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

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Aim. To evaluate the performance of three multiplex short tandem repeat (STR) systems (Amp*fl*STR Profiler™, Amp*fl*STR Profiler Plus™, and Amp*fl*STR COfiler™), and a megaplex STR system (PowerPlex™ 16) on DNA extracted from the skeletal remains. By performing a microbial DNA challenge study, we also evaluated the influence of microbial DNA on human DNA typing.

Methods. A subset of 86 DNA extracts isolated from 8-50 years old bone and teeth samples, corresponding to 20 identification cases from mass graves in Croatia and Bosnia and Herzegovina, and to 4 paternity cases involving deceased parents in Spain, were analyzed by the above systems.

Results. Bone samples with no detectable human DNA (tested with Quantiblot), as well as teeth samples with detectable human DNA, were successfully amplified. Surprisingly, even in highly degraded samples, PowerPlex™ 16 offered very robust amplification for the both Penta E and Penta D markers. We observed a few non-specific extra peaks of 202 and 308 base pairs, which appeared to match 16S rRNA of the *Pseudomonas halodenitrificans*.

Conclusion. Amp*fl*STR Profiler™ Kit, Amp*fl*STR Profiler Plus™ Kit, the Amp*fl*STR COfiler™ Kit, and the *PowerPlex*™ 16 system are very sensitive multiplex STR amplification systems, which can be successfully used to obtain a multilocus STR profile from old teeth and bone samples with minimal amounts (pg) of human DNA or even with no detectable human DNA.

Key words: *base sequence; Bosnia and Herzegovina; Croatia; DNA; DNA bacterial; DNA fingerprinting; forensic medicine; molecular sequence data; paternity; polymerase chain reaction; Spain; war crimes*

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 (if pre-mortem inked prints are available); dentition (if premortem dental records are available); 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, as well as our work on some other forensic bone or 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 dental records with 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 and DNA identification was requested.

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 DNA contamination. Various methods have been used to improve the identification of skeletal remains by DNA technology. Most of these systems include either short tandem repeat (STR) analysis 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. On the other hand, the most common strategy used for parentage testing in cases involving deceased parents is the analysis of exhumed teeth and bone samples from the alleged father.

The AmpliType® PM+DQA1 PCR Amplification and Typing Kit (Applied Biosystems, Foster City, CA, USA), which we used at the beginning of identification process, soon 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.

Material and Methods

Laboratory Organization

To minimize the risk of contamination, the bone and teeth samples' extractions and amplifications were set up in different laminar flow cabinets (with dedicated equipment) in a dedicated Pre-PCR laboratory, which was separated from the Pre-PCR laboratory where the reference samples' extractions and amplifications were set up. Ultra-violet (UV) irradiation and treatment with 10% bleach were 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 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 additional 105 DNA extracts from bloodstain reference samples (Split University Hospital).

2. Ten DNA extracts from teeth samples (10-30 pg/μL nuclear human DNA, as revealed by Quantiblot).

3. Thirty-six DNA extracts from bone samples (Instituto Nacional de Toxicología) and additional 40 DNA extracts from bone samples (Split University Hospital). In all cases, Quantiblot (Applied Biosystems) 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 procedures: protocol A and protocol 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 (2-3 mm deep) with a K9 Foot control unit, type 900 (KaVo Elektrotechnisches Werk, Vertriebsgesellschaft GmbH, Leutkirch, Germany). The samples were cleaned from sawdust first with cotton presoaked in 5% commercial bleach, and then with standard dental carbon brushes. Sawing time (contact with the bone) was limited to up to 3 s, since the longer exposure may cause heating of the bone resulting in rapid DNA damage. Approximately 2.0-3.0 g of each bone specimen was obtained. Bone fragments were weighed in a weight boat and placed into the laminar flow hood.

