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TECHNICAL NOTE

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Study of Short- and Long-Term Storage of Teeth and Its Influence on DNA

ABSTRACT: DNA degradation can interfere with the resolution of forensic cases. Allelic dropout often reduces the opportunity for adequate comparisons between degraded and reference samples. This study analyzed DNA degradation in 24 extracted teeth after storage at room temperature for 0, 2, 5, and 10 years. DNA concentration, quantified by dot-blot hybridization, declined significantly for the first 2 years, but there was no significant further degradation from the second to the tenth year of storage. COfiler™ analysis was used and the allelic dropout ratio for the amelogenin locus relative to CSF1PO locus was also estimated. Statistically significant differences were found between fresh teeth and teeth from the 2- and 5-year groups but not from the 10-year group. Under our storage conditions most of the DNA degradation occurred during the first 2 years. Further research is needed to control for individual and external factors that could affect DNA.

KEYWORDS: forensic science, forensic odontology, DNA degradation, short tandem repeat, CSF1PO, D16S539, D7S820, THO1, TPOX, D3S1358, amelogenin, teeth

Problematic samples containing only degraded DNA are an obstacle to the resolution of practical forensic cases that involve trace amounts of evidence, historical cases with a long postmortem interval, or recent items recovered under extreme ambient conditions at sites with hot and humid weather. Samples collected from degraded exhibits can offer limited or no opportunities for meaningful analysis or conclusions. Higher molecular weight markers fail to amplify and the reduced information content of partial short tandem repeat (STR) profiles results in lower discriminating power (1). Earlier work showed that artificial degradation of DNA by sonication and DNase I treatment led to relatively stable fragment lengths up to 200 bp, whereas longer DNA fragments, in general, could not be amplified, resulting in partial STR profiles (1–3).

DNA degradation can occur when samples have been exposed to light, humidity, elevated temperatures, and bacterial and fungal contamination (1–3). In addition, the length of the postmortem interval and the conditions the samples are exposed to during this time can affect the analyst's ability to recover typable DNA (4–6).

Because teeth are valuable sources of postmortem DNA evidence (7–10), the behavior of dental DNA during different postmortem intervals is an important consideration. The authors have been involved in cases in which antemortem dental data for presumptive victims were not available, and have turned to DNA analysis for identification purposes. But due to the extreme conditions of mass casualty incidents or decomposition in cases involving single found bodies, even the relatively protected DNA that is embedded in teeth can be affected over prolonged periods. This paper reports the results from extracted human teeth stored for four different periods (0, 2, 5, and 10 years) on the recovery of typable DNA.

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Materials and Methods

Twenty-four healthy teeth extracted from adult patients at the public Oral Health Service and private dental clinics in Granada (Spain) were studied. The teeth were extracted for valid clinical reasons (periodontal disease, malocclusion, or orthodontic treatment). Each tooth was donated to the authors by the dentists who treated the patients. The protocols for sample collection were approved by the appropriate Ethics Committee for Research Involving Human Subjects, and the study was conducted in accordance with the ethical standards set out by the Declaration of Helsinki.

Immediately after clinical extraction, any retained soft tissue or bone adhering to the tooth was removed. Each sample was air-dried; no chemicals were used to clean the teeth. The samples were divided into four groups consisting of six teeth each, which were stored at room temperature for different periods (0, 2, 5, and 10 years); thus, the samples were collected over a period of 10 years and processed for DNA at the same time. The sample set included 3 incisors (1 from the 0-year group and 2 from the 5-year group), 1 canine (from the 10-year group), 6 premolars (2 from the 2-year group and 4 from the 5-year group), and 14 molars (5 from the 0-year group, 4 from the 2-year group, and 5 from the 10-year group).

A high-speed surgical handpiece with a diamond burr was used to remove the enamel from each tooth, and the samples were individually decontaminated (11). They were washed with a 3% sodium hypochlorite solution for 5 min. They were then rinsed with sterilized water to remove any residual bleach and dried under a 256-nm ultraviolet light source (Philips TUV 30 W, Microzone Corp., Nepean, Ontario, Canada) for 10 min.

The whole teeth were pulverized after freezing with liquid nitrogen (SPEX CertiPrep® Freezer Mill, Stanmore, London, UK). The mass of each sample was recorded before and after pulverization (data not shown). The dental powder was transferred to 1.5-mL conical tubes, demineralized with 0.5 M EDTA, pH 8.0, plus 35 μL 10% (wt/vol) sodium dodecylsulfate, and 100 μL proteinase

K (20 mg/mL) to lyse the cell and nucleus walls and to denature proteins. Samples were incubated at 37°C for 12–18 h. After this step, 50 µL proteinase K (20 mg/mL) was added, and the samples were incubated again at 37°C for 2 h and then centrifuged. Each sample was extracted three times with phenol/chloroform/isoamyl alcohol (25:24:1). The upper aqueous layer was transferred to a 1.5-mL tube and extracted once with chloroform/isoamyl alcohol (24:1), then washed with distilled water three times in a Centricon-100 concentrator (Amicon, Millipore, Toronto, Ontario, Canada) and concentrated with 1x Tris–EDTA, pH 8.0, to a final volume of 100 µL. The samples were stored at −20°C pending further analysis.

