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Effect of environmental factors on PCR-DNA analysis from dental pulp

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Abstract This study was designed to observe the results of DNA typing on teeth subjected to aging, different temperatures and various environmental factors. A total of 570 teeth were studied. The study included the analysis of the PCR-based polymorphisms HLA DQA1, D1S80, HUMTH01, HUMFES/FPS and the XY homologous gene amelogenin. In general the best results were obtained with the XY homologous gene amelogenin, followed by the two STRs studied (HUMTH01 and HUMFES/FPS). The small fragment sizes and the method of detection used after PCR amplification are the main factors explaining this fact. In general, teeth submerged in water gave the poorest results. Teeth exposed to outdoor conditions provided better results than teeth buried in sand or soil, but even in these cases good results were obtained. Up to 4° C, temperature had only a slight influence on the results. Positive results were obtained in most cases at high temperatures (400° C for 2 min) which are rarely reached in practical casework. Positive typing results for the XY homologous gene amelogenin and the STRs were obtained from teeth 10–30 years old. The usefulness of dental pulp for identification purposes is exemplified in some actual cases.

Key words DNA polymorphisms · PCR · Dental pulp · Identification

Introduction

Forensic odontological analysis is a necessary approach for individual identification in situations such as skeletal remains, burnt bodies or victims of mass disasters. Since the hard dental tissue physically encloses the pulp, which is itself a high cellular density tissue, teeth provide a suitable source of DNA for the purpose of forensic identification. Prior to DNA typing, individual dental identification was usually carried out by comparing dentition and antemortem records of the deceased. Physical identification has the limitation that the antemortem records are not always available. Although variable number of tandem repeat polymorphisms (VNTR) are highly informative, their analysis as restriction fragment length polymorphisms (RFLPs) has many limitations in typing forensic material which has highly degraded DNA. The analysis of dental remains has been greatly facilitated by the introduction of the polymerase chain reaction (PCR) for DNA typing. The use of PCR-based polymorphisms (HLA DQA1 and 3′ApoB) for dental identification using human dental pulp was proposed by Pötsch et al. (1992). Methodologies for access to the tooth interior and subsequent DNA extraction were systematically investigated by Smith et al. (1993). The effect of environmental conditions on DNA typing using single locus probes (SLPs) was studied by Schwartz et al. (1991), but a systematic study of these enviromental effects on PCR-based polymorphisms was clearly needed.

This study was designed to observe the results of DNA typing on teeth subjected to aging, different temperatures and various environmental factors. The study included the analysis of DNA polymorphisms amplified by PCR, such as HLA DQA1, D1S80, two STRs (HUMTH01 and HUMFES/FPS) and sex typing (XY homologous gene amelogenin).

Material and methods

Samples

Teeth were obtained from patients of oral surgeons (n = 570). The samples were rinsed with distilled water, air dried and inmediately frozen at –20° C (after extraction) until being exposed to the experimental conditions. Another six teeth which had been extracted 10–30 years previously were also obtained. In addition, four teeth were obtained from forensic casework. All of the teeth were molars and premolars. Control samples were obtained from all the donors for all the samples except for the forensic casework samples. In each case six teeth were examined for the study of the different environmental factors.

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Conditions of exposure

To examine the effect of temperature 198 teeth were removed from the freezer and mantained at 4° C, 20° C and 40° C for periods ranging from 2 weeks to 36 months. To examine the effect of immersion in water 96 teeth were submerged in the sea and in a river for periods ranging from 15 days to 6 months. A total of 72 teeth were buried outdoors in a garden and in a sand dune (Pontevedra). They were placed approximately 20 cm under the ground for periods of 2 weeks to 6 months.

For outdoors air exposure 36 teeth were exposed to open air for periods varying from 2 weeks to 6 months. For incineration experiments we incinerated 168 teeth by placing them in a dental ceramic furnace (Programat P95, Ivoclar) for 1 and 2 min at 75° C, 100° C, 200° C, 300° C, 400° C, and 500° C. At 100° C and 200° C an extra 5 and 10 min was added.

For aging (old samples) experiments six teeth were examined which had been kept at room temperature for 10–30 years. The forensic casework samples were three teeth from cremated bodies and one from an exhumed body (buried for more than 50 years).

