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Histological Analysis and Ancient DNA Amplification of Human Bone Remains Found in Caius Iulius Polybius House in Pompeii

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Thirteen skeletons found in the Caius Iulius Polybius house, which has been the object of intensive study since its discovery in Pompeii 250 years ago, have provided an opportunity to study either bone diagenesis by histological investigation or ancient DNA by polymerase chain reaction analysis. DNA analysis was done by amplifying both X- and Y-chromosomes amelogenin loci and Y-specific alphoid repeat locus. The von Willebrand factor (vWF) microsatellite locus on chromosome 12 was also analyzed for personal identification in two individuals showing alleles with 10/11 and 12/12 TCTA repeats, respectively. Technical problems were the scarcity of DNA content from osteocytes, DNA molecule fragmentation, microbial contamination which change bone structure, contaminating human DNA which results from mishandling, and frequent presence of Taq DNA polymerase inhibiting molecules like polyphenols and heavy metals. The results suggest that the remains contain endogenous human DNA that can be amplified and analyzed. The amplifiability of DNA corresponds to the bone preservation and dynamics of the burial conditions subsequent to the 79 A.D. eruption.

Key words: anthropology, forensic; base sequence; dental enamel; DNA fragmentation; human identification; polymerase chain reaction; satellite DNA; sex determination (genetics); von Willebrand factor

The spectacular new application of molecular biology techniques provides researchers the possibility of studying ancient DNA (aDNA) recovered from human, animal, and plant remains. The benefit achievable from genetic information available in those materials lies in the understanding of how cultural activities affect genetical property in man-related species, including man himself. The knowledge of aDNA sequences allows further studies in fields such as inherited and acquired diseases (pathogen sequences). The study of aDNA, together with paleopathological analysis, can produce objective information to help answer a number of questions which until now were inconceivable, such as the origin of humankind, development of ancient societies, spreading of diseases, and allele haplotyping.

Pompeii and Herculaneum

In collaboration with the Pompeii Archaeological Superintendence, our research group initiated the study of ancient DNA extracted from human bone remains buried in Pompeii and Herculaneum during the Vesuvius eruption of 79 A.D. From a molecular genetics point of view, these remains are of exceptional scientific value because they offer a unique random sampling of the human population which died during this historically documented catastrophe. These random samplings allow the comparison of nucleotide sequences of aDNA of significant genetic loci found in an ancient population with nucleotide sequences found in today's population of the same geographic area.

The acquisition of genetic data by gender obtained by aDNA analysis, compared with anthropo-metric evaluation, together with specimen evaluation coming from the physical and environmental parameters, can provide conclusive evidence that the DNA samples under study are indeed authentic endogenous molecules. In this report we present the main results from our analysis of the skeletal remains found in the house of Caius Iulius Polybius in Pompeii.

Inhabitants of the House of Caius Iulius Polybius

Analysis of aDNA obtained from the bone remains from Pompeii was initiated as a part of a multidisciplinary research project started by the Archaeological Authorities of Pompeii in 1995. It was aimed toward a more detailed documentation on the house of Caius Iulius Polybius, which is a very large and well preserved house despite the earthquake of 62 A.D. It is located in Via dell' Abbondanza.

Thirteen skeletons, whose bone fragments are the focus of this study, were found in different rooms

(1). The position of some skeletons at the time of their discovery, as can be seen in the archaeologists' sketches (Figs. 1 and 2), suggests that they belonged to the people who were closely related (Fig. 1). Figure 2 shows a skeleton of a young woman with the remains of an unborn child nearby, probably her child. Today's molecular biology allows us to try to reconstruct genealogical pedigrees by studying genetic markers such as nuclear satellite DNA sequences (2-6).

Figure 1. Drawings from the excavation notebooks of the Caius Iulius Polybius house. The skeletons shown were found in the room marked GG. The approximate height of the skeletons is indicated.

Figure 2. Drawings from the excavation notebooks of the finding of a skeleton of a young women and the remains of an unborn child found by her side.

Key Methods of Identification

An accurate gender determination comes from the study of genetic markers such as the amelogenin loci, which code for one of the structural proteins found in dentine. These loci are present on both the X- and Y-chromosomes, although with differences in their nucleotide sequence. Further confirmation of the molecular data on gender is provided by analyzing the alphoid repeat sequences locus, specific for Y chromosome (7,8).

