TECHNICAL NOTE

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Comparison of Three DNA Extraction Methods on Bone and Blood Stains up to 43 Years Old and Amplification of Three Different Gene Sequences

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ABSTRACT: Extraction of amplifiable DNA from degraded human material in the forensic context remains a problem, and maximization of yield and elimination of inhibitors of the Polymerase Chain Reaction (PCR) are important issues which rarely feature in comparative studies. The present work used PCR amplification of three DNA sequences (HLA DPB1, amelogenin and mitochondrial) to assess the efficiency of three methods for extracting DNA (sodium acetate, magnetic beads and glass-milk) from 32 skeletal samples and 25 blood stains up to 43 years old. The results, analyzed using multivariate statistics, confirmed that the extraction method was crucial to the subsequent detection of amplification products; the glass-milk protocol performed better than sodium acetate, which was better than magnetic beads. Successful amplification also depended on gene sequence, multiple copy mitochondrial sequences performing best; however, with the singly copy sequences, the longer HLA DPB1 (327 bp) being detected just as often as the shorter amelogenin (106/112 bp). Amplification products were obtained more frequently from blood stains than bone, perhaps reflecting differences inherent in the material, and from younger compared with older specimens, though plateauing seemed to occur after 10 years. PCR inhibitors were more frequent in sodium acetate extracts.

KEYWORDS: forensic science, DNA extraction, glass-milk, polymerase chain reaction, HLA, amelogenin, mitochondrial DNA, bone, bloodstains, MANOVA

The role of DNA in the identification of human remains in forensic and historical cases has increased with the discovery and standardization of extremely polymorphic genetic systems (1). However, in spite of recent advances in molecular biology, the extraction of DNA, particularly from old and degraded material such as bone, remains a problem, and maximization of yield and elimination of inhibitors of the Polymerase Chain Reaction (PCR) are among the most important aspects of this work which still need to be considered. Reports of amplification and sequencing of DNA from ancient remains (2–4) are particularly interesting in view of the difficulties frequently encountered when extracting DNA from material which is only decades old (even though some notable successes have been reported (5–7)). If one assumes that DNA is a fairly robust molecule and that relatively short sequences can survive under appropriate conditions, the method used for its extraction could be crucial. Experience accumulated from almost a decade of using PCR suggests that the method of extraction could affect both the quantity of genetic material obtained and also the quality of the extract.

Relatively few studies have been performed that formally compare different extraction procedures. Preliminary experiments, which examined the relative efficiency of Chelex, phenol-chloroform and non-organic extraction of bloodstains (8,9), showed that all methods seemed reliable and apparently gave satisfactory results. A similar investigation, which compared the phenol-chloroform and Chelex techniques, concluded that although Chelex was simple and fast, inhibitory substances were not eliminated (10). In the case of bone samples, the efficiency of water elution (11), boiling, rapid lysis, standard organic extraction and prior decalcification were assessed (12–14); again, all methods appeared to give satisfactory results.

A recent study on forensic bone showed that, as judged by amplification of selected sequences of the HLA DRB1 gene, acetate extraction was possibly better than phenol-chloroform: amplification products were obtained from 2 forensic specimens (3 and 9 months postmortem) with the former method, but from neither specimen when extracted using a standard and carefully performed phenol-chloroform technique (15). Newer, and apparently satisfactory, protocols have been developed for specific archaeological situations; for example, the use of silica based techniques for ancient bones and insects embedded in amber (3,16). Several investigations have been performed on teeth (17) under the assumption that the pulp chamber was likely to be a better environment for cellular DNA survival than bone tissue (and therefore an easier target for DNA extraction): the results gave some support for this view. A few investigations have also assessed the type of

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informative DNA surviving in blood stains and bones of different ages under different environmental conditions. It was shown that restriction fragment length polymorphisms (RFLP) and PCR products could be retrieved from blood stains exposed to ultraviolet light, humidity, soil contamination and heat (37°C) for up to five days (18); from compact bone buried in soil or immersed in water for up to 3 months, from bone from 11-year-old mummified remains (12); and from tooth pulp kept at various temperatures and under different soil conditions and pH values (17). Short tandem repeats (STR) and amelogenin sequences were used successfully to identify victims of the Waco mass disaster, though here the DNA was extracted from bone marrow (and muscle) rather than from dry bone (19,20).

In the present study, the efficiency of three methods for extracting DNA has been compared using multivariate statistical procedures. The methods were precipitation with sodium acetate (15), the commercial 'Dynabeads DNA Direct' kit (Dynal AS, Oslo) and the use of silica particles (glass-milk) (3). A series of 32 bone samples and 25 blood stains ranging in age from one week to 43 years was studied. PCR was used to amplify three short sequences of DNA from the HLA DPB1 locus (327 bp), the amelogenin gene (106/112 bp) and the mitochondrial genome (120 bp); these were chosen as they show variation in length and copy number and are of forensic and anthropological interest. The quantity and purity of DNA in each extract were assessed spectrophotometrically to determine whether there was a correlation between these measurements and successful amplification. Finally, extracts which failed to give amplifiable DNA were tested for the presence of PCR inhibitors.

