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DIRECT EVIDENCE OF ANCIENT DNA FROM HUMAN BONES UP TO TWELVE THOUSAND YEARS OLD BY PROBE HYBRIDIZATION

ABSTRACT: Efforts from many research groups, often in a hunt for the oldest sequences, showed that ancient DNA was a poor substrate for the enzymes used in molecular biology, present in tiny amounts, hard to purify, copurifying with inhibitors of polymerase chain reaction (PCR) and frequently damaged. Inside ancient DNA extracts, the quantity of the DNA of interest and its genuine specie, have never been evaluated before, but with quantitative PCR. Herein, direct DNA quantitation method was applied. This method should be a useful tool for ancient DNA study as it is currently for forensic material. Among techniques performed on forensic samples, we chose a chemiluminescent method based on probe hybridisation to a human alpha satellite locus D17Z1. We thought that such a rapid and sensitive method, might be extended to the ancient human DNA applications, especially as the study of nuclear ancient DNA is growing up. We report now the successful quantitation of nuclear human DNA in DNA extracts prepared from very ancient bones. Twenty-four different human specimens, up to 12,000 years old, were analysed and the quantity of human DNA content determined for fourteen DNA extracts on twenty-nine tested.

KEY WORDS: Ancient DNA – DNA Quantitation – Hybridization – Nuclear DNA

INTRODUCTION

Ancient DNA, extracted from archaeological specimens, is often retrieved in very limited amount, chemically damaged, rarely pure, copurifying with inhibitors of polymerase chain reaction (PCR) (Higuchi *et al.*, 1984, Pääbo *et al.* 1989a, Hagelberg *et al.* 1989, Hänni *et al.* 1995, Pääbo *et al.* 1989b, Höss *et al.* 1993, Audic *et al.* 1997). From such a material, PCR experiments must be considerably optimised, increasing, in turn, the risk of contamination by modern DNA sequences; several ancient DNA sequences obtained from PCR product were controversial (Golenberg *et al.* 1990, DeSalle *et al.* 1992, Cano *et al.* 1993). Besides all methods used to authenticate ancient DNA sequences (Lindahl 1993, Handt *et al.* 1994a, Richards *et al.* 1995, Poinar *et al.* 1996, Beraud-Colomb *et al.* 1997, Stoneking 1995), ancient DNA field needs a straightforward method to identify the origin of DNA extracted; direct evidence of ancient DNA has never been described. Classical methods for quantitative DNA estimation such as spectrophotometry and ethidium bromide coloration were attempted. No results have been obtained by spectrophotometry due to the brown coloration of most samples and to the presence of all the organic components in the extracts of ancient material (Audic *et al.* 1997, Hagelberg *et al.* 1991), whereas results obtained by ethidium bromide coloration method showed a very low accuracy: the amount of DNA was estimated according to the intensity of the smear in acrylamide gels compared to a set of standards with known DNA concentration (Tuross 1994). Quantity of the ancient DNA of interest and its genuine specie, have never been evaluated, but with

quantitative PCR (Handt *et al.* 1994b). With this indirect technique, serial dilutions of a competitor template containing a small insertion or deletion in the selected DNA fragment, is added to a constant amount of extracted DNA. Then, PCR reaction is performed to estimate the amount of DNA (Handt *et al.*, 1994b; Krings *et al.*, 1997).

Because ancient DNA and forensic fields have to face similar questions upon the origin of DNA retrieved, genetic studies in ancient human and forensic material include several common strategies. There is no way to be sure, for some particular forensic specimens, that the material submitted to further genetic analysis is really human and the characterisation of DNA extracted is a quite necessary first step. Among the techniques performed on these forensic samples, we chose a chemiluminescent method based on probe hybridisation to a human alpha satellite locus D17Z1 (Waye *et al.* 1986), we thought that such a rapid and sensitive method, very useful in forensic field, might be extended to the ancient human DNA applications, especially as the study of nuclear ancient DNA is growing up (Beraud-Colomb *et al.* 1995, Lawlor *et al.* 1991, Kurosaki *et al.* 1993, Filon *et al.* 1995, Schultes *et al.* 1999, Greenwood *et al.* 1999).

