DNA Typing from Skeletal Remains: Evaluation of Multiplex and Megaplex STR Systems on DNA Isolated from Bone and Teeth Samples

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INTRODUCTION

Various forensic techniques are used today to identify a human corpse, depending on the circumstances and the state of remains. The four most common methods are identification of the remains by a living person who knew the deceased by direct facial recognition or recognition of special features, such as scars or marks (tattoos); matching of fingerprints; dentition; and DNA analysis.

We describe here our work on the identification of persons killed in wars in Croatia and Bosnia and Herzegovina between 1991 and 1995 whose remains were found in several mass graves. We also describe our work on other forensic bone and teeth samples. In every case, forensic examiners performed a detailed examination of the clothing and belongings of the dead, described special features, analyzed skeletal remains to estimate sex and height, and compared premortem and postmortem dental records (1). In addition, X-ray comparisons were performed for bone morphology as well as the superimposition of the skull and photographic images. Unfortunately, the standard forensic identification methods were not sufficient in 30–35% of all victims, so DNA identification was requested.

We describe here our work on the identification of persons killed in wars in Croatia and Bosnia and Herzegovina between 1991 and 1995 whose remains were found in several mass graves.

The problem that forensic scientists most often face when working with DNA extracted from bones and teeth samples recovered from mass graves or mass disasters is either DNA degradation or contamination. Various methods have been used to improve the identification of skeletal remains by DNA technology. Most of these involve either short tandem repeat (STR) or mitochondrial DNA analysis. The ability to analyze, by polymerase chain reaction (PCR)-based methods, trace amounts of human DNA isolated from old teeth and bone samples (2–4) offers the opportunity to identify unknown skeletal remains by a comparative genetic analysis with their presumptive relatives.

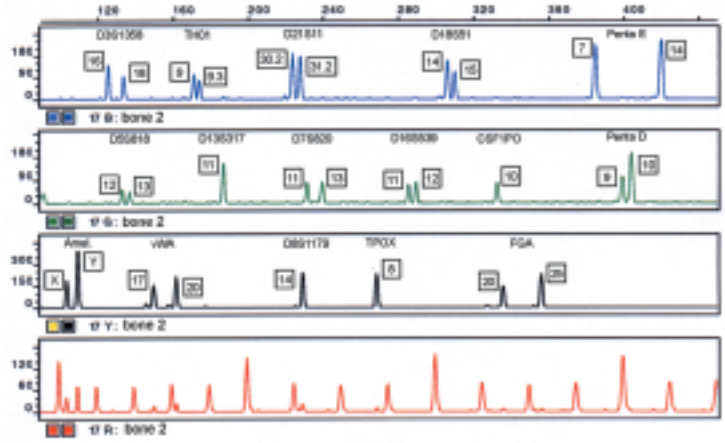
The AmpliType® PM+DQA1 PCR Amplification and Typing Kit, which we used at the beginning of the identification process, proved useless in 75% of all cases (5). Common problems with this system were either amplification difficulties or nonspecific hybridization that caused ubiquitous data.

In the majority of analyzed bone and teeth DNA extracts, we observed the presence of minimal amounts (pg) of degraded human DNA mixed with high amounts of microbial DNA. Therefore, we have also evaluated the influence of this "junk" microbial DNA on human DNA typing by performing a microbial DNA challenge study.