Materials and Methods

A series of 32 bone samples was collected. Six fresh femoral heads (specimens 1–6) were obtained from 3 male and 3 female patients undergoing hip replacement surgery at the Northern General Hospital in Sheffield. Four vertebral bodies from forensic cases (specimens 7–10) were examined 3 to 6 years postmortem and came from the skeletal remains of 4 male individuals found on the outskirts of Milan between 1989 and 1991 and studied at the Medico-Legal Institute in Milan; they had been stored refrigerated at 4°C. Twenty-two vertebral bodies (specimens 11–32) were 13–43 years old and came from 17 males and 5 females who had died between 1952 and 1982 and had been interred; following exhumation in the early 1990's, the skeletons were kept at ambient temperature for 5 years before the specimens were collected and stored at −80°C.

The 25 blood stains were made on cloth/cotton gauze and were roughly 3 cm in diameter. Eleven one-week-old (5 male and 6 female) and 7 five-year-old stains (3 male and 4 female) were prepared at the Trent Regional Blood Transfusion Centre (these were specimens 33–43 and 44–50, respectively); a further 7 blood stains (specimens 51–57), ranging in age from 11 to 26 years (4 male and 3 female), were taken from a collection routinely prepared from murder victims at the Medico-Legal Institute in Milan. The blood stains were stored under clean, dry conditions at room temperature. All the specimens were obtained with patients' or Magistrates' consent or according to Police Mortuary Regulations and after Ethical Committee approval for the work had been granted.

DNA Extraction

Approximately 1 cm^3 of powdered bone (prepared by pulverization in liquid nitrogen) or 1 cm^2 of shredded blood stained cloth from each specimen were subjected to the three extraction methods.

Sodium Acetate

The protocol used here has been previously described in detail (15). Briefly, the sample was incubated with 3 mL of extraction buffer (10 mM Na_2EDTA, 50 mN NaCl), with the addition of 25 μL of proteinase K (20 mg/mL) and 50 μL of 10% sodium dodecyl sulfate (SDS), overnight in a water bath at 42°C. One mL of saturated sodium acetate was added and the tube shaken manually for 30 s and then centrifuged at 4000 grams for 10 min. Four mL of isopropanol were added to the supernatant, mixed and the tube centrifuged again. The pellet remaining, consisting of DNA, was washed in 70% ethanol, dried and reconstituted in sterile distilled water.

Dynabeads DNA Direct Kit

The sample was continuously mixed in a polypropylene tube containing 3 mL of White Cell Lysis Buffer (10 mM Tris-HCl [pH 7.6], 10 mM Na_2EDTA, 50 mM NaCl) for 3–4 h; 15 μL of 0.5% Nonidet P-40 (Sigma, UK) and 50 μL of Proteinase K (20 mg/mL) were added prior to overnight incubation in a water bath at 42°C. 200 μL of magnetic beads from the kit were added and the samples incubated at room temperature for 5–10 min before being subjected to magnetic force for 90–120 s. The supernatant was discarded and the bead/DNA complex washed twice with 200 μL of the supplied wash buffer before being resuspended in 60 μL of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM Na_2EDTA) and incubated at 65°C for 5 min in order to separate the complex. The supernatant was immediately collected and stored at −20°C until needed.

Glass-milk

The sample was incubated in 1 mL of extraction buffer (4 M Guanidine thiocyanate. 0.05 M Tris-HCL pH 7.6, 0.01 M EDTA, 1% Triton X-100) at 60°C for 1 h before centrifuging at 4000 g for 5 min, 500 μL of supernatant were added to 500 μL of extraction buffer and 40 μL of silica suspension (12 g washed silica, 2 mL sterile filtered distilled water, 120 μL of 10 M HCl), and rotated for 10 min at room temperature, centrifuged at 4000 g for 15 min, washed twice with wash buffer (extraction buffer with 150 μL of sterile filtered distilled water added), twice with 70% ethanol and centrifuged at 4000 g for 5 min. The resulting silica pellet was dried for 10 min at 56°C, resuspended in 65 μL of sterile filtered distilled water and left at 56°C with occasional mixing; the suspension was centrifuged at 4000 g for 3 min and the supernatant transferred to a clean microcentrifuge tube. The remaining silica pellet was resuspended, centrifuged for a second time and the supernatant removed and pooled with the previous one.

The DNA solution (i.e., the pooled supernatants) was finally centrifuged at 6000 grams for 5 min and the supernatant removed and stored at −20°C.

Amplification

The extracts were subjected to PCR amplification for three gene systems (HLA DPB1, amelogenin and a 120 bp region of mitochondrial DNA) in a 'Tempcycler' PCR machine (MJ Research, USA).