We report now the successful quantitation of nuclear human DNA in DNA extracts prepared from very ancient bones.

MATERIAL STUDIED AND METHODS

Human specimens

24 different bones were analysed: five independent specimens from the archaeological site of Taforalt, in western Morocco settled by man 12,000 years before present (B.P.); two specimens from San Benedetto, an Italian Neolithic archaeological site; five specimens from Geili, Khartoum in Sudan, selected from Meroitic period (3rd century B.C.), thus dated 2,300 years B.P.; two specimens from Su Sercone, an Italian archaeological site dated about 2,000 years B.P.; one specimen from Dolianova, Italy, dated 1,400–1,600 years B.P.; four specimens from Volonne, an 8th century, high Middle Age archaeological site in Italy, i.e., 1400 years B.P.; one specimen from Fostat, a 13th century archaeological site in Egypt; two specimens from Tiya an archaeological site in Ethiopia, dated as 600 years; one recent specimen from Ghilarza, Italy, and one recent specimen from Kapanda, in Angola.

Animal specimens

Eight bones described by archaeozoologist as from *Bos taurus* and one from *Bos primigenus* were analysed. The eight *Bos taurus* specimens from the archaeological site of Lattes, in Hérault, France were dated between 2,500 and 1,600 years. One *Bos primigenus* specimen was collected from the archaeological site of Le Portel, in Dordogne, France, and dated about 60,000 years.

Laboratory precautions

All experiments were performed in a laboratory where the study and amplification of human DNA were not performed. All buffers and water were autoclaved and purified before use by filtration through disposable centricon 30 microconcentrators (Amicon). A "quality control" PCR containing 10 U. Pfu polymerase without added DNA was performed on random aliquots from a large amount of PCR premix containing all the necessary components, except DNA and Pfu polymerase. The preparation of bone samples and the DNA extractions were performed in one room, and DNA extracts were stored in a freezer in a separate room. Preparation of buffers and PCR set-up were performed in another separate room in a dedicated sterile hood under constant UV illumination (254 nm germicinal lamp) between use. Dedicated pipettes with aerosol resistant plugged tips were used throughout. PCR experiments were performed in a dedicated thermocycler in a fourth room of the laboratory. For each set of PCR experiments, including set of nested PCR, reaction blanks and mock extraction controls were conducted. For each protocol of extraction, human and animal specimens were treated. Animal specimens were performed as extraction controls of human specimens.

DNA extraction

Samples of bone were extensively cleaned by cutting off 1–2mm from the entire bone surface with scalpel blades. Bone powder was produced by sawing the sample with handsaw and disposable blades. Then, extraction was performed as previously described (Beraud-Colomb *et al.* 1995).

PCR experiments

Primers

The sequence of primers used to study one hypervariable mtDNA region of the human D-Loop is 5' GAC TCA CCC ATC AAC AAC CGC 3' (V110), 5' CGT ACA TTA CTG CCA GCC ACC ATG 3' (V115), 5' CTT TGG AGT TGC AGT TGA TGT GTG 3' (V240), 5' GAT AGT TGA GGG TTG ATT GCT G 3' (V2C), 5' CGG AGG ATG GTG GTC AAG GGA CC 3' (V392). The sequence of primers used to study one hypervariable region of the bovine D-loop is 5' GCC CCA TGC ATA TAA GCA AGT AC3' (Bov 1), 5' GTT TCA CGC GGC ATG GTA AT 3' (Bov2AS) and 5' TGA GAT GGC CCT GAA GAA AG 3' (Bov 4AS).