After being washed in a deionized water three times, each time for 30-40 s, and twice in the 80% ethanol, bone fragments were again washed once, separately, in a 50 mL conical tube filled with 5% commercial bleach (10 s). During this process, the bones were gently agitated in either water or ethanol solution. Bone fragments were poured into clean, labeled weight boats and allowed to air dry in the laminar flow hood for 24 h. The samples were placed in the steel-plated chambers, crushed with a hammer, and pulverized into a fine powder in liquid nitrogen. We found it useful if the steel chamber and the bone were kept separately at least 15-20 min in the liquid nitrogen before the pulverizing process. The bone should be pulverized into a very fine powder before the addition of extraction buffer. The pulverized bone was stored in a cool, dry, dark environment until the next step. For a longer storage, pulverized bone was stored at –20ºC. Three mL of the extraction buffer (10 μmol/L Tris, pH 8.0; 100 μmol/L NaCl; 50 μmol/L ethylenediaminetetraacetic acid [EDTA], pH 8.0; and 0.5% sodium dodecyl sulphate [SDS]) and 100 μL of 20 mg/mL proteinase K (PK) were added to the specimens and the reagent blank (15 or 50 mL 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 56ºC. The following morning a second sample of 2 mL of extraction buffer with PK was added and incubation was extended for additional 5 h. Afterward, the sample was thoroughly mixed and extracted with 5 mL of phenol/chloroform/isoamyl alcohol (25:24:1). The procedure was repeated at least twice, until an upper aqueous layer was completely clear.

The aqueous layer was extracted with 3 mL n-butanol and thoroughly mixed, then centrifuged 2 min at 4,950 G in a Beckman GPR Centrifuge (Beckman Coulter, Inc, Fullerton, CA, USA) (6,400 RPM) or the IEC Centra MP4 (IEC International Equipment Company, Needham Heights, MA, USA) (5,000 RPM). The lower aqueous layer was transferred to the corresponding Centricon-100 concentrators (Note: pipetting any residual n-butanol should be avoided and pipet tip wiped with a Kim-Wipe to remove any carry-over n-butanol). Centricon-100 concentrators were centrifuged in the IEC Centra MP4 at 1,000 G (2,600 RPM) for approximately 30 min. After discarding the filtrate, 2 mL of sterile TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0) were added to the sample reservoir. A tube was centrifuged at 1,000 G (2,600 RPM) in the IEC Centra MP4 for 30 min or longer, until approximately 40-50 μL of retentate remained. The filtrate was discarded and the procedure was repeated twice.

The retentate (40-50 μL) was pipetted up and down 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 100 μL. Samples were stored at 4ºC if amplified within 3 weeks or at –20ºC if stored for a longer time. In most cases, 1 μL of the filtrate obtained after DNA extraction was used for the amplification by PCR. When working with DNA extracted from the skeletal remains, the pipettor should not touch the sides of the sample reservoir. We also noticed that better data were obtained if the volume was not spun down to recover DNA. Therefore, one should pipet the liquid off of the membrane, add a sample of TE buffer, pipet vigorously to remove DNA from the membrane, and transfer it to a clean tube.

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 g of compact bone were grounded into a fine powder by use of a 6750 freezer mill. The following parameters were programmed on the mill: 15 min precooling, 1 min grinding, 2 min cooling, and 1 min grinding with an impact frequency of 10 impacts/s. 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 min. 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 g of the teeth or bone powder was mixed thoroughly with 2 mL of 0.5 mol/L EDTA (pH 8.0) containing 1 mg proteinase K, plus 0.5% SDS, and 0.04 mol/L dithiothreitol (DTT), and incubated at 56ºC overnight. The mixture was then centrifuged and each mL of the supernatant was extracted once with 200 μL phenol/chloroform/isoamyl alcohol (25:24:1). The resultant aqueous phases were washed three times with 2 mL of TE buffer (10 μmol/L) by the use of two Centricon-100 microconcentration devices per sample. DNA extracts were concentrated to 80-150 μL.

DNA Quantitation

Total DNA was evaluated by agarose gel electrophoresis and ethidium bromide staining. Human DNA was determined by slot-blot hybridization with the primate-specific D17Z1 alfa-satellite probe by use of Quantiblot.