HLA DPB1

PCR was performed using primers specific for the whole of the second exon of the HLA DPB1 gene (21); the sequences of the

primers were: DPB AMP-A: GAC AGT GGC GCC TCC GCT CAT and DPB AMP-B: GCC GGC CCA AAG CCC TCA CTC. The reaction mixture for each amplification contained 9.6 μL of sterile filtered distilled water, 2 μL of PCR standard reaction buffer (670 mM Tris-HCl (pH 8.8), 166 mM NH_4SO_4, 0.1% Tween), 1.5 μL of $MgCl_2 \cdot 6H_2O$ (20 mM), 1 μL of dNTPs (4 mM), 0.4 μL of each sequence specific primer (25 μM) and 0.1 μL of Taq polymerase (5U/μL, Advanced Biotechnologies). To this mix, 5 μL of DNA extracts or controls were added making a final volume of 20 μL per reaction. The reaction commenced with an initial step at 90°C for 5 min followed by 35 cycles of 1 min at 95°C, 30s at 60°C, and 1 min at 72°C with a final step of 72°C for 5 min. The products were separated by electrophoresis in 25 mL of 1.5% agarose gel (Seakem) containing 10 μL of Ethidium Bromide (1 mg/mL), at 95 Volts for 15 min. The 327 bp amplified product was identified under UV light by comparison with a molecular weight marker (pUC BM21 *Hpa* II, *Dra* I, and *Hind* III). The gel was photographed with a Polaroid camera.

Amelogenin

Five μL of DNA extract were added to 5 μL aliquots of PCR reaction mixture (39 μL of sterile filtered distilled water, 10 μL of dNTPs (4 mM), 10 μL of $MgCl_2$ (20 mM), 20 μL of ×10 ABIV buffer, 1 μL of Taq polymerase (5U/μL) and 10 μL of each primer (10 μM) for a sequence within the first intron of the amelogenin gene (22)). The sequences of the primers were Amel-A: CCC TGG GCT CTG TAA AGA ATA GTG and Amel-B: ATC AGA GCT TAA ACT GGG AAG CTG. PCR commenced with an initial step at 94°C for 2 min followed by 10 cycles of 94°C for 10 s and 65°C for 1 min and then 25 cycles of 94°C for 10 s, 62°C for 40 s, and 72°C for 30 s. The polymorphic products of 106 bp and 112 bp derived from the X and Y chromosomes, respectively, were separated by gel electrophoresis at 100 V for 90 min in 25 mL of 5% agarose gel stained with 15 μL of Ethidium Bromide (1 mg/mL). The number of distinct bands was visualized with UV light and photographed: males gave 2 bands and females 1 band.

Mitochondrial DNA

PCR was performed employing the same reaction mixture and controls as for HLA DPB1 amplification, but the oligonucleotide primers were used to amplify a 120 bp fragment from region V of mitochondrial DNA (4); they were: Mit-A: ATG CTA AGT TAG CTT TAC AG and Mit-B: ACA GTT TCA TGC CCA TCG TC. The reaction profile consisted of an initial step of 5 min at 95°C followed by 35 cycles of 95°C for 1 min; 57°C for 1 min and 72° for 1 min; the final step was at 72° for 5 min. The products were demonstrated by UV visualization after electrophoretic separation on 1.5% agarose as for HLA DPB1.

Controls

All the work was performed following standard procedures to avoid contamination, such as appropriate washing and sterilization of crushing devices, the use of positive displacement pipettes and the inclusion of positive and negative control samples with each test run. Mock extractions were carried out in parallel: for the sodium acetate procedure, this involved bovine bone, while for Dynabeads, sterile filtered distilled water took the place of the test sample. Glass-milk extractions were repeated without test material, and also on 20 human bone specimens which had been severely burnt. As a further negative control, sterile filtered distilled water blanks were included with each PCR. Positive controls for HLA DPB1 and mitochondrial sequences consisted of a series of DNA samples extracted from human blood ranging in concentration from 32-356 ng/μL; for amelogenin, extracts from known males and females were used.

Test for PCR Inhibitors

Samples where amplification was unsuccessful were tested for PCR inhibition by performing another reaction with a combination of the positive control and the extract (2.5 μL of each). A negative result would confirm the presence of an inhibitor in the extract, whereas a positive result, although not excluding an inhibitor (it would depend on relative quantities of positive control DNA and DNA inhibitors), would suggest absence or degradation of the sequence.

Quality Measures of Extracts

The quantity and purity (quality) of DNA in each extract was assessed using a spectrophotometer (GeneQuant II, Pharmacia Biotest, Cambridge). The absorbance (A) of 20 μL of extract in 980 μL of sterile filtered distilled water was measured at 260, 280, and 320 nm, the reference sample being 1000 μL of sterile filtered distilled water. At A_{260}, one optical density unit corresponds to approximately 50 μg/mL of double stranded DNA, whereas the ratio A_{260}/A_{280} provides an estimate of the purity of the nucleic acid; pure preparations of DNA have a ratio of approximately 1.8, which is significantly reduced by contamination with protein or phenol (23). The protein content in each extract in mg/mL was given by the relation $1.55 \times (A_{280}-A_{320}) - 0.76 \times (A_{260}-A_{320})$ (GeneQuant II user manual).

