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Procedures to recover DNA from pre-molar and molar teeth of decomposed cadavers with different post-mortem intervals

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ABSTRACT

A task-force to resolve 26 pending forensic caseworks was carried out. We tested four different protocols to extract DNA from molar and pre-molar teeth from 26 cadavers with post-mortem intervals from 2 months to 12 years. We compared the amount of DNA and DNA profiles with the time elapsed between death and laboratory procedures. Molar or pre-molar teeth were removed from the corpses, cleaned, and DNA was extracted using 2 or 12 h of incubation on lysis buffer and filtered using concentration column or precipitated with isopropanol. DNA profiles were obtained using PowerPlex16™ System PCR Amplification Kit, AmpFlSTR® Yfiler™ and/or mtDNA sequencing. Complete DNA profiles comparison and statistical evaluation allowed unambiguous identification of the 26 victims. No significant differences were observed in the amount of DNA obtained with the distinct incubation times. The use of concentration column resulted in an increased amount of DNA when compared to isopropanol. However, the lower concentration of DNA obtained with isopropanol seemed to have been compensated by the higher purity. No significant differences in the number of amplified *loci* were found. A non-significant tendency was found between the amount of total DNA recovered and the time elapsed between death and laboratory procedures. The increase of post-mortem time did not interfere in the analysed autosomal *loci*. In conclusion, molar and pre-molar teeth were shown to be good candidates to obtain satisfactory DNA profiles, suggesting the high potential of tooth samples as source for DNA typing independently of the decomposed corpse's time or laboratory procedures.

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1. Introduction

Forensic identification of victims is essential for humanitarian reasons, but also for civil or criminal investigations. Identification of a corpse is essentially based on anthropology, odontology, fingerprints, radiology, and/or DNA typing.[1] However, it can be complicated when the corpse is old, completely destroyed from mass disaster or putrefactive, skeletonized, drowned, or burned. In these cases, identification is usually difficult[1–3] since the elements used by pathologists, anthropologists and/or odontologists (such as fingerprints, sexual characteristics, physical constitution, ethnic group, stature and/or dental arch) can be modified by degradation, hampering a conclusive result. Given this scenario, forensic specialists looking for better preserved

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tissues to obtain DNA with good quality and amount[4,5] have turned to DNA analysis.[6–9]

An excellent alternative is the use of cells from inside molar and pre-molar teeth. Regarding the molar and pre-molar mineralized inert structure, size, and location,[4,10–14] they preserve cells with high molecular weight DNA for longer periods even when the body is in an advanced state of decomposition.[15,16] The dental enamel makes them resistant to adverse circumstances that could degrade the DNA, such as humidity, high temperature, or the action of fungi and bacteria.[17] Such degradations and contaminations increase with the corpse decay and with post-mortem time span.

Four different protocols were tested to recover DNA from pre-molar and molar teeth from 26 cadavers in bad decomposition stages with different post-mortem intervals. We compared the amount of DNA obtained and the DNA profiles with the time elapsed between death and laboratory procedures.

2. Materials and methods

2.1. *Questioned samples and DNA extraction*

Forensic Laboratory of the Department of Legal Medicine of Instituto-Geral de Perícias (Rio Grande do Sul, Brazil) received 26 questioned samples that were unidentified due their advanced stage of decomposition. A task-force with the objective to resolve these 26 pending caseworks was carried out.

Molar or premolar teeth were removed from corpses, cleaned with sterilized water only (we did not use abrasive, bleach, sandpaper, nor any mechanisms of deep cleaning), and stored for at least 24 h at −80 °C (SANYO UltraFreezer, Tokio, Japan). For each evaluated corpse data were recorded regarding subject's age and sex, corpse condition, local where the corpses were found, and estimated post-mortem time.