Amplifications

Amplifications were performed in 50µl of PCR buffer containing 10 mM KCl, 10 mM $(NH4)_2SO_4$, 2mM $MgCl_2$, 0.1% Triton X-100, 0.1 mg BSA/ml, 20mM Tris-HCl (pH 8.75), 200µM dNTPs, 20 pmol each primer, 2µl or 5µl of the extracted DNA, and 5–10 U Pfu DNA polymerase (Stratagene, USA). The same PCR profile was used for all primer combinations: an initial 94°C denaturation step for 5 min, followed by 94°C for 1min, 55°C for 1 min and 72°C for 1min, for a total of 40 cycles, finishing with a

final elongation step at 72°C for 6 min. When PCR amplification were not efficient enough for direct sequencing of PCR product, nested PCR were performed.

PCR product analysis

We directly sequenced PCR product, since we previously showed by cloning and direct sequencing of the PCR product we retrieve the same DNA sequences (Beraud-Colomb *et al*. 1995), as others authors did (Horai *et al.* 1989, Hagelberg *et al.* 1991, Höss *et al.* 1996).

Quantitation

We used the human DNA quantification kit Quantiblot (Perkin-Elmer, USA) (Walsh *et al.* 1992).

Samples DNA were immobilised on a Biodyne B nylon membrane (Gibco BRL, France) with the slot blot apparatus (Gibco BRL, France). DNA was hybridised with a biotinylated D17Z1 probe (Waye *et al.* 1986) then the enzyme conjugate: HRP-SA was bound to the hybridised probe. D17Z1 probe is complementary to a primate-specific alpha-satellite DNA sequence at the locus D17Z1. A stringent wash was done to remove non-specifically bound probe with a washing buffer (1,5X SSPE, 0,5X SDS).

Chemiluminescent detection was performed with ECL detection kit. Membranes were exposed 15 minutes, finally 1 hour and then overnight on a Kodak XAR5 film.

DNA was quantified by comparing the signal intensity of the DNA test sample with the signal intensity obtained for the DNA standards spotted in parallel. We tried different volumes of DNA solution, and 15µl was the optimum and maximum quantity we could load without losing sensibility.

Extraction mocks and extraction from animal specimens were treated like human samples, with an optimal load of 15µl in each slot. The darkness of the bands was manually estimated or red with a densitometer. Once the quantity of DNA was estimated, the concentration of the sample can be calculated (*Table 1*).

To analyse samples in wich no DNA was detectable in 15µl, we have tried to bypass this limit by concentrating DNA with ethanol precipitation. No positive results were obtained in this way.

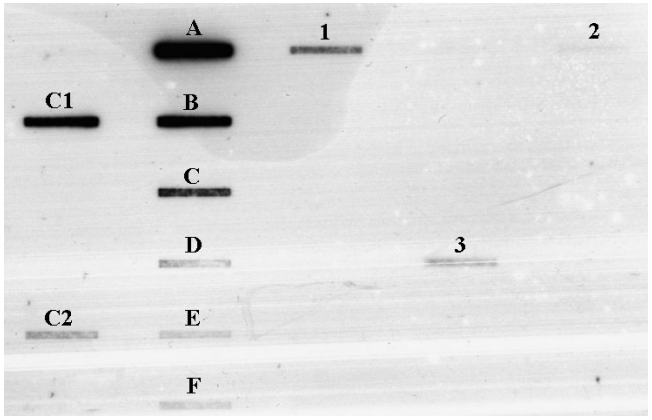
To evaluate the specie-specificity of the probe in our experimental conditions, we try to quantify DNA from non-human current specimens (*Bos taurus, Papio papio* and *Rattus norvegicus*). None of the samples (with amount of DNA ranging from 1.5µg to 30 ng from *Bos taurus*, 1.5µg to 7.5ng from *Rattus norvegicus* and to 30 ng to 0,15ng from *Papio Papio*) shows any signal in our experimental condition despite, according to the kit manual, D17Z1 probe may hybridize with large amount (30–300 ng) of DNA from a wide range of species with signal equal to or below the one obtained for 0.15 ng of human DNA (Waye *et al.* 1989).