LABORATORY ORGANIZATION

To minimize the risk of contamination, the bone and teeth DNA extractions and amplifications were set up in different laminar flow cabinets (with dedicated equipment) in a dedicated pre-PCR laboratory, separate from the pre-PCR laboratory set for reference sample extractions and amplifications. Ultraviolet (UV) irradiation and treatment with 10% bleach was used to eliminate possible DNA contaminants from cabinets and laboratory surfaces. All reagents, plastic tips provided with filters, and tubes were sterilized by autoclave and exposed to UV light before use. Safety glasses and

Panel A



Panel B

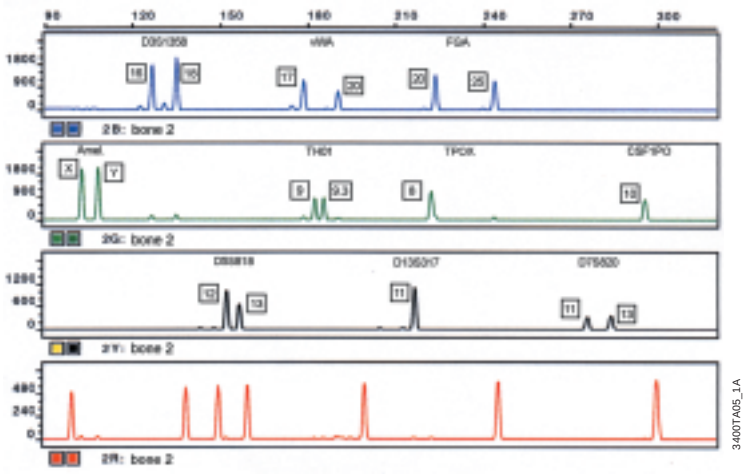


Figure 1. DNA extracted from 12-year-old bone sample discovered in a cave. DNA was amplified using the PowerPlex® 16 System **(Panel A)** and the AmpFlSTR® Profiler® Kit **(Panel B)**.

disposable laboratory caps, coats and gloves were mandatory items during extraction and amplification. Both extraction and PCR reagent controls were run in every case to properly monitor the occurrence of contamination. The DNA profile of every person from the laboratory was available for comparisons. Two different investigators on each case performed at least duplicate extractions.

ANALYZED DNA SAMPLES

We analyzed the following samples:

1. Twenty-one DNA extracts from blood and bloodstain reference samples (Instituto Nacional de Toxicología) and 105 DNA extracts from bloodstain reference samples (Split University Hospital).
2. Ten DNA extracts from teeth samples (10–30pg/µl nuclear human DNA, as revealed by QuantiBlot® analysis).
3. Thirty-six DNA extracts from bone samples (Instituto Nacional de Toxicología) and 40 DNA extracts from bone samples (Split University Hospital). In all cases, QuantiBlot® analysis revealed no detectable nuclear human DNA.
4. Thirty-two DNA extracts from different microorganisms (bacteria and yeasts).

DNA ISOLATION

The Split Laboratory for Clinical and Forensic Genetics and the Instituto Nacional de Toxicología followed two different protocols: A and B, respectively.

Protocol A. This protocol is a modification of previously described procedures (6,7). All bones discovered either at mass graves or crime scenes were cleaned from the remnant soft tissue and all soil traces. Additionally, the bone surfaces were brushed in warm water with mild detergent. After sampling, the bones were rinsed with distilled water several times and left to air-dry. The external and internal surfaces of the bone specimens were removed by linear sawing (2–3mm deep) with a K9-Installation EWL 900-with foot control unit (KaVo Elektrotechnisches Werk, Vertriebsgesellschaft GmbH, Leutkirch, Germany). The samples were cleaned from sawdust with cotton presoaked in 5% commercial bleach and standard dental carbon brushes. Sawing time (contact with the bone) was limited to up to 3 seconds, since longer exposure may cause heating of the bone, resulting in rapid DNA damage. Approximately 2–3 grams of each bone specimen was obtained.

