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CASE REPORT

Ilenia Pietrangeli,[1] B.S.; Vincenzo Caruso,[1] M.D.; Liana Veneziano,[2] Ph.D.; Aldo Spinella,[3] Ph.D.; Giovanni Arcudi,[4] M.D.; Emiliano Giardina,[1] Ph.D.; and Giuseppe Novelli,[1,5] Ph.D.

Forensic DNA Challenges: Replacing Numbers with Names of Fosse Ardeatine's Victims*

ABSTRACT: The Fosse Ardeatine massacre was a mass execution carried out in Rome on March 24, 1944 by Nazi German occupation troops during the Second World War as a reprisal for a partisan attack conducted on the previous day in central Rome. The 335 civilians were taken to the "Cave Ardeatine" and they were shot. Only 323 corpses out of 335 have been identified. The aim of this work is the genetic and anthropological analysis of the remains exhumed from grave number 329 of Fosse Ardeatine's Shrine to assess their identity. So far, such remains have been supposed to belong to MM but mitochondrial analysis excluded a biological relationship to two living maternal relatives. Our analysis indicated that remains recovered in grave number 329 do not belong to MM. This result suggests that genetic analysis of the remains should be also applied to the other 12 unknown corpses to elucidate their identity.

KEYWORDS: forensic science, mitochondrial analysis, anthropological, skeletal remains, mass disaster, DNA analysis

The identification of skeletal remains, murders or victims of mass disasters where bodies are undiscovered for many years is a problem of modern forensic medicine (1,2). The powerful techniques offered by molecular DNA analysis used in combination with conventional methods (i.e., anthropological and anthropometrical) increases the probability of obtaining a correct identification provided that suitable relatives can be found for comparison. When environmental insults severely degrade the integrity of nucleic acids leading to an insufficient amount of DNA to obtain a reliable identification, mtDNA analyses can be successfully performed (3,4). The high copy number of mtDNA molecules in each cell and its circular structure make it less prone to degradation.

More than 60 years after the end of the Second World War, many victims still have not been identified and today there is a growing public interest in DNA analysis for the identification of skeletal remains. Considering the temporal gap of 60 years, DNA analysis seems to be the only viable approach, as demonstrated by several research groups in Europe recently involved in identification by DNA typing of human remains of that period. Palo et al. (5) identified a large number of Finnish soldiers who lost their lives in the Soviet Union, and Marjanovic et al. (6) identified 15 remains out of 27 individuals found in two small mass graves uncovered in Slovenia. Both the studies are still ongoing to obtain as many identifications as possible.

Here, we report the results of anthropological and mitochondrial analysis of the remains exhumed from grave number 329 of Fosse Ardeatine's sacrarium. On March 23, 1944, in Rome, during the Nazi occupation, a group of patriots, belonging to the "Freeing National Committee," carried out an attack in Via Rasella, against an SS Company of the Bozen Battalion. Thirty-two men from the German Police were killed and many were injured. On that same day the German Command ordered that one communist should be shot for each German man killed in the attack.

Men, women, the elderly, and children were immediately arrested in the streets, houses, and shops of Rome and some of them were shot straight away. The massacre was not carried out against those who were responsible for the attack in Via Rasella, but against people of every age, social class, and religion, representative of every slice of the Italian population. On March 24, 1944, General Maeltzer from the German Command ordered that 335 men were taken from the two prisons of Regina Coeli and Via Tasso; SS Colonel Dollmann and Lieutenant Colonel Kappler were instructed to compile the lists of the massacre. The 335 civilians were brought to the "Cave Ardeatine," close to Rome, where, on March 24, 1944 they all were killed and the quarries were blown up. On July 26, 1944, the corpses were extracted to allow the forensic identification of the victims. Thus, 323 corpses out of 335 have been successfully identified. Merely one corpse out of the 75 Jews of the Juden List that Kappler filled in has not been identified, the one of MM, a salesman who was born in Rome on July 1, 1916. He was a partisan, and for this reason was arrested on February 18, 1944, and taken first to the prison of Via Tasso and then transferred to the one of "Regina Coeli." On June 6, 2006, MM's brothers, PM and AM asked for the possibility to identify the corpse contained in the grave 329 of the Fosse Ardeatine's sacrarium (upon which an unknown person put a David's star) given that they believe it belongs to their brother MM. In February 2007, the grave number 329 was excavated and bone fragments

[1]Department of Biopathology and Diagnostic Imaging, Centre of Excellence for Genomic Risk Assessment in Multifactorial and Complex Diseases, School of Medicine, University of Tor Vergata, Rome, Italy.
[2]Institute of Neurobiology and Molecular Medicine, National Council Research, Rome, Italy.
[3]Direzione Centrale Anticrimine, Servizio di Polizia Scientifica, Rome, Italy.
[4]Department of Public Health and Cell Biology, University of Tor Vergata, Rome, Italy.
[5]Fondazione Livio Patrizi, Rome, Italy.

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were recovered. To perform the mtDNA comparison, the two living maternal relatives (PM and AM) have been used as maternal references.