Samples preparation and DNA extraction

After removing teeth from experimental conditions of exposure, they were rinsed with distilled water and air dried. Access to dental pulp was performed using three different methods (Smith et al. 1993): most of the teeth were crushed whole and the others were either opened by conventional endodontic access or cut by a transversal section. Dental pulp was retrieved using a fine forceps and resuspended in water. DNA extraction was then performed using a chelating resin (Singer-Sam et al. 1989).

DNA was quantified using the DNA DipStick kit (Invitrogen Corp.).

Primer sequences

HLA DQA1 (Saiki et al. 1989)
5′GTGCTGCAGGTGTAAACTTGTACCAG3′
5′CACGGATCCGGTAGCAGCGGTAGAGTTG3′

D1S80 (Kasai et al. 1990)
5′GAAACTGGCCTCCAAACACTGCCCGCCG3′
5′GTCTTGTTGGAGATGCACGTGCCCCTTGC3′

HUMTH01 (Edwards et al. 1991)
*5′GTGGGCTGAAAAGCTCCCGATTAT3′
5′ATTCAAAGGGTATCTGGGTCTTGG3′

HUMFES/FPS (Polymeropoulos et al. 1991)
*5′GGGATTTCCCTATGGATTGG3′
5′GCGAAAGAATGAGACTACAT3′

X Y homologous gene amelogenin (Sullivan et al. 1993)
*5′CCCTGGGCTCTGTAAAGAATAGTG3′
5′ATCAGAGCTTAAACTGGGAAGCTG3′

(* Primers 5′-end labeled with fluorescent dye)

Oligonucleotides were synthesized following the phosphoramidite method in a 380A DNA synthesizer and purified through an OPC column (Applied Biosystems, Foster City, Calif.).

Amplification conditions

PCR amplification of the systems was performed using 10 µl (usually from 2–5 ng) of the extracted DNA solution in a 25 µl reaction volume following the previously described conditions for D1S80, HLA DQA1 (Lareu et al. 1993), HUMTH01, HUMFES/FPS (Pestoni et al. 1995), XY homologous gene amelogenin (Sullivan et al. 1993).

Detection systems

Non-denaturing polyacrylamide gel electrophoresis and silver staining were used for visualisation of D1S80 system (Lareu et al. 1993). HLA DQA1 alleles were analysed using the reverse dot-blot method with the HLA DQa AmpliType kit (Perkin/Cetus) (Lareu et al 1993). HUMTH01, HUMFES/FPS and XY homologous gene amelogenin was typed using the ALF DNA sequencer under the electrophoretic conditions described by Pestoni et al. (1995).

Results

The results of the markers (HLA DQA1, D1S80, HUMTH01, HUMFES/FPS and XY homologous gene amelogenin) are summarized in Tables 1–3.

Table 1 Results of HLA DQA1 and D1S80 systems under different conditions of exposure and time periods

Time	4° C	20° C	40° C	Conditions of exposure				
				fresh water	seawater	outdoors	sand buried	soil buried
HLA DQA1								
2 weeks	6/6	6/6	6/6	3/6	6/6	6/6	6/6	5/6
1 month	5/6	6/6	6/6	1/6	1/6	6/6	4/6	5/6
3 months	5/6	6/6	6/6	1/6	0/6	4/6	3/6	4/66
6 months	4/6	5/6	5/6	1/6	1/6	4/6	2/6	2/6
12 months	6/6	5/6	5/6					
24 months	6/6	6/6	6/6					
36 months	5/6	6/6	6/6					
D1S80								
3 months	6/6	6/6	6/6	2/6	2/6			
6 months				1/6	1/6	3/6	2/6	3/6
12 months	6/6	5/6	4/6					
36 months	5/6	4/6	3/6					
Total number of teeth	60	60	60	36	36	30	30	30

positive results/sample number

Table 2 Results of HUMTH01, HUMFES/FPS and XY homologous gene amelogenin loci under different conditions of exposure and time periods