In a laminar flow hood, bone fragments were washed with deionized water three times (30–40 seconds each), twice with 80% ethanol and once in a 50ml conical tube filled with 5% commercial bleach (10 seconds). Fragments were poured into clean, labeled weigh boats and allowed to air-dry in the laminar flow hood for 24 hours. The samples were then placed in steel-plated chambers, crushed with a hammer and pulverized into fine powder in liquid nitrogen. It was useful to keep the steel chamber and the bone separate for at least 15–20 minutes in the liquid nitrogen before the pulverizing process. The pulverized bone was stored in a cool, dry and dark environment until the next step. For longer storage, pulverized bone was stored at –20°C. Three milliliters of the extraction buffer (10µmol/L Tris [pH 8.0], 100µmol/L NaCl, 50µmol/L EDTA [pH 8.0] and 0.5% SDS) and 100µl of 20mg/ml proteinase K were added to the specimens and the reagent blank (15ml conical tubes were used). One hour after the incubation, the samples were mixed thoroughly and the caps of the tubes were resecured. The bone dust was suspended in the reagents and incubated overnight at 56°C. The following morning a second sample of 2ml of extraction buffer with proteinase K was added and incubation was extended for an additional 5 hours. The sample was then thoroughly mixed and extracted with 5ml of phenol/chloroform/isoamyl alcohol (25:24:1). The procedure was repeated at least twice, until the upper aqueous layer was completely clear.

The aqueous layer was extracted with 3ml n-butanol and thoroughly mixed, then centrifuged for 2 minutes at 4,950 × *g* (6,400rpm). The lower aqueous layer was transferred to the corresponding Centricon®-100 concentrators. The concentrators were centrifuged at 1,000 × *g* (2,600rpm) for approximately 30 minutes. After discarding the filtrate, 2ml of

sterile TE buffer (pH 8.0) were added to the sample reservoir. The tube was centrifuged at 1,000 × *g* (2,600rpm) for 30 minutes or longer, until approximately 40–50µl of retentate remained. The filtrate was discarded and the procedure was repeated twice.

The retentate was pipetted 8–10 times and transferred directly to a sterile, labeled microcentrifuge tube. The membrane was rinsed with a volume of TE buffer necessary to adjust the final volume to approximately 100µl. Samples were stored at 4°C if amplified within 3 weeks or at –20°C for longer storage. In most cases, 1µl of the filtrate obtained after DNA extraction was used for PCR amplification.

Protocol B. DNA was extracted from bone and teeth samples by proteolytic digestion followed by phenol/chloroform purification and Centricon®-100 filtration. In the case of bones, both the outer and the inner medullar surfaces were first removed by sanding, and approximately 1–3 grams of compact bone were ground into fine powder using a 6750 freezer mill. The following parameters were programmed on the mill: 15 minutes precooling, 1 minute grinding, 2 minutes cooling and 1 minute grinding with an impact frequency of 10 impacts/second. In the case of teeth samples, the outer surfaces were first extensively washed with distilled and sterile water and then each side was irradiated with UV light for 30 minutes. The teeth sample (two pieces, if possible) was crushed to a fine powder by hammering the washed dental pieces between two steel plates or by using a 6700 freezer mill programmed as previously described. Each sample of approximately 0.5 grams of the teeth or bone powder was mixed thoroughly with 2ml of 0.5mol/L EDTA (pH 8.0) containing 1mg proteinase K plus 0.5% SDS and 0.04mol/L DTT, then incubated at 56°C overnight. The mixture was then centrifuged and each milliliter of the supernatant was extracted once with 200µl phenol/chloroform/isoamyl alcohol (25:24:1). The resultant aqueous phases were washed three times with 2ml of TE buffer (10µmol/L) using two Centricon®-100 microconcentration devices per sample. DNA extracts were concentrated to 80–150µl.

DNA QUANTITATION

Total DNA was evaluated by agarose gel electrophoresis and ethidium bromide staining. Human DNA was determined by slot-blot hybridization with primate-specific D17Z1 alpha-satellite probe using the QuantiBlot® assay.

