Int J Legal Med (2000) 113 : 98–101 © Springer-Verlag 2000

ORIGINAL ARTICLE

R. Lleonart · E. Riego · M. V. Saínz de la Peña
K. Bacallao · F. Amaro · M. Santiesteban · M. Blanco
H. Currenti · A. Puentes · F. Rolo · L. Herrera
J. de la Fuente

Forensic identification of skeletal remains from members of Ernesto Che Guevara's guerrillas in Bolivia based on DNA typing

Received: 19 January 1999 / Received in revised form : 15 April 1999

Abstract We report the positive identification of several members of the guerrillas led by Ernesto "Che" Guevara on the 1960 s in Bolivia by means of DNA fingerprinting. Successful DNA typing of both short tandem repeat loci and the hypervariable region of the human mitochondrial DNA was achieved after extracting total DNA from bones obtained from two burial sites. Given the size of the Cuban database for the STR allele frequencies, a conservative approach was followed to estimate the statistical significance of the genetic evidence. The estimated probabilities of paternity for the two cases in which the paternity logic was applied were higher than 99%. One case was analyzed using mitochondrial DNA and could not be excluded from the identity proposed by the forensic anthropology team. A fourth case was identified by exclusion, on the basis of the positive identification of the other remains, the historical and other anthropological evidence.

Key words Short tandem repeat · DNA typing · Polymerase chain reaction · Paternity analysis

Introduction

The growing knowledge in the field of molecular biology has boosted the power of human identification techniques. Advances such as the use of the polymerase chain reaction have allowed the forensic investigator to positively identify persons based on the analysis of very small amounts of tissue.

In March 1995 a commission was created by the Bolivian government to find the remains of the Che Guevara's guerrilla members which were buried somewhere in the countryside during the 1960 s [Anonymous (1997), Granma June 26:3]. Soon the group became international, including forensic scientists from Argentina, Cuba, and Bolivia. After several months of historical research and work in the field, remains from several bodies were found in two burial sites. The site on Cañada del Arroyo contained the potential remains of the Bolivians Jaime Arana Campero "Chapaco" and Francisco Huanca Flores "Pablito", the Peruvian Lucio Edilberto Galván "Eustaquio", and the Cuban Octavio de la Concepción de la Pedraja "Moro". The second site in Florida contained the remains of the Cuban Carlos Coello "Tuma". In order to further assess the identity of the remains, DNA testing was considered imperative for the case of the two Cubans and the Peruvian Lucio Edilberto Galván "Eustaquio".

Until now the most promising approach to analyze the polymorphisms of the DNA and thus establish the genetic identity of remains is the one based on the short tandem repeats (STR)[1–3]. These loci are repeats of three to seven bases and are highly polymorphic in human populations [4–6]. The analysis of STR by the use of the polymerase chain reaction (PCR) has aided the process by allowing the use of small amounts of DNA.

Such DNA typing using the standard forensic specimens (fresh tissue, sperm, hairs) has been reported to be reasonably straightforward [7]. However, this is not the case when only very old samples are available, such as old bones and teeth. Several protocols have been described to obtain PCR competent DNA for STR and mitochondrial analysis from forensic samples [8–11] but many factors can adversely influence the performance of the technique when such materials are used [12].

Using a procedure based on the use of protease, detergent, organic extraction and a final step of spin dialysis, DNA was obtained which allowed successful typing from

R. Lleonart (✉) · E. Riego · L. Herrera · J. de la Fuente
Mammalian Cell Genetics Division, Center for Genetic, Engineering and Biotechnology, P.O. Box 6162, Havana, Cuba
e-mail: orgsup@cigb.edu.cu, Fax +537–218070

M. V. Saínz de la Peña · M. Santiesteban · H. Currenti
A. Puentes
Laboratorio Central de Criminalística, Zulueta 465, Habana Vieja, Havana, Cuba

K. Bacallao · F. Amaro
Instituto de Medicina Legal, Apartado 10600, Havana, Cuba

M. Blanco · H. Currenti · A. Puentes · F. Rolo
Laboratorio de Investigaciones sobre Sida, Havana, Cuba

bones that had been buried for 30 years in the tropical conditions of Bolivia. The analysis confirmed the identities proposed by the forensic anthropologists. The statistical significance of the genetic evidence was estimated from the allelic frequencies observed on a sample of the Cuban population (manuscript in preparation).