The four different protocols used to extract DNA are demonstrated in Fig. 1. Each tooth was grinded using IKA Works A11 Basic Analytical Mill (IKA® Processing Equipment), and the resulting powder was weighed in a precision balance (Adventure™; AR3130, OHAUS® Corp. pine Brook, NJ, USA) and separated into four 2 ml tubes. Around 0.6 g (max = 1.02 g; min = 0.34 g) of teeth powder was used for each DNA extraction. DNA was extracted using 600 μl of lysis buffer [100 mM NaCl, 10 mM EDTA (ethylenediaminetetraacetic acid), 2% SDS (sodium dodecyl sulphate), 10 mM Tris–HCl (pH 8), 24 μl of 20 mg/ml proteinase K (Invitrogen, Carlsbad, USA), and 48 μl of 1 M DTT (dithiotreitol; Invitrogen, Carlsbad, CA, USA)]. Samples were incubated at 56 °C for 2 or 12 h. For precipitation, 700 μl of UltraPure™ (phenol:chloroform:isoamyl alcohol, 25:24:1, Invitrogen, Carlsbad, CA, USA] were added, vortexed, and centrifuged for 7 min at 15,000 × *g*. The upper aqueous layer was placed inside a Microcon™-100 concentrator (Millipore, Beverly, MA, USA) and centrifuged at 500 × *g* until only a few micro-liters remained. Microcon™-100 filtering was repeated twice by adding 400 μl of DNA-free H_2O. Fifty micro liters of DNA-free H_2O was added, the columns were inverted, and the kept was collected by centrifugation at 1000 × *g* for 3 min. The final sample was transferred into a new micro-centrifuge tube and stored at −20 °C. Alternatively, the upper aqueous layer was precipitated by adding an equal volume of isopropanol, centrifuged at 7000 × *g* for 7 min, and washed twice with 70% ethanol, centrifuged, and dried in a dry bath at 95 °C for 5 min followed by proteinase K inactivation at 95 °C for 5 min. Extracted DNA was re-suspended in a volume of 50 μl of DNA-free H_2O and stored at −20 °C.

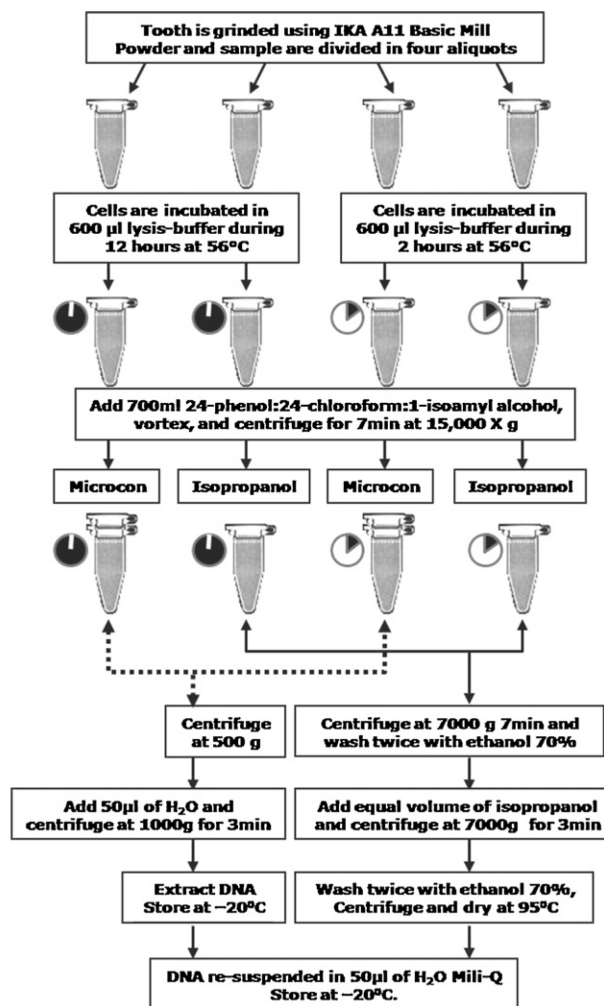

Fig. 1 – Four different protocols to extract DNA from molar and pre-molar teeth.

2.2. *DNA quantification*

DNA concentration was measured with GeneQuant *pro* spectrophotometer (Amersham Biosciences, Cambridge, England) at λ = 260 nm from 10 μl of total DNA volume. Purity of DNA was assessed using the ratio of OD260 nm/280 nm.