DNA REPURIFICATION PROCEDURE

DNA from each substrate that failed to amplify initially or after standard inhibitor troubleshooting strategies (heat soak, hot start, BSA, extra *Taq* and extensive dilution) were subjected to NaOH treatment (8). Approximately 30–50µl of DNA were placed into a Microcon®-100 unit along with 200µl of 0.4mol/L NaOH. The volume was reduced to 5µl by centrifugation at 500 × *g* and the eluate was discarded. The chamber was refilled with 400µl of 0.4mol/L NaOH and centrifuged once more as described (8). The sample was neutralized by washing once with 400µl of Tris and recovered in 15µl of 10mmol/L Tris (pH 7.5). The quantity and quality of DNA were determined by standard agarose gel electrophoresis and the QuantiBlot® assay.

DNA extracts that showed inhibition of *Taq* DNA polymerase were further purified using the QIAamp® DNA blood midi kit following the manufacturer's protocol (9).

DNA AMPLIFICATION AND TYPING

Amplifications were performed on the Perkin-Elmer thermal cyclers 480, 2400, 9600, or 9700 using the PowerPlex® 16 System[a,b] (10), the AmpFlSTR® Profiler® PCR Amplification Kit, the AmpFlSTR® Profiler Plus™ PCR Amplification Kit or the AmpFlSTR® COfiler™ PCR Amplification Kit according to the manufacturers' protocols (11–13). Typing of PCR products was performed on the ABI 377 DNA sequencer with 5% Long Ranger® gels or the ABI PRISM® 310 Genetic Analyzer. The recommended parameters for GeneScan® analysis were followed. Automatic assignment of genotypes was performed with the Genotyper® software and the PowerTyper™ Macro (used with the PowerPlex® 16 System).

RESULTS

BONE SAMPLES WITH NO DETECTABLE HUMAN DNA

The first case we analyzed was DNA extracted from a 12-year-old bone sample discovered in a cave. Figure 1 shows successful DNA amplification with the PowerPlex® 16 System and the AmpFlSTR® Profiler® PCR Amplification Kit.

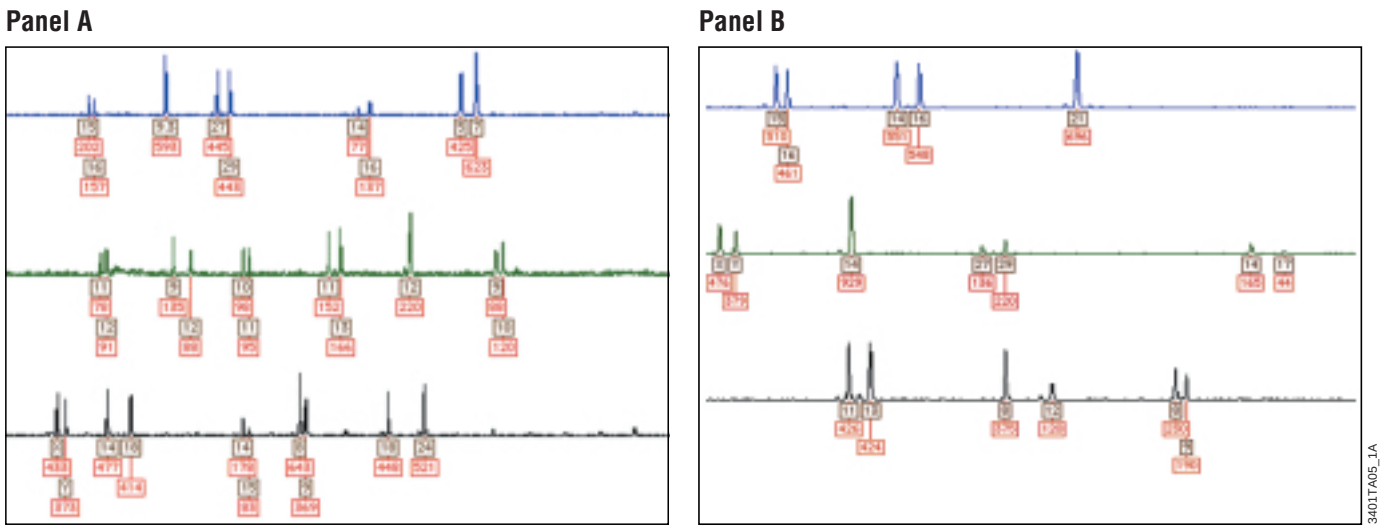


Figure 2. DNA was isolated from an 8-year-old bone sample with no detectable amounts of nuclear human DNA. Multilocus profile obtained with the PowerPlex® 16 System **(Panel A)** and AmpFlSTR® Profiler Plus™ **(Panel B)**. Note the extensive dropout of some alleles.