Materials and methods

DNA extraction and purification from bone

Bone samples were obtained, coded by a third party and delivered to two different laboratories (1. Center for Genetic Engineering and Biotechnology, Havana,Cuba, and 2. the AIDS Research Laboratory, Havana, Cuba) to be typed blind. The whole procedure was organized following strict standard rules to avoid PCR contamination.

Bone fragments were reduced to powder by the use of a Universal Mill M-20(AGEM, Cuba). From these 5 g powder was decalcified in 0.5 M EDTA pH 7.5, 3 times for 12h each and washed twice in sterile distilled water. The remaining pellet was suspended in lysis buffer (10 mM Tris-HCl pH 8, 10 mM EDTA, 100 mM NaCl, 2% SDS, 0.5mg/ml Proteinase K). After 12 h at 56 °C with slow shaking, Proteinase K was added to a final concentration of 1mg/mL, and incubation continued for additional 24 h. The lysate was extracted in phenol/chloroform (1 : 1) once and the aqueous phase was extracted with ether and heated for 10 min at 55 °C to eliminate organic traces. The remaining DNA solution was concentrated by Centricon 100 (Amicon, USA) to a final volume of 40–100 µl and washed twice with sterile distilled water. The DNA concentration in the final extracts was determined by dot blot hybridization to a human satellite DNA probe following the instructions of the manufacturer (Quantiblot, Perkin Elmer, USA).

STR typing by PCR

Various amounts (from 0.1 to 2 µl) of the extracted DNA were used in PCR reactions containing primer sets to amplify the STR loci HUMCSF1PO, HUMTHO1, HUMTPOX, HUMF13A01, HUMFESFPS, HUMBFXIII, HUMHPRTB, HUMVWFA31, and HUMLIPOL [1,13]. PCR reactions also contained 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM each dNTP, 12.5 pmol each oligonucleotide, and 0.5–10 units of *Thermus aquaticus* (Taq) DNA Polymerase (Heber Biotec, Cuba) in a final volume of 25 µl. In cases where obvious inhibition of the enzyme was present, acetylated BSA (Biolabs, USA) was included at a final concentration of 170 µg/ml. A Minicycler (MJ Research, USA) was employed for the systems HUMBFXIII, HUMFESFPS, HUMHPRTB, HUMLIPOL and HUMVWFA31 (96 °C for 2 min, then 10 cycles of 94 °C for 1 min, 60 °C for 1 min, 70 °C for 1.5 min, then 20 cycles of 90 °C for 1min, 60 °C for 1 min, 70 °C for 1.5 min). For HUMCSF1PO, HUMF13A01, HUMTH01 and HUMTPOX the same program was used except for an annealing temperature of 64 °C.

After completion of the reaction, 3 µl was mixed with the same amount of loading buffer (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol), heated for 2 min at 95 °C and immediately loaded on a denaturing polyacrylamide gel (6% acrylamide, 0.31% bisacrylamide, 7M urea, 0.5X tris borate buffer). Electrophoresis was carried out in 0.5X Tris borate buffer [14] at constant power so that the gel temperature was kept at 55 °C. After an adequate time (depending on the marker to be resolved) the gels were satined with silver using the instructions of the manufacturer (Promega, USA). Genotyping for each sample was done by visual comparison of the resulting pattern with allelic ladders containing all the known alleles (Promega, USA).

Mitochondrial DNA analysis

The same PCR reaction conditions and sets of primers previously described [15] to amplify the hypervariable regions of the human mitochondrial DNA were used to obtain mtDNA sequences from the bone. The thermal cycling was 96 °C for 2min; then 36 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1min. The product of the correct size was subcloned into the pGEM5ZF T-vector (Promega, USA) and both strands sequenced on a Vistra 725 Automated sequencer using Texas Red labeled forward and reverse primers (Amersham, UK). Each resulting sequence was compared to the Anderson reference sequence [16] and differences were recorded.