RESULTS

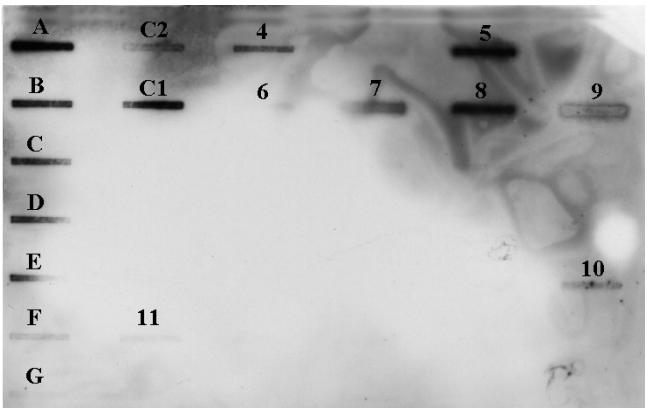
In this study, forty-one samples were analysed from twenty-four different human bones and from three extraction mocks and nine animal bones used as extraction controls. Hybridization technique allowed to quantify DNA contained in fourteen human samples (DNA extracts) prepared from ten different bone specimens up to 12,000

TABLE 1. Concentration was calculated with the quantities of estimated DNA and the amount of loaded solution. Quantities of DNA in bone were calculated using mass of extracted bone. Slot numbers are referring to *Figure 1*.

Sample (age)	Slot number	DNA Loaded in µl	DNA concentration in the sample (ng/µl)	DNA retrieved in bone (ng of DNA / g of bone)
San Benedetto I (Neolithic)	5	9	0.444	396
	12	1	0.4	
Dolianova (around 550 years)	3	3.5	0.421	388
	8	9.5	0.428	
Taforalt VIII (12,000 years)	1	15	0.133	130
Su Sercone 2 (Middle Age)	7	7.5	0.133	120
	15	5	0.12	
Volonne 140–155 (High Middle Age)	4	15	0.04	40
Ghilarza (Recent)	16	15	0.066	
	9	9	0.056	36
Tiya 5.10.I14/15 (600 years)	14	15	0.033	33
Tiya 5,8,j18 (600 years)	13	15	0.02	20
Su Sercone 1 (Middle Age)	17	7.5	0.018	15
Taforalt VI9E (12,000 years)	2	15	0.01	10



A



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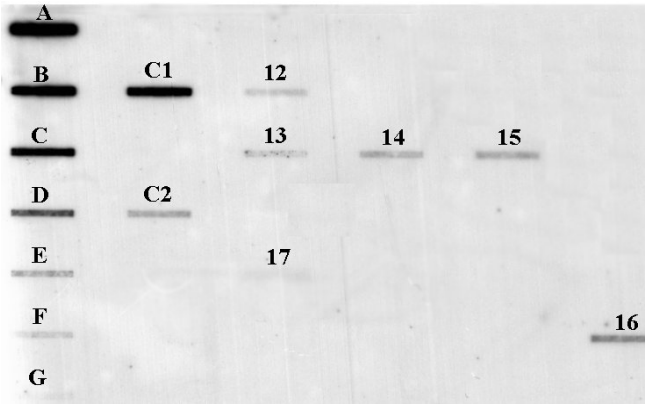