NaOH Repurification Procedure

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

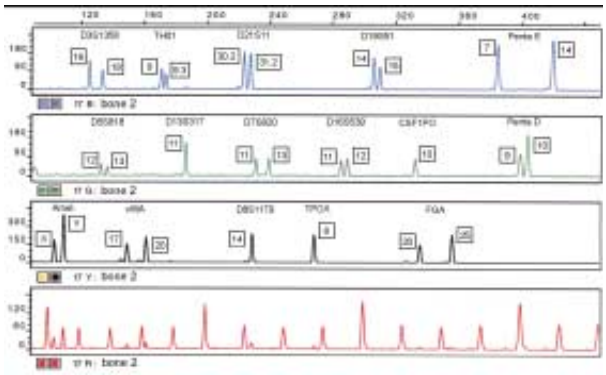

Figure 1. Electropherogram of DNA extracted from the 12-year-old bone sample discovered in the cave and amplified with the Amp*fl*STR Profiler™.

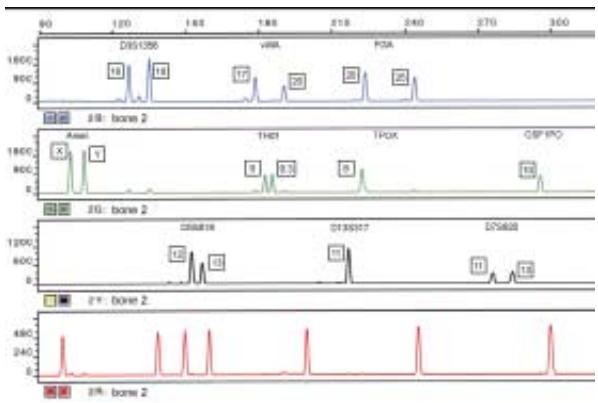

Figure 2. Electropherogram of DNA extracted from the 12-year-old bone sample discovered in the cave and amplified with the Amp*fl*STR Profiler™.

Silica-based DNA Repurification Procedure

Some DNA extracts that showed inhibition of TAQ polymerase were further purified using the QIAamp DNA blood Midi Kit (Qiagen Inc., Valencia, CA, USA) following the manufacture protocol (9).

DNA Amplification and Typing

Amplifications were performed on the Perkin-Elmer thermal Cyclers 480, 2400, 9600, or 9700 (Applied Biosystems) using the *PowerPlex*™ 16 System (Promega Corporation, Madison, WI, USA) (10), the Amp*fl*STR Profiler™ PCR Amplification Kit, the Amp*fl*STR Profiler Plus™ PCR Amplification Kit, or the Amp*fl*STR COfiler™ PCR Amplification Kit (Applied Biosystems) according to the manufacturer's protocols (11-13). Typing of PCR products was performed on ABI 377 DNA sequencer (Applied Biosystems) with 5% Long Ranger gels or on ABI Prism 310 Genetic Analyzer (Applied Biosystems). The recommended parameters for *GeneScan* analysis with a peak amplitude threshold of 50-150 relative fluorescent units (RFU) were followed. Automatic assignment of genotypes was performed with Genotyper software.

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.

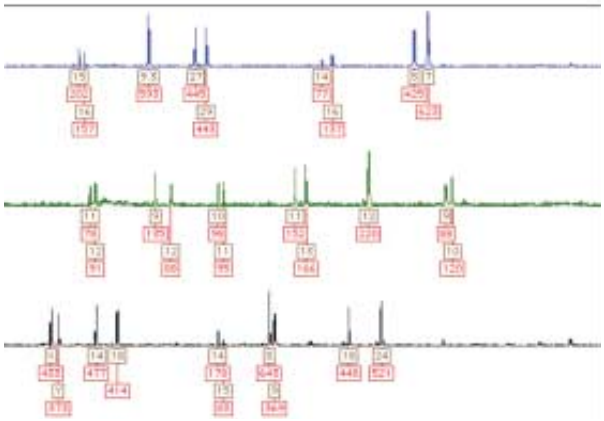

Figure 3. Multilocus STR profile obtained with PowerPlex™ 16 kit from a DNA sample isolated from an 8-year-old bone sample with no detectable amounts of nuclear human DNA. Note the extensive dropout of some alleles.