Database preparation

Human blood samples obtained at random at the Cuban Institute of Legal Medicine were used to extract DNA by the Chelex method [7]. A total of 60 samples were typed using the described STR systems and the allele frequencies were estimated by the allele counting method. Further validation of the database for population studies and forensic purposes is being submitted for publication elsewhere.

Statistical analysis

In order to estimate the significance of the observed match at the STR loci, the logic of paternity analysis was followed. The paternity index (PI) was calculated as described [17, 18] for each locus and a combined PI was obtained as described for independently segregating alleles. Using the combined PI, the probability of paternity (W) was estimated for each case [13, 17, 18].

Results and discussion

Sections of femoral shafts were used in all cases because of their good condition compared to other bones. The skeletal remains were in general very poorly preserved as expected for a soil burial site in nearly tropical conditions. The outer layers were removed under sterile conditions and pieces of about 5 g were pulverized and subjected to DNA extraction.

For each bone sample delivered to the laboratories, DNA was extracted twice along with the blank extractions required to check for the presence of contemporary DNA contamination. Both extractions where successful in all cases, although the quantity and quality of the purified DNA proved to be very variable when final extracts were checked for the presence of human DNA and Taq Polymerase inhibitors (data not shown). Due to this fact, the use of acetylated BSA was necessary in all cases and higher amounts of polymerase per reaction was also required for some samples. The presence of Taq polymerase inhibitors in DNA samples from bone has been widely reported, although not extensively studied [19–21].

From the remains found at the burial site on Cañada del Arroyo, those of the Bolivian Jaime Arana Campero "Chapaco" were successfully identified by anthropological means. For the identification of the putative remains of the Cubans Carlos Coello "Tuma" and Octavio de la Concepción de la Pedraja "Moro", the logic of paternity cases was followed as living relatives of the deceased men were available.

Table 1 Statistical evaluation of STR data corresponding to the bone DNA sample thought to belong to Carlos Coello "Tuma" and his wife and son

Locus	Genotypes			Paternal allele freq.	Number of individuals in database	Upper 95% limit of allele freq.	Paternity index (for 95% upper limit)	Probability of paternity (W, %)
	Wife	Son	Bone					
HUMVWFA31	16 18	16 17	16 17	0.297 0.121	37	0.401 0.195	2.56	71.9
HUMTH01	6 9	6 9	6 9	0.200 0.230	50	0.278 0.312	1.69	62.8
HUMTPOX	8 8	8 12	11 12	0.260 0.040	50	0.345 0.078	6.41	86.5
HUMCSF1PO	11 11	11 13	13 14	0.102 0.030	49	0.161 0.063	3.1	75.6
HUMF13A01	4 4	4 7	4 7	0.100 0.200	45	0.161 0.282	1.77	63.8
HUMFESFPS	8 12	8 11	7 11	0.010 0.456	46	0.03 0.557	0.897	47.2
HUMBFXIII	6 9	9 10	9 10	0.265 0.351	47	0.354 0.447	1.11	52.6
HUMLIPOL	10 11	10 12	10 12	0.467 0.250	46	0.568 0.338	1.47	59.5
HUMHPRTB	9 11	11 11	11 11	0.153	39	0.232	4.31	81.1
					Cumulative values:		952.4	99.89

Table 2 Statistical evaluation of STR data corresponding to the bone DNA sample thought to belong to Octavio de la Concepción de la Pedraja "Moro" and his wife and son

