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Application of DNA techniques for identification using human dental pulp as a source of DNA*

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Summary. Dental pulp tissue could be obtained in most cases from materials obtained under experimental conditions and from forensic casework (air accidents, burned and putrefied bodies). Teeth extracted during dental treatment ($n = 30$) were stored for 6 weeks and 4 years at room temperature. In addition teeth ($n = 10$) extracted from jaw fragments that had been stored for 15 years at room temperature, and teeth extracted post mortem from actual identification cases ($n = 8$) were investigated. Following extraction from dental pulp tissue the DNA concentration was measured by fluorometry. The amount of DNA obtained from the dental pulp tissue of a single tooth varied from 6 µg to 50 µg DNA. In most cases high molecular weight DNA was still present although the major portion consisted of degraded DNA. Genomic dot blot hybridization for sex determination using the biotinylated repetitive DNA probe pHY 2.1 was performed and sex was correctly classified in all cases using 50–100 ng target DNA. PCR typing of the HLA-DQα and ApoB 3′ VNTR systems from dental pulp tissue DNA was in agreement with the results obtained from blood, bloodstains, or lung tissue. In addition, Southern blot analysis of selected samples using the single locus VNTR probe pYNH24 was successfully performed. In all cases the DNA recovered from dental pulp was unsuitable for multilocus probe analysis.

Key words: Identification – Forensic odontology – Dental pulp – Sex determination – DNA – PCR – HLA-DQα typing – Apo B 3′ polymorphism – VNTR analysis

Zusammenfassung. Zur Untersuchung gelangten anläßlich einer Zahnbehandlung extrahierte Zähne nach einer Liegezeit bei Raumtemperatur von 6 Wochen ($n = 20$) bis zu 4 Jahren ($n = 10$) sowie postmortem extrahierte Zähne von aktuellen Identifikationsfällen ($n = 8$) und einem Flugzeugabsturz des Jahres 1976 ($n = 10$). Die Zahnpulpa wurde präpariert und eine DNA Extraktion vorgenommen. Die Ausbeute betrug 6 µg bis 50 µg DNA pro Zahn. In den meisten Fällen war neben degradierter DNA noch hochmolekulare DNA vorhanden. Die Geschlechtsbestimmung erfolgte durch Dot-Hybridisierung mit der biotinylierten repetitiven DNA-Sonde pHY 2.1. In allen Fällen konnte das Geschlecht mit 50–100 ng Ziel-DNA zutreffend bestimmt werden. An genetischen Markern nach DNA-Amplifikation durch PCR erfolgte eine Typisierung der HLA-DQα und ApoB 3′ VNTR Merkmale. Die Resultate zeigten Übereinstimmung mit den Genotypen, die an Blut, Blutspuren oder Lungengewebe ermittelt wurden. Southern Blot-Analysen mit Minisatelliten-DNA-Sonden ergaben auswertbare Resultate mit der Einzellocus-Sonde pYNH24, während eine Multilocus-Sonden-Hybridisierung der aus den Zahnpulpen isolierten DNA nicht gelang.

Schlüsselwörter: Identifikation – Forensische Odontologie – Zahnpulpa – Geschlechtsbestimmung – DNA – PCR – HLADQα Typisierung – ApoB 3′ – VNTR-Typisierung

Introduction

Forensic odontological examination is the primary means of identification in situations where exposure, time elapsed since death and destruction of the body (fire, explosion etc.) has made other means of identification impossible, but it does have its limitations e.g. when the remains are fragmented and only small portions of jaw bearing teeth can be recovered. Under experimental conditions and from actual forensic cases (air accidents, burned and putrefied bodies) it was observed that dental pulp tissue could be extracted in most cases [20, 21]. Dental pulp consists of a "stromal" tissue containing nerves, blood

Abbreviations: BCIP: 5-bromo-4-chloro-3-indolylphosphate; RFLP: Restriction fragment length polymorphism; VNTR: Variable number of tandem repeats; AMP-FLP: Amplified fragment length polymorphism

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and lymph vessels. The pulpa cells are irregularly stellate in shape and contain a variably placed nucleus. Therefore dental pulp tissue is an important material for identification by: ABO blood typing, GC-subtyping, sex determination and confirmation of genetic markers such as the HLA-DQα type or determination of polymorphic marker systems such as the apo B 3′ VNTR polymorphism. It is possible to identify sex in human tissues, bloodstains and pulpal cells for up to one year by the detection of fluorescent Y-chromosomes in interphase nuclei after staining with quinacrine dihydrochloride [3, 9, 10, 27, 28, 29, 30, 32]. Recent advances in molecular biology methods have significantly increased the ability to detect genetic markers and variations at the genomic level for forensic purposes [see 22].