2.3. *PCR amplification conditions*

Samples were amplified using ABI 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). STR genotyping was performed using PowerPlex16™ System PCR Amplification Kit (nDNA; Promega Corporation, Madison, WI, USA) version

3.1. Y-STR genotyping was conducted using AmpFlSTR® Yfiler™ Amplification Kit (Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction (PCR) was carried out according to the manufacturer's instructions. Fluorescence detection of genotypes was performed with ABI Prism® 3100-Avant Genetic Analyzer and by using Data Collection v.2.0, and Gene Mapper ID v.3.2 analysis software (Applied Biosystems, Foster City, USA). For mtDNA analysis we amplified HV1 (Primer L15996: 5′-CTC CAC CAT TAG CAC CCA AAG C-3′; Primer H16401: 5′-TGA TTT CAC GGA GGA TGG TG-3′) and HV2 (Primer L29: 5′-GGT CTA TCA CCC TAT TAA CCA C-3′; Primer H389: 5′-CTG GTT AGG CTG GTG TTA GG-3′) regions. Considering that nucleotide positions within the mtDNA are numbered from 1 to 16,569 using the L-strand from control region, HV1 region spans positions 16,024 to 16,365 (342pb) and HV2 covers positions 73 to 340 (268pb). PCR products of mtDNA were purified from residual primers with Exonuclease I (EXO I; Amersham Biosciences - E70073Z; GE HealthCare©) and Shrimp Alkaline Phosphatase (SAP; Amersham Biosciences - E7009; GE HealthCare©), and sequenced directly by cycle sequencing. Hyper variable segments were sequenced with BigDye Terminator Cycle Sequencing kit from Applied Biosystems on ABI PRISM™ 3100-Avant DNA sequencer. ABI PRISM™ 3100-Avant Genetic Analyzer was used for separation and detection of the fluorescence-labelled chain termination products. The sequences of mtDNA were manually checked using CHROMAS[18] and aligned with CLUSTAL-X.[19]

2.4. *Reference samples and ethics approval*

DNA profiles obtained from the teeth of the deceased were compared to the DNA profiles of reference samples obtained from close relatives. Relatives' genomic DNA was extracted from leucocytes by a standard method.[20] DNA analyses of relatives were performed as described above.

This project was approved by the Research Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (Tel. +55 51 33203345; protocol #1107/05) and the consent or assent to take part in this study was obtained from Forensic Laboratory of Instituto-Geral de Perícias of Rio Grande do Sul, Brazil.

Table 1 – Data of 26 investigated victims.

Case number	Tested tooth	Subject age	Subject sex	Corpse condition	Local where corpse was found	Post-mortem time[a]
1	PM	nr	M	Putrefactive	Under ground	12y1m
2	ML	nr	M	Putrefactive	Under ground	5y7m
3	PM	nr	M	Putrefactive	Under ground	5y3m
4	PM	22	M	Skeletonized	Outside	4y7m
5	PM	44	M	Drowned	River	3y
6	ML	43	M	Putrefactive	Woodland area	3y8m
7	ML	43	M	Putrefactive	Outside	3y1m
8	ML	51	M	Putrefactive	Outside	2y1m
9	ML	16	M	Skeletonized	Woodland area	7y
10	ML	44	M	Burned	Outside	1y9m
11	PM	51	M	Putrefactive	River	1y8m
12	ML	33	M	Putrefactive	Woodland area	2y
13	PM	33	M	Putrefactive	Under ground	1y6m
14	PM	51	M	Skeletonized	Under ground	3y4m
15	ML	48	M	Putrefactive	Under ground	2m
16	PM	46	M	Putrefactive	River	2y3m
17	ML	32	M	Putrefactive	River	2y3m
18	PM	nr	M	Putrefactive	River	1y1m
19	ML	26	M	Putrefactive	River bank	5m
20	ML	50	M	Skeletonized	Woodland area	1y10m
21	ML	56	M	Drowned	River bank	2y4m
22	PM	59	M	Putrefactive	Under ground	3y3m
23	PM	23	M	Putrefactive	Outside	6m
24	ML	22	M	Putrefactive	River bank	4m
25	ML	18	M	Skeletonized	Under ground	4m
26	ML	23	F	Putrefactive	Edge of river	3m
Total	*n* (%)	M ± SD	*n* (%)	*n* (%)	*n* (%)	M ± SD
	ML: 15(58) PL: 11(42)	38 ± 13	M: 25(96) F: 1(4)	Burn.: 1(4) Drow.: 2(8) Putr.: 18(69) Skel.: 5(19)	Edge: 1(4) Out.: 5(19) River: 5(19) RBank: 3(12) Under: 8(31) Wood.: 4(15)	2y7m ± 2y6m

PM: premolar tooth; ML: molar tooth; nr: not reported; *n*: number of cases; %: percent of cases; M: mean; SD: standard deviation.
[a] Time estimated in years (y) and months (m) between the Person's disappearance and the DNA analysis.