Microsatellite sequences (2-3-4-5-6), such as the vWF (von Willebrand factor) locus (3), being highly polymorphic markers, are used for genetic linkage, mapping and personal identification. In fact, the analysis of these loci, inherited in mendelian fashion, are widely used in forensic medicine, including paternity testing, although a number of different microsatellite loci have to be tested to get reliable results.

In the aDNA study, microsatellite analysis is very useful in solving possible interrelationships or problems of exogenous DNA contamination.

DNA analysis makes it also possible to document the presence of people native to Africa in Pompeii and Herculaneum by determining the nucleotide sequence of hypervariable segments belonging to the region that controls the mitochondrial (mt) DNA replication machinery, currently believed to be the most accurate continent-specific marker (9-15). The most promising approach would be to align the sequence of hypervariable segments with the published results and determine the nearest neighbors by phylogenetic analysis (16).

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) represents the unique method of selectively studying specific DNA fragments starting from minute amounts of short DNA fragments to the aDNA (17-20).

The main problem with aDNA analysis concerns the authenticity of the extracted DNA. It stems from the possibility of contamination by recent exogenous DNA, due mainly to the mishandling of the findings at the archeological site or to laboratory contamination. While the latter one can be avoided by careful precautionary procedures and more sophisticated instrumentation and conditions, the proof of authenticity can be obtained only by independent experimental approaches that may vary widely. Concomitant evidence on aDNA authenticity may be gathered by procedures other than PCR, among them nitrogen content, bone preservation detection by microscopy, and amino acid racemization testing.

Moreover, analyzing loci with a high degree of hetherozygosis, like microsatellites sequences and finding allele pairs different from individual to individual, might exclude exogenous contamination from at least the molecular biology laboratory operator. In this last case, if contamination occurred, identical alleles would be found in all the samples.

Average Life of Nucleic Acid Molecules

Another problem, specific to the aDNA field of study, concerns the half-life of the DNA molecules, which is highly dependent on the preservation conditions of the finding. In fact, nucleic acids undergo spontaneous decomposition; the phosphodiesteric bonds of the RNA are particularly sensitive to hydrolysis. As for the DNA, the mechanism which mainly affects its degradation is depurinization (21). On the other hand, there are many physical factors, such as temperature, pH, oxidative stress, and dehydration, known to directly cause alterations in the DNA double helix, which can substantially modify its half-life. High temperature and an acidic pH induce the breakage of DNA filaments at the N-glycosidic bonds, while anoxia and dehydration favor their conservation (21,22).

Histological analysis performed along with the aDNA amplification provides additional criterion to readily ascertain whether the DNA extracted is exogenous or endogenous despite the fact that tissues stored for thousands of years under the ground can undergo alteration processes caused by physical (temperature), chemical (pH), and biological factors (fungi and bacteria) (23).

Methods

pH

The pH of the material that presumably covered the bone findings of Pompeii was measured using samples of the pyroclastic material which covers the skeleton of an equid still present today in the Casti Amanti house stable, adjacent to the Caius Iulius Polybius house. The technique consisted of mixing two parts of material with five parts of distilled water and stirring at room temperature for at least 24 hours before the pH measurement was found to be 7.5. This slightly alkaline value is in agreement with other values for aDNA in bone findings (24).

Histology

Cross sections from long bones, no thicker than 3 mm, were dehydrated and embedded in resin. Slides were ground to an uniform thickness (about 80 mm) by increasingly fine sandpaper and/or by a petrographic grinding wheel. All sections were observed by a Leitz (Orthoplan) light microscope under both transmitted and polarized light.

aDNA Analysis: Pre-Treatment of Samples

Although the bones had certainly been handled previously, the fragments were handled using disposable gloves during this study. The bone was first scraped to remove the surface contamination. Areas to be sampled were treated with 5% sodium hypochloride (26) and exposed to UV light for 10 min at 20 cm distance. UV light introduces, in exogenous DNA eventually present on bone surface, covalent bonds between two adjacent thymine bases giving the intrastrand pyrimidine dimer that prevents the amplification of such DNA.

The phases of sampling, extraction, and amplification were carried out in separate rooms.

aDNA Extraction and Amplification

DNA extraction has been performed following a silica-based purification method (27). Amelogenin loci amplifications were performed as reported elsewhere (28). The amplification reactions were carried out using dCTP labeled with 32P.