Application of DNA techniques in forensic odontology may offer a new tool when identification by usual forensic means fails due to effects of heat, trauma or autolytic processes [12].

Materials and methods

Samples. Teeth were obtained from patients of oral surgeons ($n = 30$) and stored at room temperature for 6 weeks ($n = 20$) and 4 years ($n = 10$). Sterile gauze pads with the patients' blood obtained during dental treatment were used as references. From actual forensic identification cases (burned corpses; $n = 3$, putrefied bodies; $n = 2$ and high impact crashes; $n = 3$) teeth were extracted at autopsy, and blood or lung tissue was also obtained. Teeth ($n = 10$) were extracted from jaw fragments collected after an air accident in 1976 and which had been stored at room temperature for 15 years. Only information on the sex of the individuals was available in these cases.

Sample preparation and characterisation. The teeth were cut and the contents of the dental cavities were scraped out with a scaler. DNA extraction from dental pulp was performed using previously described methods for tissue specimens [11, 16, 26]. Ethanol-precipitated DNA was resuspended in 100 µl TE buffer. The DNA concentration was measured by fluorometry using Hoechst dye 33258 [15]. Electrophoresis was performed on a 0,9% agarose gel in TBE buffer (0.089 *M* TrisHCl, 0.089 *M* borate, 2 m*M* EDTA pH 8) at 1.5 V/cm.

Sex determination by dot hybridization. The DNA probe pHY 2.1 (DY Z 2; kindly provided by T. Cremer, Institut für Humangenetik, Universität Heidelberg) [8, 25] was labeled by nick translation with biotin-dUTP (Enzo, Neckargemünd, FRG) according to the manufacturer's instructions. Labeled probe was separated from the reaction mixture using spin columns with Sephadex G50 in 40 m*M* Tris-HCl, 1 m*M* EDTA, 0,1% SDS pH 8 (Sigma, München, FRG).

For hybridization, 3 µl of resuspended DNA in TE (containing about 50–100 ng DNA) were heated to 95°C, chilled on ice, dotted in duplicate on 2 different Immobilon N membranes (Millipore, Eschborn, FRG) and fixed to the filters for 1 h at 80°C. The membranes were prehybridized for 30 min at 65°C in 10 ml tubes containing SSCM [6 × SSC, 0,7% (w/v) non-fat milk (Glücksklee, Hamburg, FRG)] and hybridization was performed under high stringency conditions. The filters were incubated for at least 12 min at 72°C followed by an overnight hybridization at 42°C, but shorter hybridization times (4 h) were also found to be successful. After hybridization filters were rinsed with 2 × SSC containing 1% SDS (v/v) at room temperature, followed by a 10 min wash at 56°C in 2 × SSC, 0,1% SDS (v/v) and 2 × 10 min at 40°C in 2 × SSC 0,1% SDS (v/v) with slight agitation. Due to the stringency of the washing procedure it was necessary to incubate for 20 min at 45°C in SSCM to avoid background staining.

For detection the filters were briefly rinsed in 0.1 *M* Tris-HCl pH 7.5, 0.15 *M* NaCl, followed by incubation with streptavidin-alkaline-phosphatase-conjugate (1:1000) (Dakopatts, Hamburg, FRG) for 20 min at room temperature. The membranes were washed in 0.1 *M* Tris-HCl pH 7.5, 0.15 *M* NaCl. After a 5 min wash in 0.1 *M* Tris-HCl pH 9.5, 0.1 *M* NaCl, 0.005 *M* $MgCl_2$ the filters were applied to a staining gel containing 5-bromo-4-chloro-3-indolylphosphate (BCIP) as substrate according to Pflug [19]. After 1 hour the coloured dots were examined.