2.5. Statistics

Allele identification was carried out with Gene Mapper ID software version 3.2 using ABI Prism 3100 from Applied Biosystems. Comparisons between the results obtained from the different protocols were examined using ANOVA or ANOVA followed by Student Newman's Keul *Post Hoc* and Pearson's correlation (SPSS software package for Windows version 13.0; SPSS, Inc.). A *P* value of <0.05 was considered to be statistically significant. DNA profiles and statistical evaluation were used to identify the victims. A probability of relationship between questioned and reference samples were calculated using the avuncular index and PATCAN software (M.T. Zarrabeitia and J.A. Riancho, from the University of Cantabria, Santander, Spain).

3. Results

Descriptive data about tested teeth, subject's age and sex, corpse condition, local where the corpses were found, and the estimated post-mortem time of human subjects are shown in Table 1.

Looking for alternative protocols, we tested cell incubation during 2 or 12 h and DNA precipitation using isopropanol or Microcon^{TM}-100 procedure. Table 2 shows that no significant differences were observed in the amount of DNA obtained with the distinct incubation times. However, Microcon^{TM}-100 extraction resulted in an increased amount of DNA when compared to isopropanol (12 h: Micr = 9.2 ± 3.8 *vs.* Isop = 2.6 ± 2.7; 2 h: Micr = 10.4 ± 4.5 *vs.* Isop = 2.7 ± 2.3; $P < 0.001$). We also noticed that isopropanol precipitation resulted in higher DNA purity (measured by 260/280 Optic Density fraction) in comparison to Microcon^{TM}-100 (12 h: Micr = 1.42 ± 0.35 *vs.* Isop = 1.72 ± 0.31; 2 h: Micr = 1.39 ± 0.38 *vs.* Isop = 1.71 ± 0.21; $P < 0.001$) (Table 2).

DNA profiles comparison and statistical evaluation allowed the correct identification of the 26 victims. Regarding the four different DNA extraction protocols, no significant differences in the number of amplified *loci* were found ($P > 0.05$) (Table 3). Twenty samples (20/26; 77%) comprised sufficient human DNA to obtain good quality autosomal DNA profiles. In the six remaining cases, Y-STR genotyping or mtDNA identification had to be performed (Table 3). Mitochondrial DNA amplification was performed in four cases and the nucleotide sequences were

Table 2 – Comparison between results obtained from four DNA extraction protocols.

Case	μgDNA/gTeeth				OD 260 nm/280 nm			
	12 h/MicrI	12 h/Isop II	2 h/Micr III	2 h/Isop IV	12 h/Micr I	12 h/Isop II	2 h/Micr III	2 h/Isop IV
1	3.272	2.707	6.347	2.304	1.013	1.478	1.036	1.263
2	4.523	0.887	11.063	0.950	1.089	1.500	1.078	1.375
3	10.525	1.014	5.589	1.166	1.377	2.000	1.672	1.857
4	12.110	1.827	6.466	1.776	1.642	2.050	1.868	1.952
5	10.326	1.239	11.580	1.395	1.411	2.500	1.340	1.762
6	8.051	0.737	8.648	1.092	1.328	1.733	1.403	1.591
7	7.645	2.566	5.505	2.129	1.651	2.148	1.718	2.091
8	8.079	4.857	10.898	2.005	1.660	1.120	1.710	1.667
9	13.773	1.087	17.694	1.091	0.961	1.471	0.947	1.333
10	9.583	1.615	23.689	1.625	1.610	1.812	1.000	1.850
11	6.349	1.078	6.226	4.045	1.479	1.714	1.506	1.907
12	10.524	9.639	8.154	8.061	1.444	1.659	1.529	1.806
13	9.302	1.517	7.660	1.211	1.151	1.722	1.065	1.438
14	12.371	1.955	15.049	2.234	1.407	1.049	1.278	1.487
15	7.009	3.636	11.714	2.269	1.813	1.700	1.426	1.844
16	1.260	0.781	10.875	1.250	1.609	1.625	1.489	1.750
17	13.378	1.178	5.529	3.699	1.579	1.909	1.673	2.000
18	7.916	1.507	8.631	0.831	1.491	1.667	1.504	1.667
19	11.762	0.972	12.564	1.092	1.540	1.786	1.451	1.857
20	8.506	3.445	8.349	4.824	1.355	1.467	1.315	1.673
21	11.388	2.695	10.009	1.970	1.414	1.871	1.500	1.714
22	16.909	12.322	18.009	10.248	1.399	1.658	1.038	1.711
23	16.023	1.257	10.861	1.943	1.694	2.167	1.687	1.910
24	3.904	1.294	5.639	1.653	1.780	1.652	1.764	1.600
25	9.327	3.137	14.751	3.138	1.374	1.718	1.353	1.676
26	5.801	2.749	8.873	5.455	1.638	1.588	1.645	1.588
Mean	9.216	2.604	10.399	2.671	1.421	1.722	1.386	1.707
SD	3.789	2.702	4.496	2.272	0.350	0.306	0.375	0.208
	I *vs.* II	I *vs.* III	II *vs.* IV	III *vs.* IV	I *vs.* II	I *vs.* III	II *vs.* IV	III *vs.* IV
P	<0.001*	0.228	0.732	<0.001*	0.001*	0.603	0.700	<0.001*