In some cases, however, the phenomenon of random allelic dropout, a consequence of very low human DNA template input in PCR, was observed with the three multiplex STR systems (Figure 2). This was one of the main causes of failure to obtain a complete multilocus STR profile from these difficult samples.

The third case was the DNA analysis of a 9-year-old bone sample discovered in a mass grave found in Bosnia and Herzegovina. DNA was successfully amplified with both the PowerPlex® 16 System and the AmpFlSTR® Profiler® PCR Amplification Kit, respectively (Figure 3).

We also performed a simple experiment to evaluate the influence of "junk" microbial DNA on the slot-blot human DNA quantitation system to demonstrate that nondetectable human DNA does not mean the absence of human DNA. The mixture of low amounts of human DNA (pg) with high amounts of microbial DNA (µg) can interfere with the specific hybridization of human sequences in a slot-blot format (data not shown).

TEETH SAMPLES WITH DETECTABLE AMOUNTS (PG) OF HUMAN DNA

We analyzed 10 DNA extracts obtained from different teeth samples (corresponding to different identification cases from Bosnia and Herzegovina and to a Spanish paternity test in a deceased father case). Concordant results were obtained for the 13 STR Combined DNA Index System (CODIS) core loci between PowerPlex® 16 and AmpFlSTR® Profiler Plus™, AmpFlSTR® COfiler™ (Figure 4). The PowerPlex® 16 System also offered robust typing results for the Penta E and Penta D markers (Figure 2, Panel A).

