Gender Identification Differences Observed For DNA Quantification versus STR Genotyping of Mummified Human Remains-How it Relates to Human Identifications in Forensic Science.

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Abstract

DNA testing of human remains is an important identification technology, especially after major mass disasters. It is particularly useful for cases where the remains are highly fragmented such as in bombings and explosions of airplanes and buildings. Bone and tissue in these disaster circumstances can be exposed to high heat and extreme drying conditions, analogous to what is observed in mummified remains. Bone and tissue from mummified human remains found in a subterranean cave in the Gobi Desert in Mongolia were donated to the Henry C. Lee Institute of Forensic Science to determine the gender of the samples using traditional forensic DNA methods. The goals of this project were to (1) determine if DNA could be recovered, (2) quantify the recovered DNA and (3) determine if genotyping was possible. Human DNA was obtained from these samples and evaluated using the Applied Biosystems 7500 Real-Time PCR System. The results showed that quantifiable amounts of both total human and male-specific human DNA were present. When these samples were genotyped, some differences between the Real-Time PCR system and the Minifiler genotyping system were observed. An explanation for these observations and the potential value for using two different gender identification genes (AMEL and SRY) with highly degraded bone and tissue samples will be presented.

Keywords: DNA, Minifiler, Quantifiler, bone, tissue, gender identification

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Introduction

In forensic science, the collection of evidence with proper storage and handling conditions is critical to the final interpretation of the case, especially when employing DNA methods. In this study, we attempted to resolve the gender of mummified tissue and bone samples to aid in the reassociation of remains to the appropriate skeletons collected from a subterranean cave located in southern Mongolia. The original photographs of the cave indicate that the skeletons were comingled and may have been moved from their original location by native explorers or through looting. These samples presented an unusual challenge for DNA testing in that (1) they were estimated to be approximately 800 years old, (2) they were mummified and (3) they were comingled (Figure 1). The choice to work on mummified remains was related to the fact that many missing persons or mass disaster situations have similar types of badly degraded, heat damaged and very desiccated samples.

Figure 1.

Photographs of the mummified samples: 1-G (tissue, photograph 1) and 1-D (rib, photograph 3); 1A/D (additional tissue samples, photographs 2 and 4).
For gender assignment using forensic identification kits, we were fortunate in that we could use the Applied Biosystems 7500 Real-Time PCR System (Quantifiler kits) for quantification and the AmpF/STR Minifiler PCR Amplification (Minifiler) Kit, which was specifically designed for genotyping inhibited or highly degraded DNA samples. All of the commonly utilized human identification kits (Profiler Plus, Cofiler, Identifiler, Minifiler, Applied Biosystems, Inc.; and PowerPlex16, Promega Corp.) amplify the amelogenin (AMEL) locus in their assays. This locus or marker generates a 106 basepair fragment for the X chromosome (female or male) and a 112 basepair fragment for the Y chromosome (male only). While the Minifiler kit has reduced amplicon sizes (to better genotype highly degraded samples) for the other loci (D7S820, D13S317, D21S11, D2S1338, D18S51, D16S539, FGA, CSF1PO), the amplicon length is the same for the amelogenin locus. In the validation and optimization process for development of the kits, many different types of human samples are tested to evaluate kit performance. We were curious to see how our mummified bone and tissue samples would perform using the quantitative Real-Time PCR system and the qualitative Minifiler kit since they were extremely aged and very mummified. As a comparative assay, the Real-Time PCR Quantifiler system uses the SRY gene for gender identification. The SRY gene (Sex-determining Region Y) is a sex-determining gene on the Y chromosome in placental mammals and marsupials. Our expectation was that the DNA results would be equivalent for both systems. We were not, however, correct in our assumption.