Methods

Bones consisting of left tibia, left femur, distal epiphysis of right tibia, and navicular of left foot were collected. To prevent possible contamination, all stages of the work were carried out under sterile conditions, using latex gloves and mouth masks to avoid contamination by skin cells or sweat. All appliances, containers, and work areas were cleaned and irradiated with UV (>1.0 J/cm) for at least 60 min. All steps (bone anthropological examination, bone cutting, surface removing, powdering, extractions, and amplifications) were carried out in separate places.

The outside layer of exposed bones was removed with a rotary sanding tool (Dremel, Racine, WI) in order to get rid of foreign DNA contamination. Bones were crushed into small fragments (2 cm^2) using a rotary tool. We collected five fragments from the left femur. No fragment was analyzed from the left tibia, right tibia and navicular. The resulting samples were pulverized using a blender cup and blender. Samples were processed individually and extracted in parallel with a reagent blank. About 3–4 g of each bone powders were incubated in 0.5 M EDTA, pH 8.0, on a rotor at 4°C for 1 week, with fresh EDTA changes every 3–4 h, to chelate high concentrations of calcium. Then the samples were centrifuged at 2000×*g* for 15 min and the supernatants were conserved and extracted in parallel with the precipitates. DNA was extracted by adding 1X Stain Extraction Buffer (Manufactured) containing (7): 10 mM Tris–HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 2% SDS, and 39 mM DTT and proteinase K (20 mg/mL) (Invitrogen, Carlsbad, CA). Then solutions were incubated at 56°C overnight. The solutions were extracted three times with phenol/chloroform/isoamyl alcohol (25:24:1) (Invitrogen). The aqueous phases was concentrated using Centricon YM30 (Millipore, Billerica, MA) (8,9). DNA was also extracted according to the method described by Salamon et al. (9), recently used to obtain large and well-preserved DNA fragments from crystal aggregates of fossil bones: decalcified bone was incubated in proteinase K buffer (10 mM Tris base, pH 8.0, 1 mM DTT, 50 mM EDTA, 100 mM NaCl, 0.5% SDS and 20 mg/ml proteinase K) for 10–45 min at 60°C and continued overnight at 37°C. The DNA was then extracted with 1 vol of 25:24:1 phenol:chloroform:isoamyl alcohol saturated with 10 mM Tris base (pH 8.0) and 1 mM EDTA and 2 vol of 1-butanol (Invitrogen) (9). The extracted DNA was purified and concentrated by using a YM30 unit (Millipore) to a final volume of 250 μL. DNA was isolated from saliva samples collected from two maternal relatives of MM using the DNA purification kit (Promega, Madison, WI) according to the manufacturer's directions. PM and AM gave their informed consent to participate in this analysis.

DNA Quantification and Short Tandem Repeats Amplification

DNA concentration was determined using Quantifiler Human DNA Quantification Kit (Applied Biosystems, Foster City, CA) as described previously (10). The reaction was carried out in ABI 7500 Real-Time PCR System according to the manufacturer's recommendations. The AmpFlSTR® Identifiler® PCR Amplification Kit (Applied Biosystems) was used to simultaneously amplify 15 STR loci: D16S539, D2S1338, D19S433, D13S317, D8S1179, vWA, D21S11, D7S820, CSF1PO, D3S1358, THO1, D18S51, D5S818, FGA, TPOX and AMEL for gender determination. Amplification was carried out as described previously (11). The total volume of each reaction was 25 μL. The PCR amplification was carried out in 9700 Gene Amp PCR System Thermal Cycler (Applied Biosystems), according to the manufacturer's recommendations. The number of cycles was increased to 32. Electrophoresis of the amplification products was preformed on an ABI PRISM 310 Genetic Analyzer. The raw data were compiled and analyzed using accessory software GeneMapper™ 3.2 (Applied Biosystems).