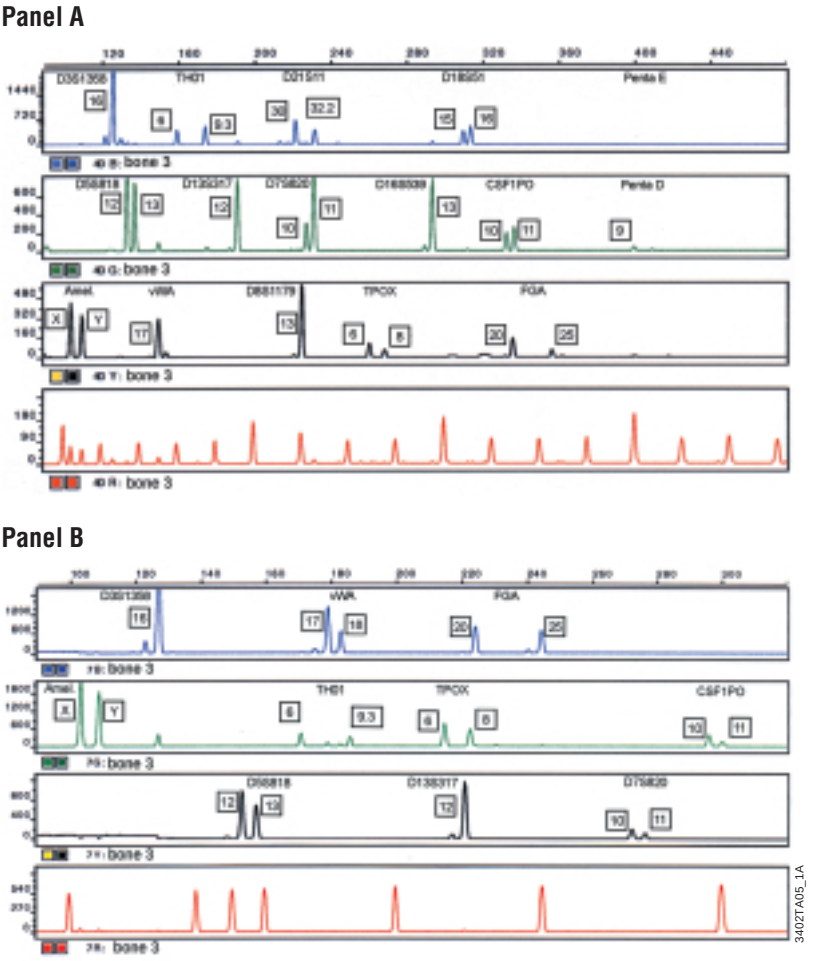


Figure 3. DNA extracted from 9-year-old bone samples discovered in a mass grave. DNA was amplified with the PowerPlex® 16 System **(Panel A)** and the AmpFlSTR® Profiler® Kit **(Panel B)**.

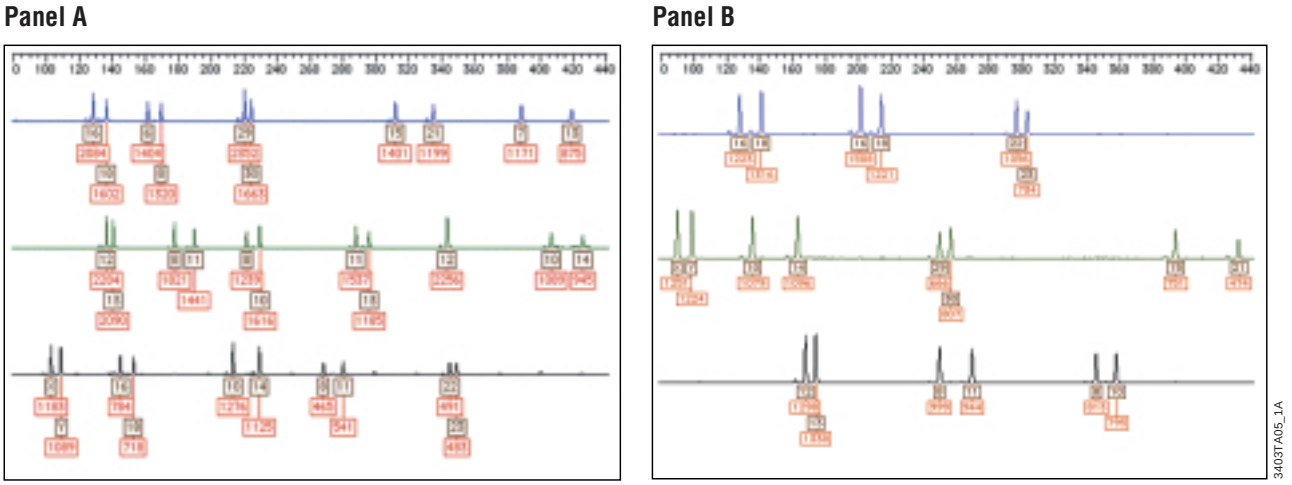


Figure 4. DNA sample isolated from an 8-year-old sample of teeth. Complete multilocus STR profile obtained with the PowerPlex® 16 System **(Panel A)** and AmpFlSTR® Profiler Plus™ Kit **(Panel B)**.