C

FIGURE 1. a, b and c are three independent slot blot analyses. A–G: DNA standards corresponding to DNA quantities spotted on the membrane (A: 10 ng; B: 5 ng; C: 2.5 ng; D: 1.25 ng; E: 0.625 ng; F: 0.3125 ng; G: 0.15625 ng of DNA). C1 and C2 are calibrators which provide DNA of known concentration to verify DNA standards. C1 has a concentration of 3.5 ng/µl and C2 0.5 ng/µl. Sample 1 is Taforalt VIII sample. Sample 2 is Taforalt VI9E sample. Samples 3 and 8 are Dolianova. Sample 4 is Volonne T140–155. Samples 5 and 12 are San Benedetto I. Sample 6 is Lattes B1. Sample 7 is Su Sercone 2. Samples 9 and 16 are Ghilarza. Sample 10 is Lattes B6. Sample 11 is Lattes B5. Sample 13 is Tiya 5.8.J18. Sample 14 is Tiya 5.10.I14/15. Sample 15 is Su Sercone 2. Sample 17 is Su Sercone 1.
For samples 1, 2, 4, 6, 10, 13, 14 and 16, we loaded 15µl of DNA; for sample 3, we loaded 3.5µl of DNA; for samples 5 and 9, we loaded 9µl of DNA; for samples 7, 11 and 17, we loaded 7.5µl of DNA and for sample 8, we loaded 9.5µl of DNA; for sample 12, we loaded 1µl of DNA sample and for sample 15, we loaded 5µl of DNA.

years old. DNA in human samples ranged from 0.44 to 0.01 ng per µl, meaning DNA retrieved in bone ranged from 396 to 10 ng of DNA per g of bone (*Table 1*). Three animal samples showed low amount of DNA ranging from 0.02 to 0.01 ng per microliters (*Figure 1* and *Table 2b*).

Three human samples (*Table 1*) were tested in duplicate, with different volumes of DNA extract loaded, and showed similar amount of DNA in both duplicates. Two samples, from two independent extractions of the same human bone specimens (Taforalt XVI A2) were found devoid of DNA, while from PCR amplification, the same polymorphic site was retrieved in the two corresponding ancient DNA sequences.

Samples are also tested in PCR, direct or nested PCR (if PCR product obtained in direct amplification was not efficient enough to permit a direct sequencing – *Table 2a*). Eight samples were amplified (two in direct PCR, six in nested PCR) with primers corresponding to mitochondrial D-loop HVR1 region. Six on eight PCR products showed polymorphisms respective to Cambridge Reference Sequence (CRS) (Anderson *et al.* 1981). Taforalt XVI A2 sample showed the same polymorphism with DNA from two independent extractions. This demonstrates authenticity of DNA extracted. All animal checks were also amplified with human primers and no PCR product was generated. With bovine primers, from two samples, bovine PCR product was generated (*Table 2b*). One of these PCR products exhibited two polymorphisms: 16118G and 16119C regarding to bovine European consensus described elsewhere (Anderson *et al.* 1982).

It is interesting to notice that most of the samples quantified (9/10) showed brown coloration and most of non-quantified samples are light (12/15). For PCR amplification, only three coloured human samples were amplified on twelve tested and 5 light samples on 13 were amplified.

DISCUSSION

Two main reasons, at least, legitimate the use of probe hybridisation for quantification of ancient DNA as of great importance. One main reason concerns the possibility of analysing ancient DNA sequences with other tools than PCR. As a matter of fact, except for the two first articles (Higuchi *et al.* 1984, Pääbo *et al.* 1985) reporting the successful direct cloning of ancient DNA sequences, in all papers on the topic, PCR is always the first step for genetic studies of ancient DNA. PCR from ancient human material requires drastic laboratory precautions and systematic controls, due to the high risk of contamination of ancient DNA sequences by modern DNA sequences. Another requirement is to reproduce the result in multiple extractions of ancient material or in different laboratories. Furthermore ancient DNA sequences must be submitted to several criteria of authenticity, like particularly phylogenetic analysis (Richards *et al.* 1995, Beraud-Colomb *et al.* 1995,

TABLE 2. "+" in "brown coloration" column means sample is brown coloured. "+" in "Amplifiability an sequencing" column means PCR products have been obtained with these samples and sequenced.
2(A): "Human" in "amplifiability and sequencing" column means that sequence of the PCR product is identified as a human sequence.
317 bp nested-PCR product was obtained with V110–V392 amplification followed by a V115–V392 amplification. 347 bp PCR product was obtained with V110–V392 amplification. 166 bp nested-PCR product was obtained with a V110–V320 amplification followed by a V100–V2C amplification. 191 bp nested-PCR product was obtained with V110–V350 amplification followed by a V110–V240 amplification. 185 bp nested-PCR product was obtained with a V110–V320 amplification followed by a V115–V320 amplification. 225 bp product was obtained by a direct amplification with V110–V320.