Locus	Genotypes			Paternal allele freq.	Number of individuals in database	Upper 95% limit of allele freq.	Paternity index (for 95% upper limit)	Probability of paternity (W, %)
	Wife	Son	Bone					
HUMVWFA31	16 16	15 16	15 17	0.162 0.121	37	0.245 0.195	2.04	67.1
HUMTH01	6 9	9 9	6 9	0.200 0.230	50	0.278 0.312	1.6	61.53
HUMTPOX	9 11	9 12	8 12	0.490 0.040	50	0.587 0.078	6.41	86.5
HUMCSF1PO	10 12	11 12	11 11	0.336	49	0.429	2.33	69.96
HUMF13A01	5 5	5 7	6 7	0.222 0.200	45	0.307 0.282	1.77	63.89
HUMFESFPS	11 11	10 11	10 13	0.293 0.065	46	0.386 0.115	1.29	56.33
HUMBFXIII	8 9	8 10	8 10	0.170 0.351	47	0.245 0.447	1.11	52.6
HUMLIPOL	10 11	10 10	10 10	0.467	46	0.568	1.76	63.76
HUMHPRTB	12 13	12 13	13 13	0.358	39	0.464	1.07	51.69
					Cumulative values:		232.6	99.57

Table 1 shows the results of the STR typing data on the sample from the potential remains of Carlos Coello "Tuma". No exclusion pattern was observed when the genotypes were compared to those from his son and wife. The statistical analysis of the data revealed a probability of paternity of 99.89% (Table 1).

The paternity analysis performed on the putative remains of the Cuban Octavio de la Concepción de la Pedraja "Moro" and his wife and son also revealed a high probability of paternity of 99.57% (Table 2). These results, when considered together with the anthropological evidences strongly argue for the proposed identities in the cases of the two Cubans, even when the estimates are very conservative. This was mainly given by the consideration of the upper 95% confidence limit for the allele frequencies due to the relative small number of individuals in the database (Tables 1 and 2).

The remains of the Bolivian Francisco Huanca Flores "Pablito", also found at the Cañada del Arroyo site, were identified by exclusion, considering the positive

identification of the other three men and the historical evidence.

The potential remains of the Peruvian Lucio Edilberto Galván "Eustaquio" could not be identified by the paternity analysis approach as only a brother was available for comparison. Given the hereditary characteristics of the human mitochondrial DNA, the analysis of hypervariable control regions from both samples (bone and brother) was done to attempt the identification of the remains. When both strands of the amplified hypervariable region 1 (HV1) were sequenced, a polymorphism was reproducibly found on the mtDNA from the brother (A→G, 16181 at the Anderson reference sequence) which was not present in the bone mtDNA. This result was unable to support neither a positive identification of the remains nor an exclusion although the burden of historical and anthropological evidence, now enhanced with additional evidence (the positive identification of other men in the same grave), allowed the forensic researchers to conclude that the remains belonged to the Peruvian Lucio Edilberto Galván "Eustaquio".

Although the analysis of the hypervariable regions in the mitochondrial DNA is already a very promising tool to exclude maternal relatedness, several events have been described which may hamper such a conclusion. Errors introduced by the polymerase while using a damaged template [22], amplification of nuclear insertions of mitochondrial sequences [23] and new mutations leading to homoplasmy or heteroplasmy [24], have been described and might explain the differences found in this study between the two potential brothers.

These results contributed to confirm the power and the usefulness of the DNA typing as a means, in some cases unique, to assess the identity of old human remains. Although the samples analyzed for this study were about 30 years old, much older bones have been successfully typed by using this procedure in our laboratory [25, and unpublished results]. The positive identification of members of the Ernesto "Che" Guevara guerrilla is important for both historical and humanitarian reasons and it is a key part of an on–going effort to find and identify all the members of that group who died in battle in Bolivia.

Acknowledgements We deeply thank V. Jiménez for oligonucleotide synthesis. We also thank Dr. Jorge González, Dr. Hector Soto, and the rest of the forensic anthropology team for fruitful discussions.