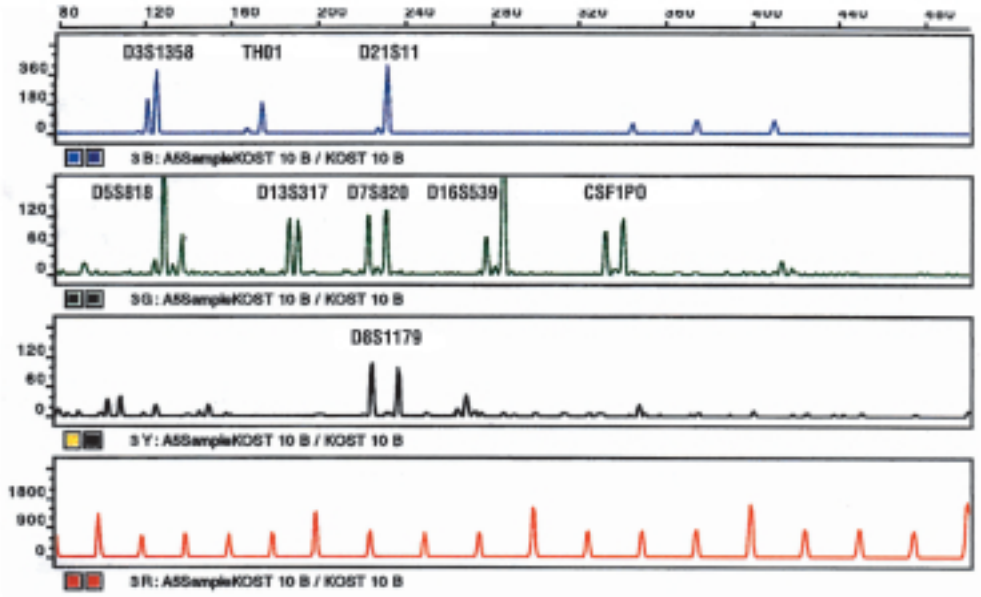


Figure 5. Multilocus STR profile obtained with the PowerPlex® 16 System from a 9-year-old bone sample after additional NaOH repurification procedure. The bone sample failed to amplify completely prior to NaOH treatment.

NONSPECIFIC PRODUCTS

In 10 DNA extracts, corresponding to 5 different identification cases from the war in Bosnia and Herzegovina, we observed the amplification of 202bp and 308bp extra peaks, during PowerPlex® 16 analysis. These were not recognized as alleles and therefore, did not influence the automatic assignment of genotypes performed using the PowerTyper™ 16 Macro software. The 202bp and 308bp fragments could be generated by a nonspecific PCR amplification of bacterial DNA template present in these samples. These extra peaks were seen in both the JOE (green) and TMR (yellow) channels, indicating that they can be generated with one primer labeled with 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxy-fluorescein (JOE) and the other labeled with carboxy-tetramethylrhodamine (TMR). To identify the source of the bacterial DNA present in the bone and teeth samples responsible for the extra peaks, we amplified and sequenced the first 500bp of the bacterial 16S rRNA gene from these samples. The sequence obtained was searched against over 11,000 bacterial sequences by use of the BLAST network service at the Swiss Institute of Bioinformatics (SIB) with the following results: *Pseudomonas halodenitrificans*: 97% sequence identities (463/476), Gaps = 1% (5/476).

During PowerPlex® 16 analysis, we also observed amplification of a nonspecific 340bp peak from the bone DNA extracts of a deceased presumptive father in a Spanish paternity case.

MICROBIAL DNA CHALLENGE STUDY

A total of 32 microbial DNA samples were amplified with AmpFlSTR® Profiler Plus™, AmpFlSTR® COfiler™ and PowerPlex® 16. None of the tested microbial DNA templates yielded any detectable PCR product within the range of size variability of the human STR markers, except in the case of *Rhodotorula glutinis* and *Morganella morganii*, which yielded green peaks of 202bp, 208bp and 150bp, respectively, with the PowerPlex® 16 System.

DNA REPURIFICATION STUDY

DNA from each substrate that failed to amplify initially, or after standard inhibitor neutralization strategies, was subject to repurification with NaOH. The bone sample that failed to amplify before NaOH treatment successfully amplified in 9 of 16 loci available in the PowerPlex® 16 System (Figure 5) after treatment.