Time	4° C	20° C	40° C	Conditions of exposure				
				fresh water	seawater	outdoors	sand buried	soil buried
HUMTH01								
3 months				4/6	4/6			
6 months				2/6	2/6	5/6	4/6	3/6
36 months	6/6	6/6	6/6					
HUMFES/FPS								
3 months				4/6	6/6			
6 months				3/6	2/6	5/6	5/6	3/6
36 months	6/6	6/6	6/6					
XY Amelogenin								
3 months				6/6	6/6			
6 months				6/6	6/6	6/6	6/6	6/6
36 months	6/6	6/6	6/6					
Total number of teeth	6	6	6	12	12	6	6	6

positive results/sample number

Table 3 Results of the PCR markers analysed after incineration with the temperatures and time used (*The same 36 teeth were used to analyse these three systems; **24 teeth were used to analyse all the systems: 6 teeth for each: 100° C–5 min; 100° C–10 min; 200° C–5 min; 200° C–10 min)

Temp	HLA DQA1		D1S80	HUMTH01	HUMFES/FPS	XY Amelogenin
	1 min	2 min	2 min	2 min	2 min	2 min
75° C	6/6	6/6	6/6	6/6	6/6	6/6
100° C	6/6	5/6	6/6	6/6	6/6	6/6
200° C	6/6	6/6	5/6	6/6	6/6	6/6
300° C	6/6	5/6	1/6	6/6	5/6	6/6
400° C	6/6	2/6	0/6	4/6	4/6	6/6
500° C	2/6	0/6	0/6	0/6	0/6	5/6
Total number of teeth	36	36	36		36*	

Temp	HLA DQA1		D1S80		HUMTH01		HUMFES/FPS		XY Amelogenin	
	5 min	10 min	5 min	10 min	5 min	10 min	5 min	10 min	5 min	10 min
100° C	6/6	5/6	6/6	3/6	6/6	6/6	6/6	6/6	6/6	6/6
200° C	6/6	1/6	4/6	0/6	6/6	3/6	6/6	2/6	6/6	4/6
Total number of teeth					24**					

positive results/sample number

Temperature and aging

During the testing period of 36 months, positive results were obtained in the majority of cases at temperatures of 4°, 20° and 40° C. Positive results were obtained for all samples in the two STRs studied and the XY homologous gene amelogenin. Results were also obtained for HLA DQA1 and D1S80 (94% and 66% respectively).

Water

Nearly 50% of samples gave positive results for the STRs in samples submerged in water. Positive results were obtained in 12% for HLA DQA1, 25% for D1S80 and 100% for the XY homologous gene amelogenin for submersion periods of 3 and 6 months.

Outdoors

Excellent results were obtained for the XY homologous gene amelogenin and the STRs after six months of outdoor exposure and slightly worse results for HLA DQA1 and D1S80 systems.

Burial

An average of 50% positive results were obtained for the 6-month-old buried samples using all the systems except for the XY homologous gene amelogenin, which had 100% positive results.

Incinerated teeth

The results of the incinerated teeth are shown in Table 3 from a sample of 144 teeth of temperatures ranging from 75 to 500° C, with two different times (1 and 2 min) for HLA DQA1 system, and 24 teeth covering 100 and 200° C at 5 and 10 min. At 100° C for 10 min good results were obtained (87%). When the temperatures were increased (200° C – 10 min) results become significantly poorer (33%). With the exception of the XY homologous gene amelogenin, completely negative results were obtained after exposure at 500° C for 2 min. The STRs gave clearly better results than HLA DQA1 which have better results than D1S80 (only 1/6 at 300° C).

Old samples

Teeth ranging from 10 to 30 years old were studied and 100% positive results were obtained for all the systems with the exception of D1S80 where only 50% positive results were observed.

Forensic casework samples

Positive results were obtained in 100% of the forensic casework for the XY homologous gene amelogenin and the STRs (HUMTH01 and HUMFES/FPS). HLA DQA1 and D1S80 failed in the body exhumed after approximately 50 years.

In addition, three special cases were examined, one tooth with an endodontic treatment, one tooth after removal of the whole dental pulp in the laboratory and one sample which consisted of only 1 mm of the apex of the tooth. These samples were maintained at 20° C for 36 months. Positive results were obtained for all three cases using the HLA DQA1 analysis.

Discussion

Although most of the teeth were crushed whole, similar amounts of DNA were recovered and similar results obtained using the two other methods (conventional endodontic access and transversal section). The method of choice for DNA extraction in teeth is in our opinion the transversal section since the anatomy of the teeth is preserved. This could be of importance for further studies.