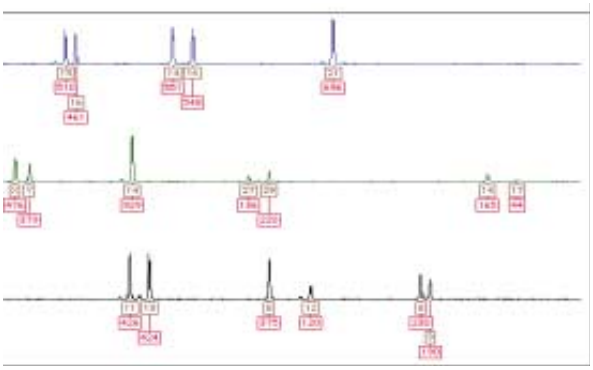

Figure 4. Multilocus STR profile obtained with Amp*fl*STR Profiler Plus™ from a DNA sample isolated from an 8-year-old bone sample with no detectable amounts of nuclear human DNA. Note the extensive dropout of some alleles.

Figures 1 and 2 show successful DNA amplification with PowerPlex™ 16 kits and Amp*fl*STR Profiler™ PCR Amplification Kit, respectively.

In some cases, however, the phenomenon of random allelic dropout, which is a consequence of very low human DNA template input in the PCR reaction, was the most common artifact observed for the three multiplex STR systems (Figs. 3 and 4). This was one of the main causes of failure to obtain a complete multilocus STR profile from these difficult samples.

The third case is 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 systems using PowerPlex™ 16 kits and Amp*fl*STR Profiler™ PCR Amplification Kit, respectively (Figs. 5 and 6).

We also performed a simple experiment to evaluate the influence of the "junk" microbial DNA on the slot-blot human DNA quantitation system to demonstrate that non-detectable human DNA, as revealed by Quantiblot, does not mean the absence of human DNA. Figure 7 shows that the mixture of low amounts of human DNA (in the range of pg) with high amounts of microbial DNA (μg) can interfere with the specific hybridization of human sequences in a slot-blot format.

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 (STR CODIS) core loci between Amp*fl*STR Profiler Plus™ (Fig. 8) and Amp*fl*STR COfiler™ (not shown), and PowerPlex™ 16 (Fig. 9). PowerPlex™ 16 also offered very robust typing results for the Penta E and Penta D markers (Fig. 3).

Non-specific Products

In 10 DNA extracts (2 from teeth samples and 8 from bone samples), corresponding to 5 different identification cases from the war in Bosnia and Herzegovina, we observed, during PowerPlex™ 16 analysis, the amplification of some extra peaks of 202 and 308 base pairs (bp), which were not recognized as alleles and therefore did not influence the automatic assignment of genotypes performed by use of PowerTyper 16 Macro software (Promega). These 202 and 308 bp fragments could be generated by a non-specific PCR amplification of bacterial DNA template present in these samples. These extra peaks were seen in both the JOE (green) channel as well as in the TMR (yellow) channel, 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) (Fig. 10). In attempting to

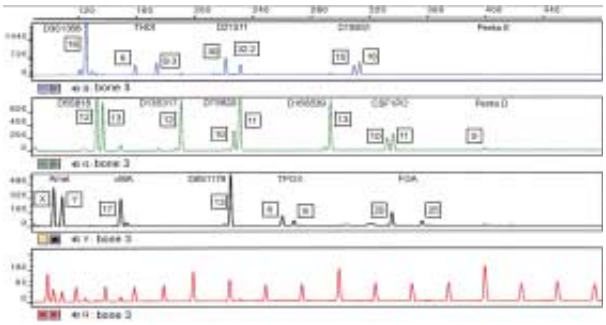

Figure 5. Electropherogram of DNA extracted from the 9-year-old bone samples discovered in the mass grave and amplified with PowerPlex™ 16 kit.

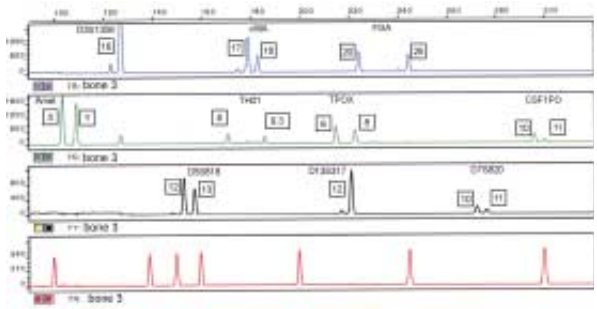

Figure 6. Electropherogram of DNA extracted from the 9-year-old bone samples discovered in the mass grave and amplified with Amp*fl*STR Profiler ™.