Mitochondrial DNA Amplification and Sequencing

Both hypervariable mtDNA regions (HVI and HVII) were sequenced for all samples collected. PCR amplification of degraded remains is generally more successful if smaller sequences are amplified. Because of that we amplified each region separately using three overlapping pairs of primers. The hypervariable region I (bases 16023 to 16365) was amplified by primer set FBIA1- B1: F15971/R16410 (440 base pairs), primer set PSI: F15971/R16258 (288 base pairs), and primer set PSII: F16144/R16410 (267 base pairs). The hypervariable region II (bases 73 to 340) was amplified by primer set FBIC1-D1: F29/R389 (361 base pairs), primer set PSIII: F15/R270 (256 base pairs), and primer set PSIV: F155/R381 (227 base pairs) (12,13). PCR amplifications was carried out separately for each primer set using 5–10 μL of extraction product in 30 μL reaction volume containing final concentration of 1X True Allele Master Mix (Applied Biosystems) and 0.2 μM of each primer (Invitrogen). The amplicons were denaturized at 95°C (5 min) and then put through 32 reaction cycles: 95°C for 30 sec, 61–63°C for 40 sec and 72°C for 40 sec. Amplicons were purified by enzymatic reaction (1U Exonucleasi I and 2U Alkaline phosphates) (Ambion, Austin, TX) and directly sequenced by cycle sequencing with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using the same primers as for the amplification. The sequencing reactions were carried out in a final volume of 20 μL containing 4 μL BigDye Terminator RR Mix, 2 μL 5X Big Dye Sequencing Buffer, 10 pmol primer, and 4–6 μL PCR product. Cycling was performed (after a first denaturation step of 96°C, 1 min) for 28 cycles of 10 sec at 96°C, 5 sec at 50°C and 2 min at 60°C. Each template was sequenced in both forward and reverse directions using the amplification primers. Sequencing reaction products were purified from residual dye terminators using CentriSep columns (Princeton Separations, Adelphia, NY). Electrophoretic separation was carried out on ABI Prism 310 Genetic Analyzer. Sequences were aligned and compared to the revised Cambridge Reference Sequence (rCRS) (14,15) using the sequence analysis and alignment software SeqScape (Version 2.0, Applied Biosystems), following international guidelines for mtDNA typing (16,17). The mtDNA profiles were exported as mutation reports from SeqScape program. The double evaluation and electronic export of the profiles ascertained the quality of the profiles in terms of interpretation of heteroplasmy.

Results and Discussion

Anthropometrical Consideration

Anthropometrical analysis suggested that all bones collected belong to the same skeleton, although showed a severe state of degradation, probably due to the wet and acid soil condition of Fosse Ardeatine's sacrarium. The whole left tibia was very friable with a maximal length of 32.1 cm, while the left femur lacking distal head was ineffective for anthropometrical purposes. The distal epiphysis of right tibia and navicular of left foot were very friable and ineffective for anthropometrical measures. We tried to estimate

TABLE 1—*Variations in HVI and HVII regions respect rCRS observed in mtDNA extracting from grave n. 329, AM and PM.*

HVI Region 16023–16365	16126	16172	16192	16270	16294	16311	HVII Region 72–340	73	152	263	309.1	315.1
rCRS	T	T	C	C	C	T	rCRS	A	A	A	–	–
Grave n. 329	C/T	T	C	C	C/T	T	Grave n. 329	G	G	A	–	C
AM	T	C	T	T	T	C	AM	G	A	G	C	C
PM	T	C	T	T	T	C	PM	G	A	G	C	C

the height of the individual from long bone length. The tibia is one of the commonly used long bones for stature estimation. We used the Trotter-Gleser methods (18,19) for estimating height. The maximal height of the individual must had been around 156.68 ± 3.27 cm. This value has been calculated using the following formula: 2.52 cm × length of tibia + 75.79 ± 3.37 cm = 156.68 ± 3.27 cm.

STRs and Mitochondrial Analysis

STR analysis under different PCR conditions was carried out for all samples, even in the case of negative results of quantification. As expected, all samples did not give evidence of positive STR amplification. Thus, to carry out a genetic comparison between samples, we decided to perform a mtDNA comparative analysis (3,4). Complete sequences of HVI and HVII regions have been obtained from left femur remains following the above described protocols. The mtDNA sequence of remains was compared with two maternal relatives of MM: PM and AM (Table 1). The mtDNA sequence of remains showed two heteroplasmic sites in HVI region (16126 and 16270) in all extracts from the left femur. The sequence heteroplasmy was defined as a situation in which two nucleotides were observed in a single position (20,21). Heteroplasmy is quite common (22) especially when only small amounts of mtDNA are accessible (23). Sequence analysis of HVII region from bone remains revealed three nucleotide variations compared with rCRS (Table 1). Identical mtDNA sequences were observed from blood of PM and AM showing five differences to rCRS in HVI and four differences in HVII. Comparative analysis of mitochondrial sequences showed seven different nucleotide variations between the bone remains and two maternal relatives of MM. An exclusion is evident when two or more nucleotides differ between the sequences of two different samples. As the samples did not originate from the same person or one maternal lineage (24,25), our analyses indicate that the grave number 329 does not hold MM, but one of the other 11 unknowns.

Conclusions

The Fosse Ardeatine massacre was a mass execution carried out in Rome on March 24, 1944 by Nazi German occupation troops during the Second World War. The 335 selected civilians were killed. Only 323 corpses have been successfully identified. Here, we report the first effort to attribute a name to one of the 12 unknown corpses. Molecular and anthropological analyses were challenging because of the poor state of the remains, but we excluded that grave number 329 contains MM. It should be emphasized that this kind of identification can be performed only if suitable relatives are available for comparison. Many of these relatives are aged, which makes it more urgent to solve these cases. These results confirm the usefulness of molecular genetics for forensic medicine and strongly suggest its further application to identify the 12 unknown corpse remains and replace the numbers on the graves with the names of 12 civilians killed in March 1944.

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Additional information and reprint requests:
Giuseppe Novellim, Ph.D.
Laboratorio di Genetica Medica
Azienda Ospedaliera Policlinico Tor Vergata Viale Oxford 1
00133 Rome
Italy
E-mail: novelli@med.uniroma2.it