For the amplification of alphoid Y-specific sequences, the following procedure was used: 10 µL of aDNA extract were amplified in 50 µL reaction mixtures containing 1.5 mmol/L MgCl2, 50 mmol/L KCl, 10 mmol/L Tris/HCl (pH 9), 0.1% Triton X-100, 200 µmol/L each dNTP, 0.2 µmol/L primer, 1.25 U of Taq polymerase, 2 µCi of 32P dCTP. Amplification cycles were as follows: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min. Thirty five amplification cycles were started by an initial denaturation step (94 °C for 4 min). Contemporary male and female human samples as positive controls and two negative controls for PCR and extraction procedures, respectively, were routinely included for each amplification.

For the amplification of the wWF locus, the following procedure has been carried out: 15/20 µL of aDNA extract were amplified in 50 µL reaction mixtures containing 1.5 mmol/L MgCl2, 50 mmol/L KCl, 10 mmol/L Tris/HCl (pH 9), 0.1% Triton X-100, 200 µmol/L M each dNTP, 0.08 µmol/L primer, 2.5 U of AmpliTaq Gold polymerase. Amplification cycles were as follows: 95 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min. Forty amplification cycles were started by an initial denaturation step (95 °C for 10 min). Two negative controls for PCR and extraction procedures, respectively, were included. PCR product (1 µL) was reamplified in the same conditions utilizing 5 µCi (1 µCi/mL) 32P dCTP.

PCR inhibition test was performed by adding DNA of the lambda phage strain CI857S7 to each aDNA sample extract as described elsewhere (20). The presence or absence of inhibitors was detected by the lack or occurrence of amplification of a 350 base-pair DNA fragment of the phage.

Results

Qualitative histological analysis (25) was used to evaluate the degree of bone preservation. In this way, the following four classes have been defined from (Table 1): 1) good preservation, when the bone microstructure was completely preserved and the different structural elements (osteons, osteon fragment, and interstitial lamellar system) were clearly distinguishable under transmitted light and polarized light through the whole area of the bone section examined; 2) intermediate preservation, when the bone microstructure was partially altered (Fig. 3); 3) poor preservation, when the most or overall bone microstructure was altered; and 4) very poor preservation, when the overall microstructure was altered.

Table 1. State of bone preservation by histological analysis, PCR amplification data on sexing for amelogenin loci and Y alphoid repeat sequences and personal identification by wWF locus PCR

Figure 3. Cross section of the femur from the skeleton B found in Box 1 observed under crossed nicols of the polarizing microscope. The typical Maltese cross of the osteon can be seen near the periosteal border, as a proof of the integral structure of this osteon (x100).

The histological analysis of skeletons from the C. I. Polybius house showed that, in some specimens, bone microstructure was so well preserved that it was indistinguishable from that observed in fresh bone. In these specimens osteons, osteon fragments, and interstitial lamellar systems are clearly visible by light microscope under transmitted light. In addition, the integrity of the bone appeared also well preserved using polarized light, with a remarkably high birefringence (Fig. 3). Other bone samples showed a high degree of alteration or were completely altered, suggested also by the fact that the bone microstructure was generally preserved only in the external limiting (periosetal) zone of the transverse section.
Most of the time a good correlation between the preservation degree of bones and DNA amplifi- ability was observed.
The acrylamide gel electrophoresis of amplification products for Y alphoid repeat sequences and wWF locus are shown in Figures 4 and 5 respectively.
Amplification results are reported in Table 1. The amplification of loci specific for sex has been successfully carried out in eight individuals for amelogenin X and Y loci. As far as Y specific sequence locus is concerned, only in seven individuals out of nine tested, it has been possible to obtain a detectable amplification product. In some cases this last amplification confirms amelogenin results, as for the samples 1A, 2C, 3E, 5/6 A and 5/6 B; in some other cases the amplification of Y specific sequences allowed us to determine the sex of the individual in the absence of amelogenin loci amplification.

Figure 4. Gel electrophoresis of PCR products of the Y chromosome alphoid repeat sequences for sex determination. From left to right: the first two samples refer to the negative control of the experiment and of the PCR, respectively. Samples 7 and 5, from Herculaneum site, give rise to an amplification products of 154 bp specific for Y chromosome. Samples 23, 36 and 16 do not give rise to amplification. A modern male positive DNA amplification control is shown in the successive lane. The last lane shows a molecular weight marker.