Sample (age)	**Brown coloration**	**DNA in 15µl**	**Amplifiability and sequencing**	**Polymorphic sites vs reference**	**PCR Product lenght in bp**
San Benedetto I (Neolithic)	+	6	– / –		
Dolianova (around 550 years)	+	6	– / –		
Taforalt VIII (12,000 years)	+	2.1	+ / Human	16 261 T	317 (nested)
Su Sercone 2 (Middle Age)	+	2.1	– / –		
Ghilarza (Recent)	+	0.9	– / –		
Volonne 140–155 (High Middle Age)	–	0.6	– / –		
Tiya 5.10.I14/15 (600 years)	+	0.5	– / –		
Tiya 5,8,j18 (600 years)	+	0.3	– / –		
Su Sercone 1 (Middle Age)	+	0.25	– / –		
Taforalt VI9E (12,000 years)	+	0.1	+ / Human	16 172C – 16 174 T	347 (direct)
Taforalt XVI A2 (12,000 years)*	–	–	+ / Human	16 189C	166 (nested)
Taforalt XXI-6 (12,000 years)	–	–	+ / Human	none	317 (nested)
Taforalt XXIV (12,000 years)	–	–	+ / Human	16 172C	191 (nested)
Taforalt XVI A2 (12,000 years)*	–	–	+ / Human	16 189C	317 (nested)
San Benedetto II (Neolithic)	+	–	– / –		
Geili (5 specimens) (2 300 years)	–	–	– / –		
Volonne V2J (1,400 years)	–	–	+ / Human	none	225 (direct)
Others Volonne (2 specimens) (1,400 years)	–	–	– / –		
Kapanda (recent)	+	–	– / –		
Fostat (500 years)	+	–	+ / Human	16 223T	185 (nested)

2(B): "Bovine" in "amplifiability and sequencing" column means that sequence of the PCR product is identified as a bovine sequence. Positions of polymorphic sites were detected versus reference sequence [31]. 161 bp nested-PCR product was obtained with Bov1S–Bov4AS amplification followed by a Bov1S–Bov2AS amplification.

Sample (age)	**Species**	**Brown coloration**	**DNA in 15µl**	**Amplifiability and sequencing**	**Polymorphic sites vs reference**	**PCR Product lenght in bp**
Lattes B6 (2,000 years)	*Bos taurus*	+	0.3	+ / Bovine	none	161 (direct)
Lattes B1 (2,000 years)	*Bos taurus*	+	0.15	– / –		
Lattes B5 (2,000 years)	*Bos taurus*	+	0.15	– / –		
Lattes B2 (2,000 years)	*Bos taurus*	+	0	– / –		
Lattes B4 (2,000 years)	*Bos taurus*	+	0	– / –		
Lattes B3 (2,000 years)	*Bos taurus*	+	0	– / –		
Lattes B8 (2,000 years)	*Bos taurus*	+	0	– / –		
Le Portel AuFI (60,000 years)	*Bos primigenus*	–	0	+ / Bovine	16 118G – 16 119C	161 (nested)
Lattes B7 (2,000 years)	*Bos taurus*	+	0	– / –		
Extraction mocks	no bone	–	0	– / –		

Hedges *et al.* 1995). Thus, hybridisation method could be a very simple and useful technique in ancient DNA studies.

Another main reason concerns the possibility of improving the purification method of ancient DNA. Up to day there has been no comparatives studies of the DNA purification method partly because there is still no alternative to PCR to measure the amount of ancient DNA present in archaeological remains.