Figure 7. Quantiblot result showing how the human DNA standards of 300 pg (indicated by closed arrows) yield a negative signal when mixed with high amounts (μg) of bacterial DNA (indicated by an open arrow).

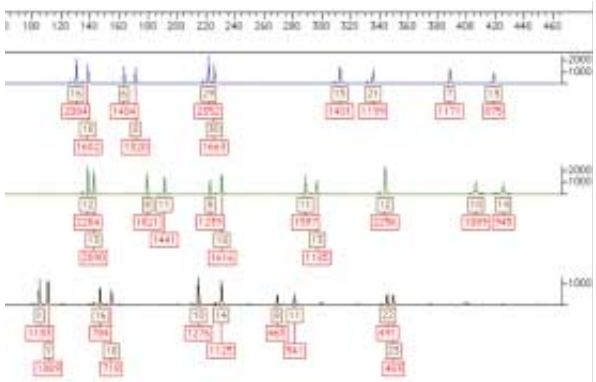

Figure 8. Complete multilocus STR profile obtained with Amp*fl*STR Profiler Plus™ from a DNA sample isolated from an 8-year-old teeth sample.

identify the bacterial DNA present in the bone and teeth samples as the tentative source of the 202 and 308 extra peaks, we amplified and sequenced the first 500 bp of the bacterial 16S rRNA gene from these DNA 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).

On the other hand, during PowerPlex™ 16 analysis, we observed amplification of a non-specific 340 bp peak from the bone DNA extracts of a deceased presumptive father in a Spanish paternity case (Fig. 11).

Microbial DNA Challenge Study

A total of 32 microbial DNA samples (*Alcaligenes faecalis, Morganella morganii, Proteus mirabilis, Providencia stuartii, Shigella sonnei, Acinetobacter calcoaceticus, Candida glabrata, Candida krusei, Cryptococcus neoformans, Lactobacillus acidophilus, Rhodotorula glutinis, Streptococcus pneumoniae, Shigella boydii, Shigella flexneri, Trichosporon beigelii, Candida guilliermondii, Bacillus subtilis, Citrobacter freundii, Bacillus cereus, Clostridium perfringens, Escherichia coli, Pseudomonas aeruginosa, Pseudomonas stutzeri, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus sanguis, Salmonella enteriditis, Micrococcus luteus, Vibrio alginolyticus, Corynebacterium sp., Candida albicans,* and *Candida tropicalis*) were amplified with Amp*fl*STR Profiler Plus™, Amp*fl*STR

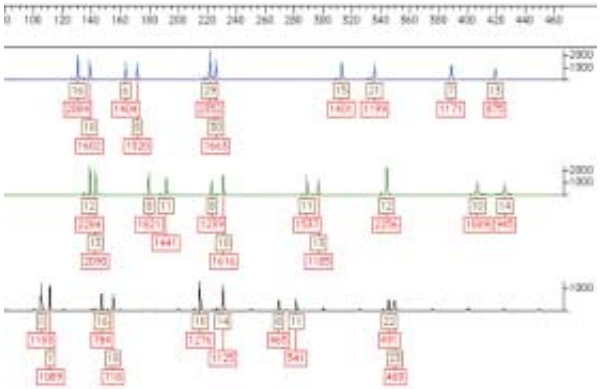

Figure 9. Complete multilocus STR profile obtained with PowerPlex™ 16 kit from a DNA sample isolated from an 8-year-old teeth sample (the same as in Fig. 8). Note the high amplification efficiency obtained for the Penta E and Penta D markers.