Statistical Methods

Basic statistical data (means, medians, and standard errors) were derived. Multivariate Analysis of Variance (MANOVA) was carried out to the examine the effects of extraction method, the 3 gene sequences, specimen group (bone or blood stains), specimen age and extract quality measures on the amplification results. For the analysis, specimen age was categorized into 4 groups: fresh, 1–10 years, 11–20 years, and >20 years. In addition, the effects of extraction procedure and extract quality measures on the presence of PCR inhibitors were considered. Where MANOVA was employed, the contributions made by individual parameters to overall significance levels were examined by univariate (ANOVA) procedures. An SPSS for Windows (Version 6.1) statistical package, implemented on an IBM-compatible PC microcomputer, was used for all analyses; statistical significance was set *a priori* at the 0.05 level.

Results

Details of specimen age, sex, and results of PCR amplification of HLA DPB1, amelogenin, and mitochondrial DNA sequences, and presence of PCR inhibitors for each extraction method are shown in Table 1. The corresponding values for the quality measures of the extracts are given in Table 2. The mock extraction controls consistently gave negative results, the sterile filtered distilled water blanks never showed amplification products and the sex determined by the amelogenin sequences corresponded to the recorded sex of the individual in every case.

TABLE 1—*Comparison of sodium acetate, magnetic beads, and glass-milk DNA extraction procedures: results of PCR amplification of HLA-DPB1, amelogenin and mitochondrial sequences and of PCR inhibitor studies.**

Specimen Group and Number	Age of Specimen in Years†	Sex	HLA-DPB1			Amelogenin‡			Mitochondrial DNA		
			Sodium Acetate	Magnetic Beads	Glass-Milk	Sodium Acetate	Magnetic Beads	Glass-Milk	Sodium Acetate	Magnetic Beads	Glass-Milk
Bone											
1	0	F	+	+	+	F	F	F	+	+	+
2	0	F	+	+	+	F	–	F	+	+	+
3	0	F	+	+	+	F	F	F	+	+	+
4	0	M	+	+	+	M	M	M	+	+	+
5	0	M	+	+	+	M	M	M	+	+	+
6	0	M	+	+	+	M	M	M	+	+	+
7	3	M	inh	–	–	–	–	–	+	–	+
8	3	M	+	–	+	–	–	M	+	–	+
9	4	M	+	+	+	M	inh	–	+	+	+
10	6	M	inh	inh	+	inh	inh	M	+	–	+
11	13	M	inh	–	–	inh	–	–	inh	–	+
12	13	M	–	–	–	inh	inh	–	–	–	+
13	13	M	+	+	+	inh	–	–	–	+	+
14	13	F	inh	–	–	inh	inh	F	inh	+	+
15	13	F	–	–	–	inh	–	–	+	+	+
16	14	F	inh	–	inh	inh	–	–	inh	–	+
17	15	M	–	–	–	inh	–	–	–	+	+
18	15	F	–	–	–	inh	–	F	+	–	+
19	16	M	–	+	–	inh	–	inh	+	+	+
20	16	M	–	–	inh	inh	–	–	+	+	+
21	16	M	–	inh	inh	inh	inh	–	+	–	+
22	16	M	–	–	–	–	–	–	+	+	+
23	16	M	+	–	+	–	–	–	+	+	+
24	16	M	inh	–	–	inh	inh	–	–	–	+
25	16	M	inh	–	+	inh	–	–	–	+	+
26	16	F	inh	–	–	inh	–	F	inh	–	+
27	17	M	–	+	–	–	–	–	+	+	+
28	34	M	–	–	–	–	–	–	+	+	+
29	39	M	–	–	inh	inh	–	–	+	+	+
30	40	M	–	–	inh	inh	–	–	–	+	+
31	42	M	–	–	–	inh	–	–	+	+	+
32	43	M	inh	inh	–	inh	–	–	inh	+	+