Figure 5. Gel electrophoresis of PCR products of vWF locus for personal identification. From left to right: the first two samples refer to the negative control of the experiment and of the PCR, respectively. Lane 3 shows two amplification products of 142 and 146 bp suggesting this individual is hetherozygote for this character. Lane 4 shows only one amplification product of 150 bp suggesting this individual is homozygote for this character. Lanes indicated with capital letters ACTG refer to the nucleotide sequence of a genome fragment of the phage M13; in this experiment it has been used as a molecular weight marker.

Table 2. Genetic loci utilized for sex determination (amelogenin and alphoid repeat sequences) and for personal identification (vWF locus), respectively. The expected molecular weights of the correspondent amplified DNA fragments are shown for each locus

Last column of Table 1 shows amplification data obtained so far for the vWF locus. Although preliminar, these data indicate that it is possible to distinguish different alleles for this locus.
The expected molecular weights of the amelo- genin and Y alphoid repeat sequences amplified fragments are reported in Table 2. The length of the amplification products was 112 bp for the amelogenin fragment specific for the Y chromosome, 106 bp for that specific for the X chromosome and 154 bp for that specific for the Y chromosome alphoid repeat sequences. The lengths of the vWF locus amplified fragment ranged from 138 bp (9 TCTA repeats) up to 162 bp (15 TCTA repeats) (Table 2).
Discussion
The results of our study of aDNA extracted from bone findings of the Caius Iulius Polybius house until now suggest that this is human DNA, that it can be amplified, and that the amplifiability of single copy genes is in good agreement with the state of bone preservation and with the dynamics of the burial conditions subsequent to the 79 A.D. eruption.
Technical problems are still encountered while studying aDNA from human remains, such as the scarcity of DNA content from osteocytes and the extent of DNA molecule fragmentation.
The scarcity of aDNA extracts does not represent a serious obstacle to the analysis since the PCR amplification reaction allows the production of up to 1011 copies of the same DNA fragment, starting from a few molecules. On the contrary, the characteristic aDNA molecular fragmentation is a limiting factor in the choice of the molecular sequence to be amplified, since only fragments no longer than 150/200 bp can be usually analyzed for the amplification.

Diagenetic alterations in bone findings of an ancient necropolis are determined, in addition to the age of the finding, by the environmental conditions of the burial, including microbial contamination (bacteria and/or fungi) which often change bone structure. In fact, when aDNA can be found by direct staining after extraction and agarose gel electrophoresis, it is likely represented by DNA from contaminating microorganisms. In the case of the bone remains from Pompeii, however, DNA was never so abundant to be visible with ethidium bromide staining. Endogenous DNA becomes visible only after amplification.

In our study, the degree of bone conservation is in agreement with the successful amplification of the extracted aDNA (Table 1). Other authors already reported a significant correlation between the bone preservation and DNA amplifiability (29).

The greatest problem that arises in this type of research is the presence of DNA contaminating the findings, mainly coming from mishandling by archaeologists, anthropologists, or molecular biologists. Special measures should therefore be taken, such as sterility of the reagents and conduction of extraction and amplification processes in separate rooms. A further precaution that should be taken is to assess the allelic asset of the operator. Yet another problem is frequent presence of Taq DNA polymerase inhibiting molecules, mainly humic acids (polyphenols) and heavy metals. In order to remove these inhibitors, specific experimental protocols are employed (30), including dialysis, ionic exchange resins, NaOH washing, and large amounts of Taq DNA polymerase.

The possibility to explore the genetic characteristics of ancient populations by amplification and sequencing endogenous ancient DNA from human remains, collected in well studied necropolis, represents a new bridge between the world of exact sciences and the world of history. This new approach that involves different research fields, such as archaeology, anthropology, genetics, and molecular biology, allows the investigation of problems that until now were thought to be inaccessible to human knowledge.