In general, there were no significant differences between the three temperatures studied (4°, 20°, 40° C) over the different time periods, and the results were similar for all three temperatures with the exception of the system D1S80 which had progressively worse results when the temperature increased (5/6, 4/6 and 3/6 after 36 months of exposure at 4°, 20° and 40° C respectively).

No significant differences were obtained when studying the effects of seawater and fresh water on the DNA typing on the teeth. In general teeth submerged in water offer the poorest results of all series. Two explanations can be given for this effect. Firstly the amount of zooplankton in seawater and freshwater in Galicia. A more likely reason is the dilution effect of water itself, which increases the rate of DNA hydrolysis compared to incubation in air or soil.

Teeth exposed outdoors gave better results than buried teeth, but even in this case, an average of 50% positive results were obtained in the 6-month-old samples. Slightly better results were obtained with STR typing.

No significant differences were noted between teeth buried in sand or soil. In general quite good results were obtained (more than 50% positive results for the two STRs after 6 months). Positive results for all the markers were obtained for an unidentified body which had been buried for more than 50 years. Better results are expected from buried bodies than in the experimental cases due to the fact that the apical foramen is not protected by the periodontal ligament and the alveolar bone in experimental cases and, therefore, it is more easily reached by bacteria and other agents.

With the exception of the XY homologous gene amelogenin, completely negative results were obtained after exposure of the teeth at 500° C for 2 min. If the exposure time to high temperatures is reduced (1 min) clearly better results were obtained. Our experience is that in real casework a temperature over 200° C is seldom obtained. At 100° C for 10 min the results were good, and therefore one can expect success in casework analysing STRs from dental pulp.

The samples from casework from three unidentified cremated bodies were also studied. All the markers used in this study gave positive results. Teeth protection by muscular and skeletal structures and the dental enamel are the main factors explaining these results.

Non-homogeneity of some results (Table 1 – better results for HLA DQA1 typing after 36 months than after 3 or 6 months) could be explained by other factors such as the age of the person, the kind of teeth and the previous state of the dental pulp. These factors were not considered since an extremely high number of samples is required to obtain reliable results.

Regarding the type of genetic maker, the best results were obtained with the XY homologous gene amelogenin followed by the two STRs studied (HUMTH01 and HUMFES/FPS).

Taking into account the 120 samples commonly used for all the markers, the amelogenin gave positive results in 117, FES/FPS in 93, TH01 in 91, HLA DQA1 in 70 and D1S80 in 57. These differences show a statistical significance ($P < 0.01$) between amelogenin and STRs and STRs over D1S80. The small fragment sizes of the systems (Fig. 1) together with the sensitivity of the method of detection used after PCR amplification (fluorescent detection) are the main factors explaining this fact.

Teeth offer a good source of DNA for the identification of old skeletal remains. Positive results were obtained in 100% for the amelogenin gene and the STRs in teeth ranging from 10 to 30 years old and good results for these

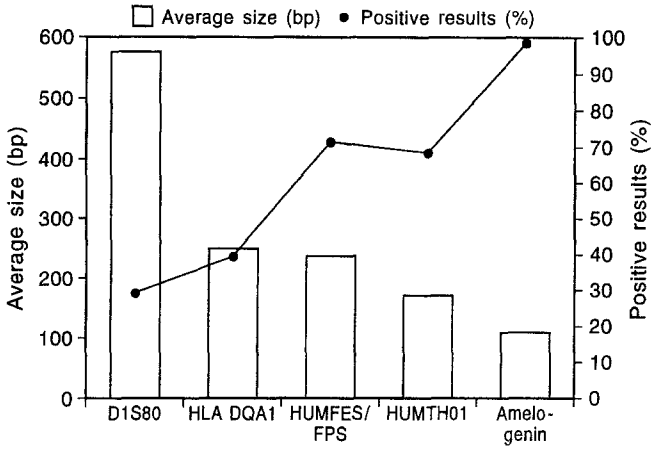

Fig. 1 Correlation between the average size of each PCR system used in this study and positive results (%)

markers were also obtained from a body buried for more than 50 years.

In conclusion dental pulp is a highly useful tissue in DNA typing for the purpose of identification. PCR-amplified DNA polymorphisms can be analysed in the dental pulp of teeth subjected to adverse environmental conditions. The size of the amplified fragment is critical. STRs therefore have advantages over AMP-FLPs and SLPs when DNA typing is performed in teeth for the purpose of forensic casework.

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