References

1. Hammond HA, Jin L, Zhong Y, Caskey CT, Chakraborty R (1994) Evaluation of 13 short tandem repeat loci for use in personal identification applications. Am J Hum Genet 55 : 175–189
2. Robertson JM, Sgueglia JB, Badger CA, Juston AC, Ballantyne J (1995) Forensic applications of a rapid, sensitive, and precise multiplex analysis of the four short tandem repeat loci HUMVWF13/A, HUMTH01, HUMF13A1, and HUMFES/FPS. Electrophoresis 16 : 1568–1576
3. Evett IW, Gill PD, Scranage JK, Weir BS (1996) Establishing the robustness of short-tandem-repeat statistics for forensic applications. Am J Hum Genet 58 : 398–407
4. Litt M, Luty JA (1989) A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am J Hum Genet 44 : 397–401
5. Tautz D (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acid Res 17 : 6463–6471
6. Weber JL, May PE (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet 44 : 388–396
7. Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10 : 506
8. Hagelberg E, Clegg JB (1991) Isolation and characterization of DNA from archaeological bone. Proc R Soc Lond B Biol Sci 244 : 45–50
9. Hochmeister MN, Budowle B, Borer UV, Eggmann U, Comey CT, Dirnhofer R (1991) Typing of deoxyribonucleic acid (DNA) extracted from compact bone from human remains. J Forensic Sci 36 : 1649–1661
10. Corach D, Penacino G, Sala A (1994) Cadaveric DNA extraction protocol based on cetyl trimethyl amonium bromide (CTAB). Acta Med Leg Soc (Liege) 44 : 35–36
11. Höss M, Pääbo S (1993) DNA extraction from pleistocene bones by silica-based purification method. Nucleic Acid Res 21 : 3913–3914
12. Höss M, Jaruga P, Zastawny TH, Dizdaroglu M, Pääbo S (1996) DNA damage and DNA sequence retrieval from ancient tissues. Nucleic Acid Res 24 : 1304–1307
13. Alford RL, Hammond HA, Coto I, Caskey CT (1994) Rapid and efficient resolution of parentage by amplification of short tandem repeats. Am J Hum Genet 55 : 190–195
14. Sambrook J, Frisch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
15. Wilson MR, Polanskey D, Butler J, DiZinno JA, Replogle J, Budowle B (1995) Extraction, PCR amplification and sequencing of mitochondrial DNA from human hair shafts. Biotechniques 18 : 662–669
16. Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin LC, Eperon IC, Nierlick DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG (1981) Sequence and organization of the mitochondrial genome. Nature 290 : 457–465
17. Morris JW, Sanda AI, Glassberg J (1989) Biostatistical evaluation of evidence from continuous allele frequency distribution deoxyribonucleic acid (DNA) probes in reference to disputed paternity and identity. J Forensic Sci 34 : 1311–1317
18. Pohl B (1982) Probability of paternity: Logic II. In: Silver H (ed) Probability of inclusion in paternity testing: A technical workshop. American Association of Blood Banks, Arlington VA, pp 61–70
19. Hagelberg E, Bell LS, Allen T, Boyde A, Jones SJ, Clegg JB (1991) Analysis of ancient bone DNA: techniques and applications. Philos Trans R Soc Lond B Biol Sci 333 : 399–407
20. Hagelberg E, Hedges R (1989) Ancient bone DNA amplified. Nature 342 : 485
21. Holland MM, Fisher DL, Mitchell LG, Rodríguez WC, Canik JJ, Merril CR, Weedn VW (1993) Mitochondrial DNA sequence analysis of human skeletal remains: identification of remains from the Viet Nam war. J Forensic Sci 38 : 542–553
22. Pääbo S, Irwin DM, Wilson AC (1990) DNA damage promotes jumping between templates during enzymatic amplification. J Biol Chem 265 : 4718–4721
23. Collura RV, Steward CB (1995) Insertions and duplications of mtDNA in the nuclear genomes of Old World monkeys and hominoids. Nature 378 : 485–488
24. Gill P, Ivanov PL, Kimpton C, Piercy R, Benson N, Tully G, Evett I, Hagelberg E, Sullivan K (1994) Identification of the remains of the Romanov family by DNA analysis. Nat Genet 6 : 130–135
25. Riego E, Lleonart R, Sainz de la Peña MV, Amaro F, Bacallao K, Santiesteban M, Rolo F, Herrera L, Blanco M, de la Fuente J (1998) Obtención de ADN útil para análisis de tipaje genético a partir de restos óseos humanos antíguos. Biotecnología Aplicada 15 : 25–29