We show in this report that it is possible to detect and quantify ancient nuclear DNA, by a slot blot hybridisation technique using a probe which corresponds to a highly repetitive satellite DNA specific for human chromosome 17 (D17Z1). The great sensitivity of this direct hybridisation technique (down to 0.15 ng of DNA by slot) appeared to be particularly interesting for critical samples such as ancient material, which are generally very poor in quantity and quality of DNA and for which an exact appreciation of the human DNA amount is needed to generate PCR reactions in optimal conditions, which should increase the confidence in results of ancient DNA sequences.

Twenty-four different human specimens, up to 12,000 years old, were studied and the quantity of human DNA content determined for fourteen DNA extracts on twenty-nine DNA extracts tested.

Although, the DNA quantitation by the D17Z1 probe does not authenticate ancient DNA, the fact that by this quantitation, fourteen DNA samples on twenty-nine human samples tested contain DNA advocate for endogenous ancient DNA presence, since only three on nine bovine samples gave a positive signal. Moreover, samples positively quantified were not from the most handled bones (as it was the case for the Taforalt collection). Finally, we point out that even if four human samples (*Table 2a*) show amount of DNA in the same range that bovine samples, six human samples show clearly higher amount of DNA.

The measured DNA concentration was in the range of 396 nanograms to 10 nanograms of DNA per gram of archaeological remain (*Table 1*), which is reliable with the amount of DNA in fresh bone (1.5–3.0 µg per gram of bone – Tuross 1994). The sensitivity of slot blot quantification permits to quantify subnanogram amounts of human genomic DNA (*Figure 1*). Despite this quite high sensitivity, ancient material may contain very damaged (maybe non-hybridisable), or very low quantity of DNA, so that absence of detection does not mean that the sample is DNA free, as demonstrated by PCR generated from samples without detectable DNA.

One specimen (Taforalt VIII: 0.133 ng per µl) among the three most concentrated DNA samples, was collected from the oldest archaeological site of this study (Taforalt, 12,000 years old – *Figure 1, Table 2a*), whereas DNA from some younger samples (including some recent ones, like bones from Kapanda, Angola) could not be quantified. This observation shows that there is no direct relation between the amount of DNA recovered from an ancient bone and its age. This observation has already been reported by ourselves and other teams when studying amplifiability of ancient DNA (Pääbo *et al.* 1989b, Beraud-Colomb *et al.*, 1995). The recovery of DNA from a sample seems more linked to the conditions of its preservation/degradation (humidity, temperature, geochemical properties and microbial infestation of the soil) than the age of the bone (Burger *et al*. 1999). However, these observations could be sharpened in the future, since at the moment we are not sure whether the extraction of ancient DNA was carried out with an optimal yield.

The absence of contamination of our positive DNA samples was checked by testing, in the same conditions of quantitation, control extractions i.e., corresponding extraction mocks and animal DNA extracts. No extraction mocks show any detectable human DNA even when 15 µl of sample, the upper allowed volume for optimum result, was loaded. By contrast two out of eight bovine specimen extracts contain human DNA, however quantity of DNA retrieved (up to 6ng in 15µl from human specimens versus 0.3ng in the same volume from bovine specimens) was significantly lower in bovine samples. These results agree with previous notes suggesting that animal specimens had better control extraction than mock extraction in human specimens studies (Richards *et al.* 1995).

Finally, using this method of quantitation we clearly observed a concomitant loss of DNA during the purification since coloured human samples contained quantifiable DNA and were poorly amplifiable, whereas most of light samples were devoid of quantifiable DNA, but were amplifiable.

Even although this method was not an authenticity criterion, human DNA quantitation in animal samples is a suitable check-up for detecting contamination by human DNA.

We have demonstrated for the first time that the quantity of DNA in ancient human bones can be directly estimated by direct evidence. Simultaneous analysis of human and bovine ancient DNA will permit to optimise and develop extraction methods of ancient DNA.

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