DNA in the samples was quantified by dot-blot hybridization with the D17Z1 alpha-satellite probe using the Quantiblot Kit (Applied Biosystems, Roche®, Foster City, CA), according to the manufacturer's recommendations. The intensity of the signal of each sample band was measured by transillumination (AC1 Auto Darkroom, UVP BioImagining Systems, Upland, CA), and DNA concentrations were calculated with LabWorks v. 4.6 software (UVP BioImagining Systems).

COfiler™ analysis (Applied Biosystems) was done with the kit reagents and protocols, and with 0.5 ng target DNA in a 50-µL reaction volume for each sample (9600 thermal cycler; Perkin-Elmer Corporation, Foster City, CA) as recommended in the AmpF*l*STR COfiler Plus PCR Amplification user's manual (12). A negative control (negative extraction) and an internal control were analyzed in parallel with each set of extractions.

Peak height and area were quantified with GeneScan® software (Applied Biosystems). We calculated peak area dropout ratio as the percentage of allelic dropout of the smallest locus (amelogenin, 106–112 bp) relative to the largest one (CSF1PO, 280–316 bp). When the marker was heterozygous, the mean for the two alleles was calculated to correct for possible imbalances between peaks. The same procedure was used to calculate peak height dropout values.

Data were exported to an Excel (Microsoft Corporation, Redmond, WA) spreadsheet and statistical analyses were performed with SPSS/PC+ software (SPSS Inc., Chicago, IL). Statistical significance was assessed by one-way analysis of variance (ANOVA). Standard error of the mean was calculated as the estimated standard deviation of the error. For significant F values *post hoc* Tukey honestly significant difference (HSD) analysis was performed.

Results and Discussion

Although much is known about the mechanism of cell death by apoptosis, little is known about the cellular degradation that follows death and putrefaction. Most studies of DNA degradation and postmortem interval have focused on the period immediately after death from a few hours up to several days (4–6). In the current study of DNA degradation, both the quantity and quality of DNA from extracted human teeth were analyzed over a period of 0–10 years.

We were able to quantify DNA in most of the samples (87.5%); however, no DNA was obtained from three samples: two teeth from the 5-year storage interval group and one tooth from the 10-year storage interval. Figure 1 shows the mean DNA concentrations according to storage interval. As expected, the highest concentration was found in teeth stored for the shortest period. Statistical analysis with one-way ANOVA showed a significant difference in DNA concentrations according to elapsed storage interval (sum of the squared deviations = 4291.499; 3 d.f.; $p \leq 0.01$). The Tukey HSD test revealed statistically significant differences ($p < 0.05$) between DNA concentration in fresh teeth (0 years) and in the other groups (2, 5, and 10 years). No significant differences were found between the 2-, 5-, and 10-year groups. The concentration of DNA declined significantly for the first 2 years; thereafter we found no significant decline in the mean concentration of DNA between 2 and 10 years.

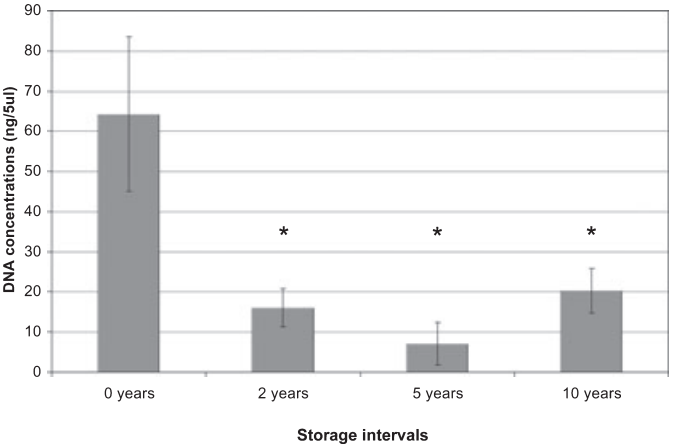

FIG. 1—*Mean values of DNA concentration and standard error of the mean bars for different storage intervals. Pairwise comparisons among mean with one-way analysis of variance showed statistically significant differences between fresh (0-year) teeth and teeth from 2-, 5-, and 10-year storage intervals;* *p < *0.05 compared to the 0-year group.*

Smaller amplicons are much more likely to amplify than larger ones in samples containing degraded DNA, since the STR loci with the largest amplicons in a multiplex amplification are the first to drop out of the DNA profile (1,13–15). To analyze DNA fragmentation we amplified 0.5 ng DNA from each tooth and analyzed it as described above. Of the entire sample, 82% of the loci were successfully typed. As expected, higher molecular weight STR loci failed to amplify more frequently. In 21% of the cases, the CSF1PO, D16S539, and D7S820 loci yielded no results; THO1, TPOX, and D3S1358 failed in about 17%, whereas the amelogenin locus failed in only 13% of the cases.