One of the key factors (other than the original quality of the bone sample) in determining the success rate of genotyping is the sample preparation and DNA extraction procedures (1-12). STR typing has been performed before on ancient DNA extracted from bones, teeth (1-6) and even hair shafts of Siberian mummies (7). Since ancient samples are anticipated to have very low quantities of DNA, it is easy to contaminate the samples during processing and special precautions need to be taken. Laboratory surfaces, tubes, water and bone processing equipment are sterilized with bleach or an equivalent DNA removal reagent and ultraviolet radiation to reduce the risk of contaminating the sample on the surface with exogenous DNA (6, 8, 10). In addition, the outer surfaces of the tissue and bone samples are sanded away and bleached so that only the internal tissues are extracted for DNA and are presumed free of contamination due to handling (4, 6). There are many different DNA extraction procedures available for ancient and low copy number DNA (4, 10); we utilized a bead-affinity kit (Invisorb Forensic Kit I; Invitek GmbH, Berlin, Germany) that included an ancient bone protocol. Bone and tissue in some mass disaster circumstances can be exposed to high heat and extreme drying conditions, analogous to what is observed in mummified remains which was the rationale for using this particular kit and approach.
Materials and Methods

Sample Preparation

Bone and tissue samples were generously donated by the National Museum of Natural History, Smithsonian Institution and washed twice with 10% bleach. Samples were then rinsed with sterile water, dried completely, and the surfaces sanded with a dremel tool. Each sample was then processed independently in a SPEX SamplePrep Freezer Mill (SPEX CertiPrep Group; Metuchen, NJ) to grind the bone samples to powder. Alternatively, the dried tissues were diced to millimeter sized pieces and extracted. All tools, equipment and surfaces were processed for DNA with control swabs to eliminate the possibility of carryover of DNA between samples. Prior to processing each new sample, tools, countertops and equipment were cleaned with either 10% bleach or DNA-Exitus Plus (Applichem, Germany). In all cases, the controls were negative. A previously genotyped positive bone control sample originating from a ten year old exhumed male was baked for 72 hours at 100°C to evaporate any volatile embalming fluid residue and mimic the natural process of mummification. This control sample was then processed exactly as the other bone samples.

DNA Extraction

Bone and tissue powder or fragments were extracted per the manufacturer’s protocol (Invisorb Forensic Kit I; Invitek GmbH, Berlin, Germany).

DNA Quantification

DNA from extracted samples was quantified using the Applied Biosystems 7500 Real-Time PCR System and the Quantifiler total human DNA or Quantifiler Y human male DNA kits available from Applied Biosystems, Inc. (Foster City, CA) per manufacturer’s instructions.

Genotyping

Each of the four samples was extracted in duplicate and each extraction was PCR amplified for a minimum of three replicates. Genotyping was performed on a 310 DNA Sequencer (Applied Biosystems, Inc.) and analyzed using Genemapper software (Applied Biosystems, Inc.). Both the Minifiler and Profiler Plus kits were used for sex typing.
Results

The Quantifiler Human DNA Quantification kit was used to detect the levels of total human DNA present in the sample, both male and female. Of thirty DNA extractions, seven contained enough DNA to be quantified. Table A shows the sample names, the count, and quantity of DNA present from the Real-Time PCR system.

Table A.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Count</th>
<th>Quantity (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Da</td>
<td>38.32</td>
<td>1.45 x 10^{-4}</td>
</tr>
<tr>
<td>1-Db</td>
<td>37.51</td>
<td>2.78 x 10^{-4}</td>
</tr>
<tr>
<td>1-Ga</td>
<td>35.44</td>
<td>1.47 x 10^{-3}</td>
</tr>
<tr>
<td>1-Gb</td>
<td>37.76</td>
<td>2.28 x 10^{-4}</td>
</tr>
<tr>
<td>1-Gc</td>
<td>30.39</td>
<td>8.57 x 10^{-2}</td>
</tr>
<tr>
<td>1-Gd</td>
<td>29.76</td>
<td>1.42 x 10^{-1}</td>
</tr>
<tr>
<td>1-Ge</td>
<td>38.69</td>
<td>1.07 x 10^{-4}</td>
</tr>
<tr>
<td>Control bone DNA</td>
<td>32.10</td>
<td>5.7 x 10^{-3}</td>
</tr>
</tbody>
</table>

*note that letters a-e is used to distinguish between separate DNA extractions*

STR testing was performed on the 1-D and 1-G DNA extractions. Each sample was genotyped multiple times with the female (X) peak being clearly distinguishable in all cases. However, in many detection runs, the male (Y) peak was present, absent, or barely distinguishable from the baseline noise (Figure 2). This factor led to some concern over whether or not, we could be mistyping samples as female when a poorly amplifying Y peak might be present and could represent the authentic gender of the sample. The variable amplification was never noted for the X peak. This type of variability is typical for what is called low copy number DNA (LCN) where multiple PCR reactions are performed and the best two of three reactions data is combined into a composite DNA profile.
From the replicates of STR amplifications, it became clear that male-specific quantification would need to be performed to clarify the DNA results. Anthropological information had been provided with these samples suggesting that 1-D was a match to a female skeleton and 1-G was a match to a male skeleton. DNA genotyping was requested to confirm this anthropological data with STR typing.