HLA-DQα typing. DNA amplification was performed by PCR according to the protocol of the supplier (Ampli Type™ HLA-DQα Kit, Perkin Elmer-Cetus, Emeryville, USA). The temperature program for all samples was denaturing at 90°C for 60 s, annealing at 55°C for 30 s and primer extension at 67°C for 30 s over 40 cycles. DNA was amplified in the presence of 5% formamide to avoid "drop out" of DQα alleles or total amplification failure [7].

Typing of the ApoB 3′ VNTR polymorphism. The extracted DNA samples were subjected to microdialysis (VSWP 02500 filter discs, Millipore, Eschborn, FRG) prior to PCR amplification. Aliquots containing 80–100 ng DNA were used as substrate for PCR. The DNA was amplified in a final volume of 100 µl containing 25 pmol of each primer (5′-ATG GAA ACG GAG AAA TTA TG-3′; 5′-CCT TCT CAC TTG GCA AAT AC-3′), 1.5 m*M* $MgCl_2$, 50 m*M* KCl. After an initial denaturation step of 4 min at 94°C, 2 U Taq polymerase (Boehringer, Mannheim, FRG) was added and 26 cycles with the following temperature program were carried out: 45 s denaturation at 94°C, annealing and extension at 58°C for 6 min according to Boerwinkle et al [2]. The amplified DNA sequences were separated by high resolution discontinous electrophoresis on polyacrylamide gels [1] followed by silver staining [23].

Southern blot analysis. Dental pulp and bloodstain DNA (2–5 µg) from selected samples were digested with the restriction enzyme Hinf I (New England Biolabs, Schwalbach, FRG) and separated in a 0.7% agarose gel in TBE buffer. Southern blotting, hybridization and radioactive detection of minisatellite polymorphisms with the ^{32}P-labelled DNA probes pYNH24 [18] and MZ 1.3 [24] were carried out under standard conditions as described [18, 24].

Results

In the present study the total yield of DNA obtained from a single tooth varied from 6 µg to 50 µg DNA. The major portion was degraded DNA, and a strong smear of low molecular weight nucleic acid was visible in the electrophorograms. The yield of DNA did not depend on the type of tooth, age or sex of the donor. There was no significant influence of storage conditions or time periods evident.

Filter hybridisation with biotinylated DYZ2 (pHY2.1) could detect about 20 pg of homologous DNA. When performing genetic dot hybridisation using 50–100 ng target DNA, the sex was correctly classified in all cases. Human female DNA gave no signal after one hour development with the BCIP-staining gel under the stringent experimental conditions described above. In some cases additional control experiments were performed as follows: after HaeIII digestion and agarose gel electrophoresis of male DNA a 2.12 Kb fragment could be detected [8, 25].

HLA-DQα typing could be performed in all cases and the results were in agreement with the genotype ob-

Table 1. Materials investigated

Material	Exposure conditions prior preparation of dental pulp	Reference samples
Teeth obtained from dental treatment ($n = 30$)	Room temperature 6 weeks ($n = 20$)	Bloodstains on gauze pads
Molars: $n = 18$ Premolars: $n = 12$	Room temperature 4 years ($n = 10$)	
Male: $n = 15$ Female: $n = 15$		
Teeth obtained from jaw fragments (airplane accident in 1976) (n = 10)	Room temperature 15 years ($n = 10$)	Forensic identification (1976)
Molars: $n = 4$ Premolars: $n = 3$ Incisors: $n = 3$		
Male: $n = 6$ Female: $n = 4$		
Teeth obtained from actual forensic identification cases ($n = 8$)	Burned corpses ($n = 3$)	Blood
	Putrefied bodies ($n = 2$)	Lung tissue
Molars: $n = 5$ Premolars: $n = 2$ Incisors: $n = 1$	High impact crashes ($n = 3$)	Blood
Male: $n = 6$ Female: $n = 2$		