TABLE 1—*Continued.*

Specimen Group and Number	Age of Specimen in Years†	Sex	HLA-DPB1			Amelogenin‡			Mitochondrial DNA		
			Sodium Acetate	Magnetic Beads	Glass-Milk	Sodium Acetate	Magnetic Beads	Glass-Milk	Sodium Acetate	Magnetic Beads	Glass–Milk
Blood Stains											
33	0	F	–	–	+	inh	inh	F	+	+	+
34	0	F	–	inh	inh	F	inh	–	+	–	–
35	0	F	–	–	+	F	inh	F	+	–	+
36	0	M	–	inh	+	–	inh	M	–	+	+
37	0	M	–	–	+	M	–	M	+	+	+
38	0	F	+	–	+	F	–	F	+	+	+
39	0	F	+	–	+	F	–	F	+	+	+
40	0	M	inh	inh	+	inh	–	M	inh	+	+
41	0	M	inh	+	+	inh	–	M	+	+	+
42	0	F	+	+	+	F	–	F	+	+	+
43	0	M	inh	–	+	inh	–	M	–	–	+
44	5	M	+	+	+	M	–	–	+	+	+
45	5	M	+	–	–	M	–	M	+	+	+
46	5	M	–	–	inh	M	–	–	+	+	+
47	5	F	+	+	+	F	–	F	+	+	+
48	5	F	+	+	+	F	–	–	+	+	+
49	5	F	+	inh	+	F	–	F	+	+	+
50	5	F	+	–	+	F	–	F	+	+	+
51	11	M	+	–	–	M	–	M	+	+	+
52	11	M	+	–	inh	M	–	M	+	+	+
53	12	F	+	inh	–	F	–	F	+	+	+
54	15	M	+	–	–	M	–	–	+	+	+
55	22	F	+	inh	–	F	–	F	+	+	+
56	26	M	–	–	–	–	–	inh	+	+	+
57	26	F	+	–	inh	F	–	–	+	+	+

*+ = presence of amplified DNA; – = absence of amplified DNA; inh = presence of inhibitor.
†For bone, 0 = fresh; For blood stains, 0 = 1 week old.
‡Results of PCR amplification expressed as sex of specimen.

TABLE 2—*Comparison of sodium acetate, magnetic beads, and glass-milk DNA extraction procedures: quality measures.**

Specimen Group and Number	Amount of DNA in Extract (μg/mL)			Amount of Protein in Extract (mg/mL)			A_{260}/A_{280} Ratio		
	Sodium Acetate	Magnetic Beads	Glass-Milk	Sodium Acetate	Magnetic Beads	Glass-Milk	Sodium Acetate	Magnetic Beads	Glass-Milk
Bone									
1	284.7	439.6	106.7	1	5.6	1.1	1.67	1.11	1.227
2	748.4	176.6	54.4	6.4	1.5	0.7	1.306	1.314	1.083
3	2415.8	208.1	38.1	32	2.3	0.6	1.09	1.189	1.056
4	1075.2	625.2	259.2	5.8	8.3	1.6	1.51	1.087	1.462
5	226.9	153	84.5	1.6	1.2	0.6	1.394	1.35	1.357
6	803.7	193.8	65.8	6.4	2	0.7	1.392	1.272	1.203
7	25.9	–	442.6	0.7	–	0	0.74	–	3.1
8	58.5	69.7	110.9	1	0.872	0.9	0.95	1.4	1.354
9	113.4	356.6	175	1.5	1.055	1.6	1.09	5.1	1.28
10	53.7	97	101.4	0.7	2.4	0.7	1.066	0.768	1.412
11	915.4	38.3	260.6	11.9	1.006	2.8	1.099	0.6	1.202
12	197.5	53.4	66.9	2.3	0.872	0.9	1.16	1.1	1.067
13	58.7	17.4	72.1	1.1	0.721	0.8	0.09	0.5	1.171
14	942.9	66	99.8	12.6	0.984	1	1.085	1.1	1.246
15	70.5	52.2	98.1	1.2	0.789	1	0.945	1.4	1.22
16	45.9	–	166.9	0.9	–	1.6	0.884	–	1.249
17	128.6	15.4	57.5	2	0.992	0.6	1.013	0.4	1.186
18	50.2	191.9	116.1	0.7	0.642	1.3	1.097	6.3	1.172
19	72	127.8	39.8	0.8	2.7	0.7	1.159	0.898	0.983
20	83.6	117.6	113.3	10.7	0.964	1.008	1.02	2	1.708
21	773.5	29.3	62.2	11	983	0.9	1.056	0.5	1.059
22	89.9	152.9	22.9	1	1.008	0	1.12	2.4	4.901
23	30.2	207.9	70.5	0.1	2.5	0.4	1.561	1.149	1.503
24	1280.9	105.1	97.4	16.6	0.973	1	1.121	1.8	1.239
25	819.2	27.1	123.4	10.4	0.896	1.3	1.111	0.4	1.219
26	168.3	166.7	124.3	1.9	1.254	1	1.174	1.6	1.319
27	43.5	136.6	84.4	0.5	2.8	0.7	1.13	0.874	1.341
28	33.8	26.1	50.4	0.5	0.873	0.7	1.083	0.5	1.079
29	34.1	73.9	28.6	0.5	0.852	0.1	1.005	1.6	1.673
30	871.8	79.6	14.7	10.3	1.7	0	1.147	0.853	1.973
31	412.9	152.3	56.1	5.9	0.823	0.3	1.05	3.4	1.455
32	128.8	32.1	160.9	1.7	1.581	1.3	1.094	0.1	1.328