To resolve this issue, the Quantifiler Y Human Male DNA Quantification kit was used; this would show only the quantities of male DNA present in a sample therefore not identifying female DNA. The same thirty samples used in the first plate were re-quantified with the male-specific quantification system. This time only two samples were positive for male DNA, both from 1-G extractions (tissue sample) and were genotypable with a male-specific STR kit (Table B).
Table B.

DNA Extractions Positive for the Presence of Male DNA Using SRY

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Count</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Ga</td>
<td>39.70</td>
<td>6.16 x 10^{-3}</td>
</tr>
<tr>
<td>1-Gd</td>
<td>30.94</td>
<td>8.68 x 10^{-1}</td>
</tr>
</tbody>
</table>

With the information gathered using the DNA quantification and STR genotyping systems described here, some theories can be developed pertaining to what could have happened with original STR genotyping data on these 14th century bone and tissue samples. First, we were able to obtain human DNA from our mummified bone and tissue samples that we believe to be from the internal portion of the samples using a kit designed for ancient bone DNA extractions. The lack of any exogenous DNA on the control swabs from the equipment and laboratory surfaces increases our confidence that the DNA detected is authentic to the samples. Second, we discovered that PCR amplification on our very challenging samples gave uninterpretable results for the Y peak expected for male samples using both the Minifiler and Profiler Plus kits. The X peak expected for female samples, however, was not variable. When we went back to a male-specific quantification system, we were able to achieve consistent results.

We conclude that DNA quantification using the Quantifiler Human and Y Human Male quantification kits is significantly more consistent for gender determination than by using STR typing alone. This is likely due to the extreme age and nature of our particular samples and the very limited amount of DNA present in the samples. For our control DNA, an exhumed bone that had been buried for ten years and still had remaining moisture was baked to dryness to remove any residual volatile constituents due to the embalming process. This sample amplified consistently as male with Profiler Plus and Minifiler kits.

The best possible explanation for our observed data is consistent with a recent publication (13) in which the SRY marker is validated for forensic gender identification. The SRY marker is the marker used in the Quantifiler kits for gender identification; SRY stand for sex-determination region Y present on the Y chromosome and results in a 96 basepair amplification product that can be used in conjunction with the amelogenin (AMEL) marker found in standard human identification multiplex kits. Sex determination with the amelogenin marker is routinely performed and results in a PCR product of 106 basepairs for the X chromosome and 112 basepairs for the Y chromosome. When extremely aged and degraded samples such as mummified bone and tissue are genotyped, allele drop-out can occur for the Y fragment of amelogenin giving false gender identification as female or variability of male then female gender based on separate PCR reactions. This is very typical in low copy number DNA samples that are
less than the optimized 1 nanogram amount of DNA for which the human identification kits were originally designed. In these cases, it may be more scientifically accurate to (a) quantify and genotype for both the amelogenin and SRY markers as well as (b) perform DNA sequencing of those regions to confirm gender.

This is particularly critical in open population samples where identification could be either male or female in samples that are disassociated from their original skeleton, thus, no anthropological data can assist in confirming gender; such was the case with our archeological specimens. Adding to our identification problems was the fact that we had no historical data to suggest whom these original nine skeletal remains that were garroted and discarded in a subterranean cave might belong to-no ancestry, no names, no historical battles to provide a connection to their identity.

Similar sorts of situations also can occur in mass disasters or explosions where fragmented remains, exposed to extreme heat and dryness, are common. Under these circumstances, it is slightly simpler to make identifications as often there is a manifest or roster of the missing, therefore, a genetically closed population exists; where there is a limit to the total number of people that need to be identified. The identification of highly fragmented and disassociated remains is still a technical challenge as personal effects; major facial and other identifying features are lost as tools for identification of the missing. Often, DNA is the only tool that can be useful; however, as our experience with mummified and fragmented remains suggests, care in the interpretation of the DNA data must be exercised as it too has some technical limitations.

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References