12 h/Micr: 12 h lysis-buffer incubation and Microcon^{TM}-100 concentration; 12 h/Isop: 12 h lysis-buffer incubation and isopropanol concentration; 2 h/Micr: 2 h lysis-buffer incubation and Microcon^{TM}-100 concentration; 2 h/Isop: 2 h lysis-buffer incubation and isopropanol concentration.

* Significant differences (Wilcoxon Test) between 12 h/Micr *vs.* 12 h/Isop and between 2 h/Micr *vs.* 2 h/Iso. SD: standard deviation.

Table 3 – DNA profiles of the 26 samples.

	DNA profiles											
	Number of autosomal *loci* analysed				Number of Y Cromosome *loci* analysed				HV1342/HV22268 regions of mtDNA			
	12 h/ Micr I	12 h/ Isop II	2 h/ Micr III	2 h/ Isop IV	12 h/ Micr	12 h/ Isop	2 h/ Micr	2 h/ Isop	12 h/ Micr	12 h/ Isop	2 h/ Micr	2 h/ Isop
1	10	15	13	12	–	–	–	–	–	–	–	–
2	11	4	15	4	–	–	–	–	–	–	–	–
3	14	14	14	13	–	–	–	–	–	–	–	–
4	9	6	3	0	–	–	–	–	Yes	–	–	–
5	14	6	0	10	17	–	17	–	–	–	–	–
6	15	14	11	13	–	–	–	–	–	–	–	–
7	15	0	15	0	–	–	–	–	–	–	–	–
8	2	0	0	4	–	–	–	–	–	–	–	Yes
9	0	6	0	8	–	–	–	–	–	Yes	–	–
10	14	10	0	14	–	–	–	–	–	–	–	–
11	15	15	15	7	–	–	–	–	–	–	–	–
12	0	0	15	15	–	–	–	–	–	–	–	–
13	9	5	1	5	–	–	–	–	–	–	Yes	–
14	15	0	15	11	–	–	–	–	–	–	–	–
15	15	15	15	15	–	–	–	–	–	–	–	–
16	15	15	15	11	–	–	–	–	–	–	–	–
17	15	15	15	15	–	–	–	–	–	–	–	–
18	11	7	13	3	–	–	–	–	–	–	–	–
19	0	0	15	6	–	–	–	–	–	–	–	–
20	15	15	15	15	–	–	–	–	–	–	–	–
21	14	5	15	14	–	–	–	–	–	–	–	–
22	14	15	15	15	–	–	–	–	–	–	–	–
23	15	15	15	15	–	–	–	–	–	–	–	–
24	3	9	0	0	17	17	17	17	–	–	–	–
25	15	12	3	9	–	–	–	–	–	–	–	–
26	15	15	15	15	–	–	–	–	–	–	–	–
Mean	11.15	8.96	10.31	9.58								
SD	5.43	5.92	6.52	5.30								
	I *vs.* II	I *vs.* III	II *vs.* IV	III *vs.* IV								
P	0.059	0.556	0.513	0.652								

Autosomal DNA fragments analysed by PowerPlex16™ System; YDNA: Y-Chromosome STR analysed by AmpFlSTR®Yfiler™; mtDNA: mitochondrial DNA analysed by Big Dye Terminator; SD: standard deviation. Statistical analysis was performed using Wilcoxon Test.

consistent with those from the reference samples obtained from presumed maternal relatives (data not shown). In all 26 cases, electropherograms of autosomal and Y-STR DNA typing showed no ambiguous peak, and no mixed DNA was observed. Despite tooth nature, subject's age or sex, corpse condition, location, or post-mortem time, the complete DNA profiles obtained from the molar and pre-molar teeth were able to be compared with the DNA profiles from reference samples.