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References

1 De Franciscis A. The Caius Iulius Polybius house. Rivista di Studi Pompeiani 1988;II:15-36.
2 Pena SDJ, Chakraborty R. Paternity testing in the DNA era. Trends in Genetics 1994;10:204-9.
3 Kimpton C, Walton A, Gill P. A further tetranucleotide repeat polymorphism in the vWF gene. Human Molecular Genetics 1992;1:287.
4 Polymeropoulos MH, Rath DS, Xiao H, Merril CR. Tetranucleotide repeat polymorphism at the human c-fes/fps proto-oncogene (FES). Nucleic Acids Res 1991;19:4018.
5 Polymeropoulos MH, Rath DS, Xiao H, Merril CR. Dinucleotide repeat polymorphism at the human CTLA4 gene. Nucleic Acids Res 1991;19:4018.
6 Gill P, Ivanov PL, Kimpton C, Piercy R, Benson N, Tully G, et al. Identification of the remains of the Romanov family by DNA analysis. Nature Genetics 1994;6:130-5.
7 Kogan SC, Doherty M, Gitscher J. An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. N Engl J Med 1987;317:985-90.
8 Human genes. Oligonucleotide handbook: II. Paris: Geneset; 1991.
9 Brown WM, George M, Wilson AC. Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci U S A 1979;76:1967-71.
10 Anderson S, Bankier AT, Barrell BG, De Bruijn MHL, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature 1981;290:457-65.
11 Wrischnik LA, Higuchi RG, Stoneking M, Erlich HA, Arnheim N, Wilson AC. Length mutations in human mitochondrial DNA: direct sequencing of enzymatically amplified DNA. Nucleic Acids Res 1987;15: 529-42.
12 Vigilant L, Stoneking M, Wilson AC. Conformational mutation in human mtDNA detected by direct sequencing of enzymatically amplified DNA. Nucleic Acids Res 1988;16:5945-55.
13 Vigilant L, Pennington R, Harpending H, Kocher TD, Wilson AC. Mitochondrial DNA sequences in single hairs from a southern African population. Proc Natl Acad Sci U S A 1989;86:9350-4.
14 Piercy R, Sullivan KM, Benson N, Gill P. The application of mitochondrial DNA typing to the study of white Caucasian genetic identification. Int J Legal Med 1993;106:85-90.
15 Torroni A, Lott MT, Cabell MF, Chen Y, Lavergne L, Wallance DC. mtDNA and the origin of

Caucasian: identification of ancient Caucasian-specific haplo- groups, one of which is prone to a recurrent somatic duplication in the D-loop region. Am J Hum Genet 1994;55:760-76.
16 Vigilant L, Stoneking M, Harpending H, Hawkes K, Wilson AC. African populations and the evolution of human mitochondrial DNA. Science 1997;253:1503-7.
17 Higuchi R, Bowman B, Freiberger M, Ryder OA, Wilson AC. DNA sequences from the quagga, an extinct member of the horse family. Nature 1984;312:282-4.
18 Paabo S. Molecular cloning of ancient Egyptian mummy DNA. Nature 1985;314:644-5.
19 Hagelberg E, Sykes B. Ancient bone DNA amplified. Nature 1989;342:487.
20 Cipollaro M, Di Bernardo G, Galano G, Galderisi U, Guarino F, Angelini F, et al. Ancient DNA in human bone remains from Pompeii archaeological site. Biochem Biophys Res Commun 1998;247:901-4.
21 Lindahl T. Instability and decay of the primary structure of DNA. Nature 1993; 362: 709-15.
22 Richter C, Park JW, Ames B. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc Natl Acad Sci U S A 1988;85:6455-67.
23 Grupe G, Dreses-Werringboer U. Decomposition phenomena in thin sections of excavated human bones In: Grupe G, Garland AN, editors. Histology of ancient human bone: methods and diagnosis, 1993. Proceedings of the Paleohistology Workshop; 1990 Oct 3-5; Gottingen, Germany. Berlin: Springer- Verlag; 1993. p. 27-36.
24 Rollo F. Prehistoric microbs: reality or fantasy? Le Scienze 1996;340;56-63.
25 Hedges REM, Millard AR, Pike AWG. Measurements and relationships of diagenetic alteration of bone from three archaeological sites. Journal of Archeological Science, 1995;22:201-9.
26 Prince AM, Andrus L. PCR: How to kill unwanted DNA. Biotechniques 1992;12:358-60.
27 Hoss M, Paabo S. DNA extration from Pleistocene bones by a silica-based purification method. Nucleic Acids Res 1993;21:3913-4.
28 Mannucci A, Sullivan K, Ivanov PL, Gill P. Forensic application of a rapid quantitative DNA sex test by amplification of the X/Y homologous gene amelo- genin. Int J Legal Med 1994;106:190-3.
29 Colson IB, Bailey JF, Vercauteren M, Sykes BC. The preservation of ancient DNA and bone diagenesis. Ancient Biomolecules 1997;1:109-17.
30 Goodyear PD, Maclaughlin-Black S, Mason IJ. A reliable method for removal of co-purifying PCR inhibitor from ancient DNA. Biotechniques 1994;16:232-5.

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