Figure 2 shows the mean dropout ratios of peak area for the amelogenin loci relative to the CSF1PO loci after different storage

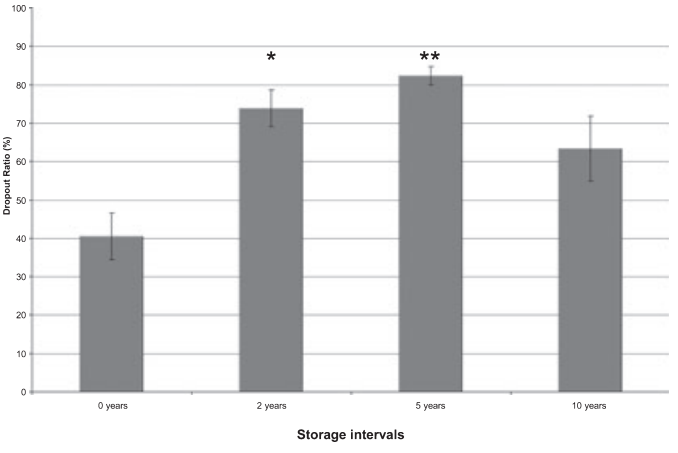

FIG. 2—*Mean values and standard error of the mean bars of the dropout ratio of peak areas for different storage intervals. The dropout ratio of the peak areas was calculated as the percentage of allelic dropout of the smallest locus (amelogenin) relative to the largest one (CSF1PO). Pairwise comparisons among mean with one-way analysis of variance showed statistically significant differences between fresh (0-year) teeth and teeth from 2- and 5-year storage intervals;* *p < *0.05 compared to the 0-year group and* **p < *0.01 compared to the 0-year group.*

intervals. Fresh teeth showed a dropout ratio of around 40%; this percentage could indicate that DNA degradation occurs during a very early period of time. The dropout ratio was 33% higher in the 2-year group, 42% higher in the 5-year group, and 23% higher in the 10-year group. Comparison of the results with one-factor ANOVA disclosed statistically significant differences (sum of the squares 4325.058; 3 d.f.; $p < 0.01$). The Tukey HSD test revealed differences in the dropout ratio between the fresh teeth group and the 2- and 5-year storage interval groups. No statistically significant differences in dropout were found between fresh teeth and those from the 10-year storage interval. Similar results were found when height peak dropouts were compared (results not shown).

The quantity and quality of DNA that can be obtained from teeth depend on several external factors, such as storage temperature, degree of humidity, and time between death and examination, and on individual factors such as type of teeth, pathological conditions, dental treatments, and pulp weight (1–8). It is difficult to control for all the conditions that can affect DNA degradation, and testing all these variables would require the examination of an enormous number of samples.

The results of DNA analysis can be influenced by the type of tooth involved (7). As expected in healthy teeth, molars and premolars yield more DNA due to the number of roots and the increasing size of the pulp chamber in distal positions. Although we did not compare DNA concentrations between different types of teeth, our samples were not significantly heterogeneous: 20 of the 24 teeth were molars or premolars and the 4 anterior teeth were distributed in three of the four storage interval groups. Moreover, we found similar DNA concentrations between anterior teeth (mean = 26.38 ng/5 μL) and posterior teeth (mean = 25.95 ng/5 μL) although this result should be considered with caution due to the sample size.

The decontamination procedure used to treat the samples is another factor that can alter DNA degradation. Of the many techniques available to decontaminate, we used a three-step procedure: physical removal of the enamel, washing with bleach, and exposure to ultraviolet light. All samples were decontaminated in the same way; however, we did not investigate whether this procedure might have affected aged teeth more than fresh ones.

For this study, we analyzed healthy teeth free of disease or previous treatments; accordingly, our results were not influenced by these factors. All samples were stored at room temperature, so the potential effect of extreme environmental conditions such as high humidity or temperature were not investigated. In that regard, our methods do not represent the full suite of environmental conditions that can ensue after a person's death.

Short tandem repeat genotyping, due to its high discriminating power, is the analytical method of choice for forensic applications. The poor quality or low quantity of extracted nuclear DNA in samples of forensic interest, however, often precludes successful STR genotyping, resulting in partial or unsuccessful STR profiles. The successful analysis of such samples would benefit from methods able to determine not only the amount of DNA, but also the presence and degree of DNA degradation. In this study, we show that most of the DNA degradation took place during the first 2 years of storage, although these results should be viewed with caution because of individual and external factors that might affect DNA. Future experiments with shorter sampling intervals within the first 2 years may be able to more precisely characterize the changes during this early period when most of the degradation occurs.

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