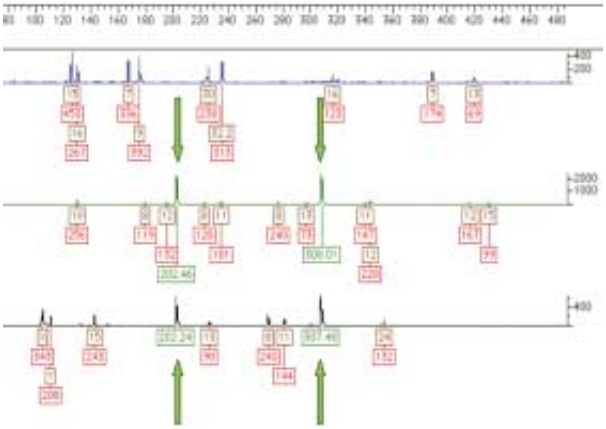

Figure 10. Non-specific 202 and 308 fragments (marked by arrows) observed from a DNA extract isolated from a bone sample. Upper arrows: extra peaks generated by primers labeled with 6-carboxy-4',5'- dichloro-2',7'-dimethoxy–fluorescein (JOE). Lower arrows**:** extra peaks generated by primers labeled with carboxy-tetramethylrhodamine (TMR).

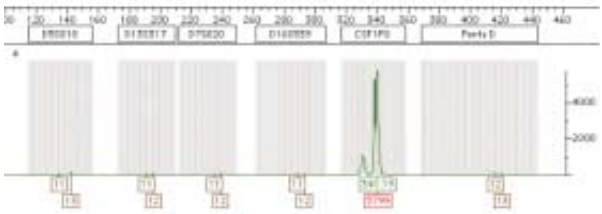

Figure 11. Non-specific 340 bp fragment observed from DNA extract isolated from a bone sample in a paternity case.

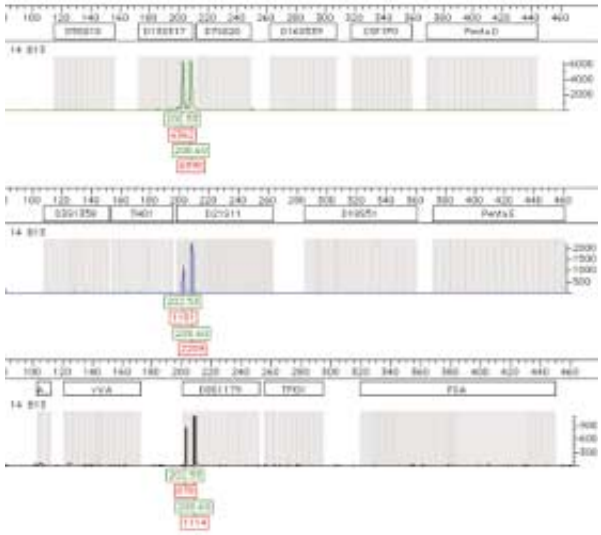

Figure 12. The 202 and 208 green products (shown in the upper row of picture) and blue and yellow pull-up (shown in the middle and the bottom row, respectively) observed after PCR amplification with PowerPlex™ 16 kit of genomic DNA from the yeast *Rhodotorula glutinis*.

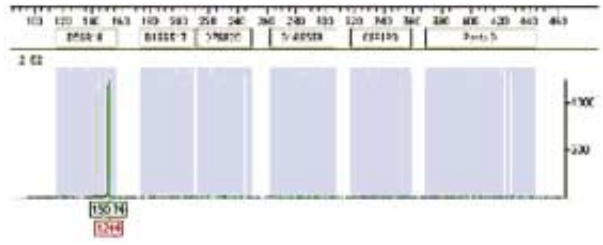

Figure 13. A 150 bp green product observed after PCR amplification with PowerPlex™ 16 kit of genomic DNA from the bacteria *Morganella morganii*.

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 PowerPlex™ 16 for *Rhodotorula glutinis* and *Morganella morganii,* which yielded green peaks of 202 and 208 bp and 150 bp, respectively (Figs. 12 and 13).

NaOH 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 (Fig. 14) successfully amplified in 9 of 16 loci available in PowerPlex™ 16 System (Fig. 15) after the treatment.