A perceptible but not significant tendency was found between the quantity of total DNA recovered and the time elapsed after death (Fig. 2). Interestingly, the increase of the post-mortem interval did not interfere in the amount of autosomal *loci* analysed (Fig. 3).

4. Discussion and conclusion

Although literature shows that some highly decomposed bodies have been successfully identified with DNA from malleable tissue, the time of corpse decomposition has been a complicating factor because DNA samples are damaged.[21] Dental pulps, in contrast, are more protected against damage. Malaver and Yunis[17] suggested that as the amount of DNA in teeth was below the limits of detection with a Quantiblot kit, it would be important to develop new protocols to accurately recover DNA from teeth in forensic caseworks. In this study, we tested different protocols to recover DNA from molar and pre-molar teeth of cadavers in bad decomposition stages with different post-mortem intervals. We were able to obtain DNA profiles from the questioned samples and to compare them with reference samples.

No significant differences were observed in the total quantity of DNA obtained in the procedures with distinct incubation times showing that short cell lysis time can be used in urgent genetic identification with good quality results.

The use of concentration column (Microcon™-100) resulted in an increased amount of DNA when compared to isopropanol. However, the lower concentration of DNA obtained by precipitation with isopropanol seemed to have been compensated by the higher purity, since the measurement by optic density fraction was higher and because no significant differences in the number of amplified *loci* were found between these protocols.

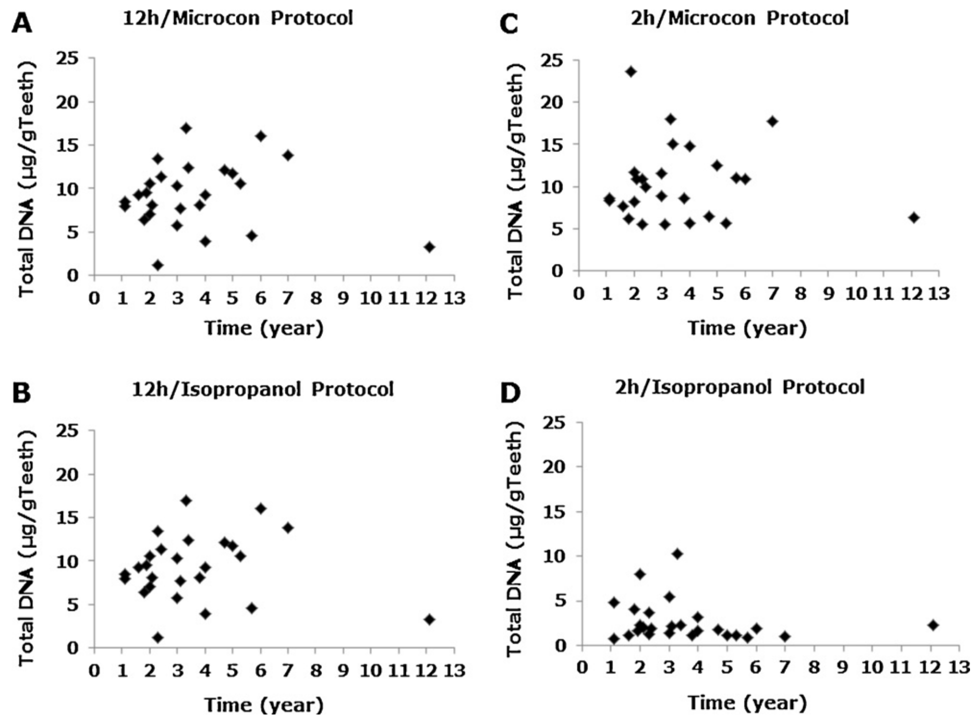

Fig. 2 – Amount of total DNA recovered using four DNA extraction protocols and time elapsed after the death of 26 corpses. (A) 12 h lysis-buffer incubation and Microcon™-100 concentration; (B) 12 h lysis-buffer incubation and isopropanol concentration; (C) 2 h lysis-buffer incubation and Microcon™-100 concentration; (D) 2 h lysis-buffer incubation and isopropanol concentration.

