10,36) and even for FISH (37,38), showing potential for adequate preservation not only of the chemical characteristics of cell components, but also of their morphological aspect. Cells obtained in this manner can therefore be utilized for different types of analysis, a fact supporting the importance of the definition of a protocol such as the one used here.

PCR amplification was used for qualitative DNA determination, as also done in other studies (16). DNA amplification by multiplex PCR for AZF (Yq11) showed that both the fixatives and the materials yielded similar good quality results. It was possible to verify the integrity of specific genes by the presence and the quality of the electrophoretic bands. The results showed well-de-

fined, strong bands positioned at the expected points for the amplified genes. In addition, there was no dispersal or migration of the material beyond or short of the expected points.

In conclusion, the proposed method of obtaining cells from autopsy cases fulfills the criteria for low cost, practicity and easy storage for a long time making it a good option for the analysis of congenital disorders mainly in places with few resources.

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