SEX DETERMINATION BY GENOMIC DOT BLOT HYBRIDIZATION AND HLA DQα TYPING BY PCR FROM FIXED TISSUES

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Introduction

Recent advances in molecular biology methods have significantly increased the ability to detect genetic variation at the genomic level for forensic purposes. However, the quality requirements for blood, fresh or frozen tissue as a source of DNA are a practical limitation for typing the victim in order to conduct investigations on unsolved cases. Since paraffin embedded specimens are easily obtainable the ability to study this material would be of great value in current forensic practice.

Materials and methods

Specimen selection:

All investigations were performed using routine forensic material. The only selection criterium was the time intervall between death and autopsy which differed between 24 h and 52 hours. From autopsy cases (n = 16) blood as well as unfixed and fixed lung tissue were under investigation.

Sampling protocols:

Each lung tissue was divided into portions which were processed according the following protocols:

1) snap frozen and stored at -20°C
2) fixation in 96% ethanol
3) fixation in 4% paraformaldehyde
4) 4% buffered formaldehyde pH 7.0
5) 10% unbuffered formaldehyde.

Incubations in the fixatives were performed for 4 and 6 weeks. After these time periods samples were processed by an Autotechnicon. About 10 paraffin sections 10 µm thick were cut using a microtome. The slides were deparaffinized and rehydrated, washed 3 times by centrifugation in xylene, absolute ethanol and SSC. Unfixed and ethanol fixed lung tissue was cut using a cryostat.

DNA extraction and purification

DNA from blood, unfixed and ethanol fixed tissue was prepared using previously described methods (4). Solutions containing paraformaldehyde, 10% formaldehyde and 4% buffered formaldehyde fixed tissue were adjusted to 1% SDS, 50 mM Tris pH 8.0, and incubated at 37°C for 2 days. The preparation was centrifuged, the supernatant discarded. The pellet of lung tissue which includes large quantities of stroma was resuspended in fresh lysis mix containing: 10 mM Tris/HCl pH 7.6, 10 mM EDTA, 100 mM NaCl pH 8, 2% SDS, 40mM DDT, 1 mg/ml proteinase K. The second incubation at 42°C was stopped after 6 days. A phenol-chloroform extraction was performed and the DNA precipitated with cold ethanol. Dot hybridization could be performed with all samples at this stage. For successful PCR

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amplification the DNA obtained from formaldehyde and buffered formaldehyde tissue had to undergo 3 additional reextraction steps of treatment at 42°C for 24 h with the above lysis buffer followed by phenol extraction and ethanol precipitation.

Sex determination by dot hybridization

Dot hybridization on Immobilon N membranes (Millipore, Eschborn, FRG) was performed as previously described (4) with repetitive DNA probe* pHY 2.1, which hybridizes specifically with the long arm of the Y-chromosome (1). DNA probe was labeled by nick translation with biotin-dUTP (ENZO, Neckargemünd, FRG). Biotinylated DNA probe was visualized by a streptavidin alkaline phosphatase conjugate (Dakopatts, Hamburg, FRG) and application to a BCIP containing staining gel according to Pflug (5).

HLA DQα typing

DNA amplification was performed according to the protocol of the supplier (Ampli Type™ HLA DQα Kit, Perkin Elmer/Cetus Emeryville, USA; 40 cycles programme)

Results

Dot Hybridization

Sex was correctly classified in all cases using 50-100 ng target DNA. In some cases additional control experiments were performed: after Hae III digestion and agarose gel electrophoresis of DNA typed as male a defined fragment of 2.12 kb could be detected.

HLA-DQα-Typing

PCR and HLA DQα-typing from ethanol fixed and paraformaldehyde fixed tissue was performed without major problems and in agreement with the genotypes obtained from blood. Complete failure of DNA amplification was seen with DNA isolated from formaldehyde fixed tissues. After additional proteinase K / 2% SDS treatments the samples could be typed successfully. In one case a complete determination of the DQα genotype was nearly missed.

Discussion

When tissue specimens are processed in forensic laboratories formaldehyde fixation times are significantly different from those in clinical pathology laboratories and often vary from a few days up to several weeks. We determined the effects of various fixatives and the fixation time on DNA extractability. It seemed likely that the fixative itself and the length of fixation are important determinants of the quality and condition of DNA present in the specimen.

With crude DNA extracted from formaldehyde fixed lung tissue a PCR product could not be obtained even when the PCR was repeated several times, or more genomic DNA was added to the reaction mixture. As initial DNA denaturation is the crucial step in PCR processing we suspect the presence of formaldehyde crosslinks as a reason for PCR failure. Formylation of nucleic acids produces Schiff bases on free amino groups of the nucleotides. Exposure of nucleoproteins to formaldehyde results in the formation of crosslinks between DNA and proteins. Formaldehyde is known to react with both the imino and amino groups of the bases

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of DNA. It is a reagent that bridges distances of 2 Å and allows crosslinking of both histone-histone and histone-DNA regions. According to the findings of Jackson (3) for reversal of histone-DNA crosslinks the samples were adjusted to 1% SDS, 50mM Tris pH 8 at 37°C for two days prior to DNA extraction. The observations suggest that obviously this pretreatment was not sufficient enough to cleave all crosslinks. Therefore prolonged proteinase K/2% SDS incubation is necessary in forensic case work to process human genomic DNA from formaldehyde fixed tissues in amplification experiments.

One of the effects of increasing the length of fixation was that only unspoolable DNA was obtained. To our opinion spooling of the DNA is not only a question of the samples to be kept concentrated enough during extraction procedure. Unspoolable DNA may also indicate that there are chemical alterations or changes in DNA structure. In agarose gel electrophoresis these samples showed that the mobility of DNA from fixed material was slightly slower than that of DNA from fresh tissue as reported by Goelz (2). In our study it was found that as long as DNA only partly can penetrate the gel PCR experiments should not be started. In one case heterozygosity nearly was missed. HLA DQα typing from formaldehyde tissue showed a strong 1.1 signal and a very faint signal for DQα 4. The typing result obtained from blood was 1.1,4. This phenomenon is still unclear. An explanation could be a preferential amplification of the DQα 1.1 allele due to persisting crosslinks in the DQα 4 allele region. This effect must be noticed in case work, when genomic DNA is amplified from material fixed for long time periods.

Sex always was correctly classified. No hybridization signal of the filters probed with pHY 2.1 was observed after one hour development on BCIP-staining gel of the DNA of human females. DNA probe pHY 2.1 hybridizes with about 2000 copies of DNA sequences from the distal part of the long arm on the Y chromosome and therefore should be easily visualized. There are another 100-200 copies also present in female genome which should not be detected under stringent hybridization conditions and washing procedures as shown by in situ hybridization. However, when high target concentrations (> 1 µg) for dot hybridization were used, weak signals in female DNA could be seen even after short development times. Therefore it is necessary for forensic application to determine the quantity of the extracted DNA prior to dot hybridization.

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