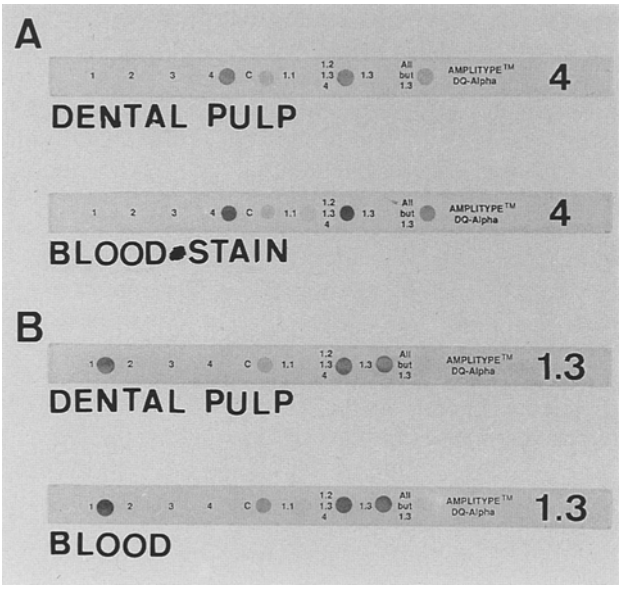

Fig. 1. A Comparison of the HLA-DQα genotype of PCR amplified genomic DNA from dental pulp and the result obtained from the corresponding bloodstain on gauze pad. Extracted tooth and bloodstain had been stored at room temperature for 4 years. **B** Results of HLA-DQα typing from dental pulp and blood. Postmortem extracted tooth (premolar) from forensic casework (high impact crash); blood taken at autopsy

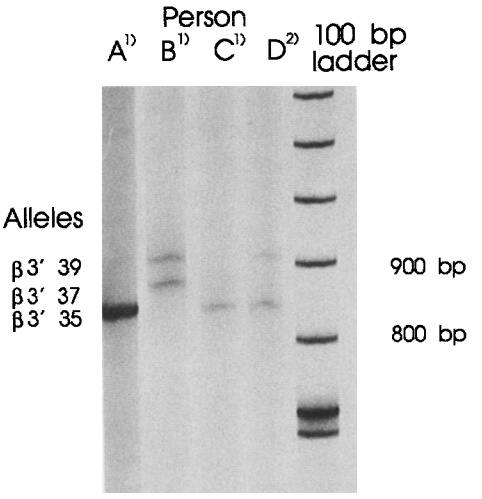

Fig. 2. Amplification products of the ApoB 3′ VNTR locus from genomic DNA of 15-year-old teeth. 1) incisor pulp 2) molar tooth pulp

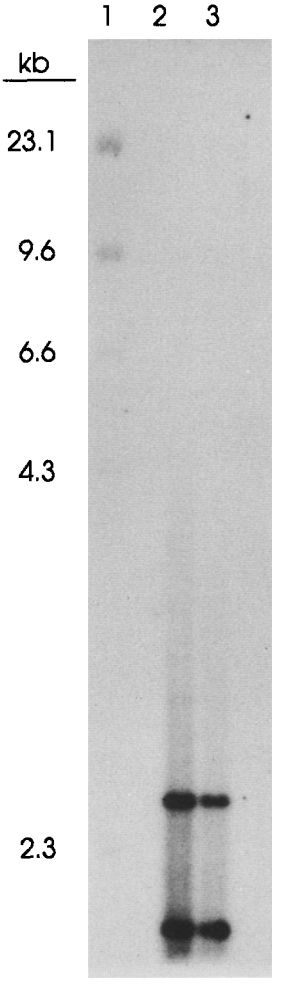

Fig. 3. Comparison of the pYNH24 fragment patterns from dental pulp *(lane 2)* and blood stain *(lane 3)* of the same individual. Molar tooth stored at room temperature for 4 years. *Lane 1*: molecular weight marker

tained from the bloodstain or lung tissue as far as available (Fig. 1). Apo B 3′ VNTR amplification was also carried out successfully. PCR results obtained from DNA extracted from dental pulp did not show any differences when compared with the band patterns obtained from

DNA isolated from corresponding bloodstains or lung tissue as far as available (Fig. 2). Southern blot analysis of selected samples of DNA recovered from dental pulp and bloodstains using the single locus probe pYNH24 was successfully performed (Fig. 3) while hybridization with the multilocus probe MZ1.3 failed in these cases (not shown).