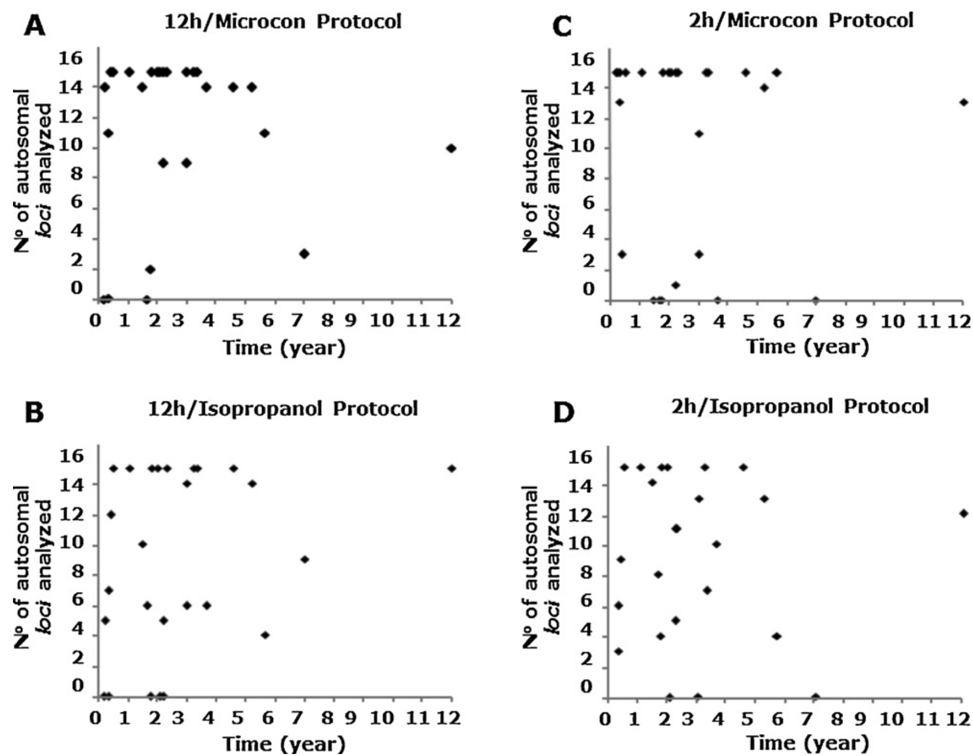

Fig. 3 – Autosomal *loci* analysed after DNA extraction by four different protocols and time elapsed after the death of 26 corpses. (A) 12 h lysis-buffer incubation and Microcon™-100 concentration; (B) 12 h lysis-buffer incubation and isopropanol concentration; (C) 2 h lysis-buffer incubation and Microcon™-100 concentration; (D) 2 h lysis-buffer incubation and isopropanol concentration; *Statistically significant.

Isopropanol was, in fact, very effective in DNA precipitation, as it has already been reported,[22] besides being low-priced.

Six samples had an apparent good amount of total DNA but resulted in poor autosomal profiles. It was probably due to unsatisfactory quality of DNA by degradation or reduced DNA quantity by microorganism DNA contamination.[23] To verify this, specific human DNA quantification by Real Time PCR analysis would be necessary.[24]

We compared the DNA amount and the DNA profiles with the time elapsed between death and laboratory procedures, but the increase of post-mortem interval did not interfere in any of these variables.

In conclusion, our work showed molar or pre-molar teeth as good candidates to obtain satisfactory DNA profiles suggesting the high potential of tooth samples as a source to DNA typing independently of the decomposed corpse's time or laboratory procedures.

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This study was financed by SENASP-IGP-RS. The study is part of the Master's Degree thesis of the first author who had a fellowship from PUCRS, Brazil.

Competing interests

We declare that we have no conflict of interest.

Ethical approval

This project was approved by the Research Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS-code #1107/05; Tel.: +55 51 33203345), and the consent or assent to take part in this study was obtained from Forensic Laboratory of Instituto-Geral de Perícias of Rio Grande do Sul, Brazil.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2012.08.014.

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