Discussion

This collaboration study aimed at improving the identification techniques based on the analysis of genomic DNA. The data indicated that the Amp*fl*STR Profiler™ Kit, Amp*fl*STR Profiler Plus™ Kit, Amp*fl*STR COfiler™ Kit, and the PowerPlex™ 16 system were very sensitive multiplex STR amplification systems, which have been applied with success to obtain a multilocus STR profile from old teeth and bone samples with minimal amounts (pg) of human DNA or even with no detectable 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. On the other hand, 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 non-specific fragments have been observed from some teeth and bone samples for the PowerPlex™ 16 system, which did not influence the assignment of genotypes. The microbial DNA challenge study demonstrated that bacteria and yeast DNA templates could be the source of these non-specific PCR products. Further research is needed to evaluate the incidence of these extra peaks in casework. The phenomenon of random allelic dropout, which is a consequence of the very low human DNA template input in the PCR reaction, was the most common artifact observed for 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 amplification reactions (with different DNA input) from at least duplicated DNA extracts.

In some cases, increasing the amount of DNA input helped to overcome this problem but in some others also bear to an increase of inhibitors rendering negative typing results. The use of silica-based purification methods has been proven to be an efficient procedure to remove or attenuate the inhibition (17,18) but, in our experience, just for some cases (data not shown). This indicates that the inhibitors could be closely associated with the DNA molecules or that the junk microbial DNA itself, which is the majority DNA component of these DNA extracts, could inhibit the PCR amplification of traces amounts of human DNA.

The procedure published by Bourke et al (8) was developed to overcome potential inhibitors of Taq Polymerase when DNA failed to amplify initially or after standard inhibitor neutralization trouble-shooting strategies (heat soak, hot start, BSA, extra Taq and extensive dilution). While working with AmpliType® PM+DQA1 PCR Amplification and Typing Kit and AmpliFLP™ D1S80 PCR Amplification Kit, we applied this procedure for the DNA extracted from the bone samples which failed to amplify with either PowerPlex™ 16 or Profiler/Profiler Plus systems. In most cases when we applied this procedure, we were able to amplify at average 5-8 loci that originally failed to be amplified. Although the mechanism of how NaOH can help amplification is not clear yet, it has been proposed that denaturing conditions would release intercalated inhibitors and that denaturing washes would 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 advise the NaOH protocol when

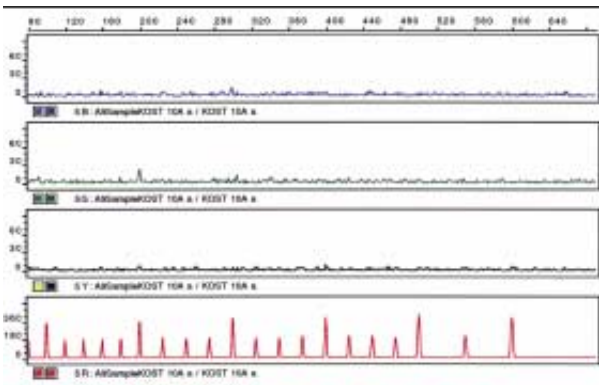

Figure 14. Incomplete STR profile obtained with PowerPlex™ 16 kit from a 9-year-old bone sample.

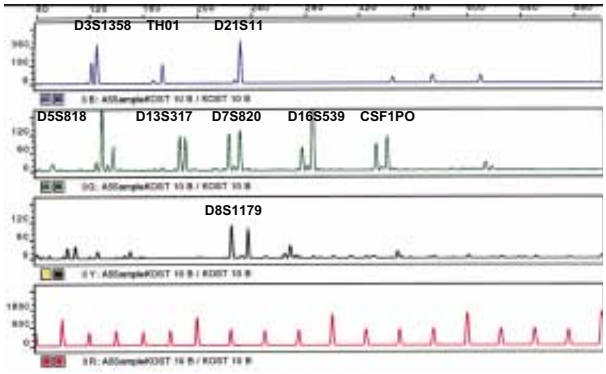

Figure 15. Multilocus STR profile obtained with PowerPlex™ 16 kit from a 9-year-old bone sample after additional NaOH repurification procedure.

the quantity of DNA is limited, since the treatment results in significant loss of DNA.

Nonetheless, it appears 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 large number of samples, since sequence specific oligonucleotide approach can provide unambiguous and reliable data that can contribute to identification and can dramatically reduce the number of samples required for sequence analysis.

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