Discussion

Dental pulp tissue is well protected in the pulp cavity as long as the teeth are firmly fixed to the alveolar bones. When teeth were extracted and stored at room temperature a rapid dehydration of pulp tissue was seen [20, 21]. This desiccation is considered to be an explanation for the surprisingly good integrity of the recovered DNA in most cases as necrotic and/or putrefactive processes are stopped as the tissue dries. Duffy et al. [11] fount that sex chromatins (both Barr bodies and F-bodies) are preserved in dehydrated human pulp for up to one year.

We observed that when the alveolar sockets are loosened and the microenvironment keeps the teeth moist pulp will putrefy but when the environment allows the pulp to mummify, DNA can be extracted and the quality is sufficient for PCR analysis and VNTR systems. It may even be applied to RFLP analysis using single locus probes.

DNA could be obtained from the pulp tissue of teeth in jaw fragments which had been stored for 15 years and sex was determined in all cases. These results indicate that the use of dental pulp as a source of DNA may solve problems of identification that occur in forensic case work. Thus sex determination from the skeletal remains of children or preadolescents, which is extremely difficult and uncertain, should be possible when dental pulp tissue is available [13]. For sex determination a simple hybridisation protocol such as a dot-blot assay can be used [14]. In this technique DNA samples are applied to filter supports without previous treatment. The DNA probe pHY2.1 hybridizes to repetitive DNA sequences from the distal long arm of the Y chromosome, its quinacrine bright region, which has previously proved useful for the detection of Y chromosomal material [8]. This probe hybridizes to about 2000 copies of the DNA sequence and can therefore be easily visualised. An additional 100–200 copies are also present in the female genome which should not be detected under the stringent hybridization and washing conditions as shown by in situ hybridization [6]. However when high concentrations of target DNA ($>1\,\mu g$) were used for dot hybridization, weak signals from female DNA could be observed. For forensic application, it seems appropriate to determine the quantity of extracted DNA prior to dot hybridization for sex determination.

Fluorometric or spectrophotometric methods often give an overestimation of the DNA yield. It would be desirable to have a method to quantitate the exact proportion of human genomic DNA in a forensic specimen of unknown composition (e.g. a mixture of fungal, bacterial and human DNA). Waye et al. [31] suggested a method using a dilution series in a slot blot assay and subsequent hybridization with a human-specific DNA probe. However this method does not seem to be appropriate for forensic case work since a considerable amount of stain material is used and is not available for further analysis e.g. for VNTR probes. In our experience, the dot blot hybridization for sex determination after fluorometry provides an estimate of the concentration of human genomic DNA. By limiting the amount of DNA used for dot blot analysis to a maximum of 50–100 ng it is possible to avoid false positive signals. We did not observe the effect that 50–100 ng degraded female DNA applied to the membrane gave a weak signal equivalent to the probe reacting with 100–200 copies of female DYZ2 DNA 2.1 fragments under the experimental conditions described above. The results demonstrate that an unknown amount of genomic DNA (less than 100 ng), as is often present in routine forensic case work, is suitable for sex typing by dot blot down to the single cell level (25 pg DNA) as shown by chromosomal in situ hybridization [6, 8]. This procedure is recommended as an additional simple intermediate control step for DNA obtained from a stain retrieved from a scene of crime.

Southern blot analysis of selected samples showing less degradation of the DNA obtained from dental pulp tissue after agarose gel electrophoresis was successfully performed using the single locus probe pYNH24. These findings confirm the study of Schwartz et al. [26], who recently reported an excellent success rate for RFLP analysis on DNA isolated from dental pulp tissue.

The use of PCR-based techniques is of increasing importance for post mortem DNA analysis in forensic case. Virtually any defined short DNA sequence can potentially be analysed easily and rapidly using the polymerase chain reaction (PCR) [5, 17]. In the present study we have shown that PCR-based analysis of the HLA-DQα and the ApoB 3′ VNTR loci can be performed successfully with DNA extracted from dental pulp tissue. The results were consistent with the genotype and band patterns obtained from blood or lung tissue. In addition the investigation of different genetic markers and the application of AMP-FLP systems [4] may help to identify a person in cases where identity cannot be determined by personal items, individual marks or by forensic odontological means. As it was found that dental pulp tissue provided valuable data even after long time periods, we conclude that the application of DNA-techniques may also be useful for potential identification of human remains, as long as relatives of the person in question (so-called reversed paternity testing) are still available.

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