TABLE 2—*Continued.*

Specimen Group and Number	Amount of DNA in Extract (μg/mL)			Amount of Protein in Extract (mg/mL)			A_{260}/A_{280} Ratio		
	Sodium Acetate	Magnetic Beads	Glass-Milk	Sodium Acetate	Magnetic Beads	Glass-Milk	Sodium Acetate	Magnetic Beads	Glass-Milk
Blood stains									
33	49.3	40.3	3.1	0.5	0.7	0	1.253	0.979	1.34
34	22.9	147.2	15.6	0.2	2.8	0	1.337	0.909	1.9
35	106.6	25.1	19.6	1.4	1	0.1	1.09	0.572	1.692
36	16.2	25.9	26.8	0	1.3	0.1	1.919	0.471	1.692
37	1.6	1.89	5.8	0.9	1.4	0.1	0.428	1.361	1.113
38	59	31.4	72.1	1.3	0.4	0.2	0.839	1.103	1.71
39	18.9	99.7	25.4	0.3	1.4	0.6	1.009	1.058	0.815
40	151.9	168.4	66.6	2.5	0.943	1.2	0.973	3	0.938
41	169.9	0	6	2.9	7.74	0	0.957	0.2	18.92
42	47.8	55.7	4.5	1.1	0.91	0.1	0.802	1.1	0.785
43	114.6	88.1	17.8	1.6	0.819	0	1.062	2	2
44	137	0	109.7	1.4	1.12	1.5	1.22	0	1.077
45	23.5	–	25.4	0.3	–	0	1.03	–	3.586
46	104.3	90.8	309	1.2	1.2	5	1.17	2.3	0.992
47	60.7	17.4	25.9	6	0.721	0.1	1.02	0.5	1.803
48	75.1	153	17.9	1	1.005	0	1.06	1	2.334
49	22.2	–	11.3	0.2	–	0	1.32	–	3.982
50	79.2	13.2	18.4	0.5	1.23	0	1.42	0.1	2.01
51	17.4	12.8	0	1.1	1.34	0	0.392	0.2	1.66
52	18.3	23	0	0.8	1.13	0.2	0.546	0.7	−2.25
53	64.1	0	134.8	1.5	1.101	2	0.788	0	1.027
54	29.3	0	36.5	0.8	1.071	0.6	0.712	0	0.986
55	42.8	0	28.6	0.7	1.06	0.2	0.949	0	1.38
56	86.2	–	457.9	2	–	0	0.809	–	3.31
57	6.3	–	23.1	0.6	–	0.2	0.226	–	1.223

*– = tests not carried out.

Discussion

It is generally agreed that current methods of extracting DNA are often unsatisfactory for old and degraded material, particularly bone. The relative paucity of published data comparing the efficiency of different DNA extraction methods and assessing the survival or detectability of different length sequences was the main reason for pursuing the present study. The investigation has shown the need to carry out proper comparisons and illustrates the complexity of considering the efficiency of three extraction procedures (as judged by the ability to detect PCR products) with respect to three gene systems and specimens of different ages and origins, which had been stored under a variety of conditions; it also shows the importance of employing valid statistical methods to assess the effects and interrelationships of such factors. Multivariate statistical tests were employed to obtain the simultaneous significance levels required for multiple comparisons within large data sets (24,25). They were calculated *en masse* and individual differences highlighted automatically using appropriate (and corrected) Bonferroni significance levels. The repeated use of univariate statistics would have been a severely flawed procedure unless appropriate allowances had been made for the multiple comparisons (the Bonferroni problem) (24,25). It should be noted that, in order not to invalidate the basic statistical assumptions, comparisons were made on the initial results without subsequent manipulation of one or other protocols (which may have been desirable in other circumstances); for example, using the glass-milk procedure, amelogenin sequences could be correctly amplified from all the forensic specimens if the original extracts were re-extracted or further purified.

Awareness of the potential problem of contamination was an important consideration during the study and necessitated meticulous attention to detail. The results of the negative controls performed with each test gave adequate assurances against the likelihood of contamination. For the HLA DPB1 and mitochondrial sequences, the consistently negative results obtained with the mock extractions and sterile filtered distilled water PCR blanks were considered as reasonable evidence that the working system had not been contaminated from an extraneous source. Further support was provided by finding 3 different HLA genotypes in 3 of the forensic samples where sequence specific oligonucleotide probing (26) was carried out. These results and the fact that the correct sex was always obtained from the amelogenin amplifications confirmed the lack of contamination and authenticity of the results. In addition, and contrary to common practice when negative test results are obtained with old material, the number of PCR cycles was not increased from the protocol in order to diminish the risk of producing false positive reactions. Ideally, since not all PCR products result in typeable patterns, one would have wished to fully type the HLA DPB1 products in every case rather than take amplifiability as the measure of successful extraction, and this would be a logical future study. However, the goal in the present work was to compare the amplification of sequences of different lengths, and HLA DPB1 was selected because it was >300 bp whereas amelogenin sequences were just over 100 bp.