DISCUSSION

This collaborative study was aimed at improving identification techniques based on the analysis of genomic DNA. The data indicated that the AmpFlSTR® Profiler® Kit, AmpFlSTR® Profiler Plus™ Kit, AmpFlSTR® COfiler™ Kit and the PowerPlex® 16 System are extremely sensitive multiplex STR amplification systems. They have been successfully used to obtain multilocus STR profiles from old teeth and bone samples with minimal amounts (pg) or even no detectable amounts of human DNA.

In our experience and according to others (14–16), the quality of DNA extracted from teeth is usually higher than that of DNA from bones. In addition, the quality of DNA obtained from long bones is higher than that extracted from skulls or ribs.

We demonstrated that high amounts of microbial DNA (µg) can interfere with the specific hybridization of human sequences in a slot-blot format, rendering false negative results on the human DNA quantitation of bone and teeth DNA samples. Some nonspecific fragments have been observed with some teeth and bone samples when using the PowerPlex® 16 System but have not influenced the assignment of genotypes. The microbial DNA challenge study demonstrated that bacteria and yeast DNA templates could be the source of these nonspecific PCR products. Further research is needed to evaluate the incidence of these extra peaks in casework. Random allelic dropout was the most common artifact observed with the three multiplex STR systems when analyzing bone DNA samples with trace amounts of human DNA. Therefore, the authenticity of the typing results must be based on the reproducibility of different PCR amplifications (with different DNA input) from at least duplicate DNA extracts. In some cases, increasing the amount of DNA input helped to overcome this problem. In other cases inhibitors rendering negative typing results also increased.

The use of silica-based purification methods has proven to be an efficient procedure to remove or attenuate inhibition (17,18).

The data indicated that the AmpFlSTR® Profiler® Kit, AmpFlSTR® Profiler Plus™ Kit, AmpFlSTR® COfiler™ Kit and the PowerPlex® 16 System are extremely sensitive multiplex STR amplification systems. They have been successfully applied to obtain multilocus STR profiles from old teeth and bone samples with minimal amounts or even no detectable amounts of human DNA.

However, this only worked in a few cases when we used the QIAamp® DNA blood midi kit. This indicates that the inhibitors could be closely associated with the DNA molecules or that "junk" microbial DNA itself, comprising the majority of these DNA extracts, could inhibit the PCR amplification of minimal amounts of human DNA.

The procedure published by Bourke *et al.* (8) was developed to overcome potential inhibitors of *Taq* DNA polymerase when DNA failed to amplify initially or after standard inhibitor neutralization troubleshooting strategies. While working with AmpliType® PM+DQA1 PCR Amplification and Typing Kit and AmpliFLP™ D1S80 PCR Amplification Kit, we applied this procedure to the DNA extracted from bone samples that failed to amplify with either PowerPlex® 16 or Profiler®/Profiler Plus™ systems. We were able to amplify on average 5–8 loci that originally failed to be amplified. Although the mechanism of how NaOH helps amplification is not clear, it has been proposed that denaturing conditions release intercalated inhibitors and that denaturing washes allow for their removal (8). Further, it is also possible that alkaline (or denaturing) conditions alone could inactivate the inhibitors, thus obviating the necessity for NaOH washes and potentially increasing the quantity/quality of DNA recovered. However, we do not recommend the use of NaOH when the quantity of DNA is limited, since the treatment results in significant loss of DNA.

It appears though that the identification of skeletal remains by STR analysis is sufficient in the large percent of analyzed cases. However, due to the high genome copy number per cell (500–1,000), mitochondrial DNA analysis often succeeds in cases where two-copy nuclear markers fail (4,7). Immobilized sequence-specific oligonucleotide probe analysis may be particularly helpful in the analysis of a large number of samples, since the sequence-specific oligonucleotide approach can provide unambiguous and reliable data that can contribute to the identification and dramatically reduce the number of samples required for sequence analysis.

ACKNOWLEDGMENT

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[a,b]Refer to patent and disclaimer statements on page 2.