The three extraction methods were chosen because sodium acetate precipitation was extremely simple and user-friendly (15), the glass-milk technique (3) was relatively new, while the Dynabeads method was the first commercially available procedure using magnetic beads, treated so that they had a high affinity for binding DNA (Dynal, Oslo). Sodium acetate extraction, unlike most established techniques, is based on the removal of non-nucleic acid material by salting-out (27). The protocol was developed from our method routinely used with fresh blood and soft tissue; it was successfully employed in a previous investigation involving forensic bone, which also showed that it was a valid alternative to phenol-chloroform extraction, with the advantage of not employing hazardous reagents (15). The Dynabeads procedure was carried out strictly according to the manufacturer's instructions and the glass-milk technique had been optimized for archaeological dry bone (3). The three extraction methods therefore seemed ideal for our comparative studies, especially since little previous work has been published that assessed the quantity and quality of DNA extracted from relatively old material (particularly bone) using different techniques (9–18).

The three gene sequences were selected both to represent material of different length and copy number and also because they were of interest in the anthropological, forensic and archaeological fields, where they play major roles in determining sex, racial affiliation, and personal identification of skeletal or otherwise unidentifiable human remains. Their PCR protocols had all been standardized to give satisfactory results with the variety of blood and tissue specimens encountered in a large transplantation laboratory and were therefore considered to be suitable for the present investigation.

Although the results (Tables 1 and 2) have helped to clarify some of the problems associated with extraction of DNA from old bone and blood stains, it should be emphasized that global conclusions relating to the performance of the methods cannot be reached as the study only compared specific protocols. Altering parameters, for example adding a clean-up step to the sodium acetate procedure, might have substantially changed its performance as judged by the measures of this study, and these are obvious avenues to follow in the future. Bearing these points (and reservations on the accuracy of the quality measures, discussed later) in mind, the analysis of the data shown in Tables 1 and 2 can be considered in detail.

Effect of Extraction Method

The method of extraction had a significant effect on the results. Overall, amplifiable DNA was obtained more often with the glass milk technique (65% of extracts) than with sodium acetate (55%) and magnetic beads (37%) (MANOVA, $p < 0.0003$). The quantity of DNA extracted (Table 2) seemed to have only a minor role in explaining these findings; in fact, the mean amount in sodium acetate extracts (256 μg/mL) was much greater than that for glass-milk (86 μg/mL). ANOVA showed that the contribution of the individual gene sequences was variable and complex. For sequences with low copy number (HLA DPB1 and amelogenin), the glass milk and sodium acetate methods were comparable; it was thought that while glass milk was better at removing PCR inhibitors (see below), a gross preparatory method such as sodium acetate was better able to maximize the yield when small amounts of DNA were present. As would be expected, glass milk was superior for the multiple copy sequences (mitochondrial DNA) where the total yield would be less important; in this instance, the sodium acetate method was only as effective as the magnetic beads.

Effect of Gene Sequence

Whether PCR amplification products were obtained depended, *inter-alia*, on the gene sequence selected for study. Mitochondrial DNA was amplified from 84% of extracts, a significantly greater proportion than the 39% for HLA DPB1 sequences ($p < 0.001$)

and the 35% for amelogenin products ($p < 0.001$). These findings presumably reflected copy number, as multiple copies of mitochondrial DNA were present per cell as opposed to a single copy for the genomic DNA (HLA DPB1 and amelogenin). One would have expected shorter sequences to be retrieved more frequently on the assumption that they were less susceptible to degradation. However, this was not the case as HLA DPB1 products (327 bp) amplified just as often as amelogenin (106/112 bp) (ANOVA $p = 0.44$).

Effect of Specimen Group

Amplifiable DNA was obtained more often from blood stains than bones (62% vs 45% of extracts: MANOVA, $p < 0.003$), this effect being mainly attributable to amelogenin and mitochondrial DNA (49% vs 24% and 91% vs 78% respectively: ANOVA, $p < 0.001$ and $p = 0.028$). These observations may be due to bone being more difficult to deal with technically as it frequently contains contaminants from both the organic and inorganic matrices, as well as exogenous substances which, in older skeletal remains, may accumulate within the trabecular structure and decrease the likelihood of successful extraction and amplification. However, adsorption onto hydroxyapatite results in a twofold decrease in the rate of depurination of DNA, which should improve its chances of being recovered from old bones (28), and it may be that purification of DNA from the hydroxyapatite crystals and other components of bone is the key to successful amplification. In the present study, storage conditions also undoubtedly played a major part. The blood stains were made specifically for this sort of investigation and were all carefully stored in clean, dry conditions at ambient temperature. The bone specimens, on the other hand, were retrieved from a variety of situations and included fresh bone from surgical procedures which was processed virtually immediately, bone from forensic cases which had been stored for up to 5 years after death at 4°C and bone from individuals who had been interred, some for nearly 40 years, before specimens were taken and stored, first at room temperature for 5 years then at −80°C. In the case of the older bones, this would certainly have meant greater exposure to microbial agents and humidity, two factors detrimental to DNA survival.

Effect of Specimen Age

Specimen age had a significant effect on the findings. Overall, PCR amplification products were obtained more frequently in extracts from younger specimens than from older ones (72% vs 65% vs 37% vs 38%, respectively, for the 4 age groups: MANOVA, $p < 0.001$); this effect was particularly evident for HLA DPB1 and amelogenin, but not for mitochondrial DNA (ANOVA, $p < 0.001$, < 0.001, and 0.093 respectively). The comments regarding storage conditions are also relevant here and were the reason for analyzing data from blood stains and bone separately. Although the blood stains, which had good storage conditions, showed an age effect overall, this was not particularly strong (MANOVA, $p < 0.015$) with a significant individual contribution only from mitochondrial DNA (ANOVA, $p = 0.018$). With the bones, however, where the age groups coincided with specimen categories of fresh, forensic, and cemetery, the overall age effect was marked (MANOVA, $p < 0.001$) with amplification products being more frequently detected in the fresh (in 98%) than in the combined forensic and cemetery specimens (23%). Significant individual contributions were made to the overall effect by HLA DPB1 and amelogenin, but not by mitochondrial DNA (ANOVA, $p < 0.001$, < 0.001 and < 0.37 respectively). It is interesting to note that the results for the 11–20 and >20 years groups were similar. Considered as a whole, the findings suggest that with good storage conditions the age effect can be reduced and that, although actual loss of DNA undoubtedly occurs, significant amounts of amplifiable DNA can survive for considerable periods of time and may even plateau after 10 years. In the case of the bone specimens, where the rate of degradation was likely to be higher because of greater exposure to water and microbial attack (28), the disadvantages of attempting to amplify single copy gene sequences are apparent, though the possibility that inhibitors from the soil and surrounding burial environment interfered with PCR amplification should be borne in mind.

Effect of Quality Measures on Presence of Amplification Products and PCR Inhibitors

The quality measures (amounts of DNA and protein and A_{260}/A_{280} ratio) had no apparent effect, either overall or individually, on the presence of amplification products for each of the gene sequences studied ($p > 0.05$ in all cases), which suggested that if there was sufficient DNA present for a PCR, the quantity in excess was of little importance. When the extracts that failed to produce amplification products were analyzed as a group, those which contained PCR inhibitors were found to have significantly more protein, whichever gene sequence was considered (MANOVA, $p < 0.05$ in all cases). However, given the range of organic compounds with complicated absorption spectra that copurify with DNA from degraded material, particularly bone, it is likely that neither DNA nor protein could be quantitated accurately by spectroscopy and thus it is not surprising that "protein" measurements alone could not be used to predict amplification. Often samples which contained large amounts of "protein" still amplified, whereas other samples with high "protein" levels were inhibited. Also, it must not be assumed that all PCR inhibitors are proteins; for example, environmental humic acids, which have been demonstrated to be common inhibitors of PCR in bone extracts, absorb strongly at similar wavelengths. The development of tests for specific inhibitors would be an important future study, particularly since the method employed in the present work was only adequate for detecting their presence, but could not confirm their absence. It therefore seems that the relatively crude quality measurements used here have less of a relationship with the subsequent ability to amplify DNA sequences than commonly thought (23). They are, in fact, unreliable and for this reason the use of spectrophotometry for assessing quality of DNA extracts was abandoned in North America for forensic studies some 8–9 years ago.

Effect of Extraction Method on Presence of PCR Inhibitors

The method used for extracting DNA was important in determining the likelihood of PCR inhibitors being present (MANOVA $p < 0.001$). Overall, fewer extracts prepared with the glass milk and magnetic beads protocols contained inhibitors (6 and 11% respectively) compared with those obtained by sodium acetate precipitation (24%). One possible explanation is that the glass-milk and magnetic bead techniques used affinity methods to capture DNA from the surrounding organic matrix, rather than attempting to remove this matrix and leave the DNA in solution, as is the case with sodium acetate extraction. However, any apparent advantage of magnetic beads should be balanced by recalling that they performed less well overall in producing amplifiable DNA. The greater incidence of PCR inhibitors in extracts prepared by sodium

acetate precipitation may partly explain why, in spite of yielding larger quantities of DNA, amplification products were obtained less frequently with this method than with glass-milk.

In conclusion and remembering its limitations, the present study seemed to show that glass milk was the best method of extraction as far as old bone and blood stains were concerned. The ability to produce amplifiable DNA (providing there was sufficient DNA present for a PCR) depended on a variety of factors, including gene copy number; specimen origin, age and storage environment; the co-extraction of PCR inhibitors; and possibly PCR design (e.g., lower susceptibility to inhibitors or better primers) and peculiarities related to specific sequences which may have made them more degradable. Non-DNA contaminants in extracts were important in adversely affecting amplification; longer sequences were just as frequently amplified as shorter ones in old and degraded material (where copy number was of greater significance) and, no doubt, other variables, which had not been considered, also affected the results. The investigation also illustrated the complex interrelationships of factors influencing successful extraction and amplification of DNA in the forensic context, and the need for specifically designed studies, employing valid statistical procedures, to unravel them.

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