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The Fingernails of Mary Sullivan: Developing Reliable Methods for Selectively Isolating Endogenous and Exogenous DNA from Evidence†

ABSTRACT: The fingernails of Mary Sullivan, the last victim of the Boston Strangler, were examined to determine if any genetic information about the murderer could be obtained. The nails were extremely friable necessitating the development of new techniques for isolating and purifying DNA. DNA yields from nails were optimized by using a NaOH-based preparation technique, which was simple, efficient, and minimized handling. Methods for selectively and thoroughly removing exogenous material on nails were also developed through use of a species-specific PCR assay, wherein mitochondrial DNA from the nail could easily be differentiated from DNA of contaminating cells.

KEYWORDS: forensic science, DNA typing, mitochondrial DNA, polymerase chain reaction, nails, fingernail, sodium hydroxide, exhumation, Boston Strangler

It is not uncommon for the forensic scientist to be confronted with unique and challenging situations—ones that are not amenable to the tools and protocols used in the day-to-day workings of the laboratory. This was the situation we found ourselves in when presented with the fingernails of Mary Sullivan—the final victim of the infamous Boston Strangler—the first of many pieces of evidence examined genetically following her exhumation in October of 2000.

A perusal of the literature and crime lab protocols indicated that current methods for DNA isolation from nails are designed to isolate substances found *on the nail* (exogenous material potentially originating from an attacker during self-defense), or to obtain DNA *from the nail* (endogenous material for identification); they are not optimized to do both. Also, these protocols deal with relatively "fresh" nails, not ones recovered following decades of underground interment. A new protocol, more discriminating and delicate than those now available, would be needed for the Sullivan nails, and thorough testing and optimization of the protocol required a system that allowed for quick and simple differentiation between endogenous and exogenous DNA.

Mitochondrial DNA (mtDNA) analysis (reviewed in 1) is often more successful than standard nuclear DNA typing when the sample in question is old or is derived from dead cellular material such as hair or nail. This then was the primary marker examined when probing Mary Sullivan's fingernails for exogenous material that might originate from her murderer. The sensitivity of mtDNA analysis and the possibility of contamination when very little target material is present underscore the importance of minimizing handling and the number of steps in DNA purification. The more elaborate protocols for DNA preparation from nail material often begin by cleaning the nail through sonication and treatment with boiling water, acetone, bleach, ethanol, and detergents (2–4). DNA isolation methods range from simple Chelex extractions (5–7), which do very little to disrupt the nail material and release DNA, to much more complex procedures that entail dicing the nail with a scalpel and incubating it in buffer containing large amounts of DTT and/or proteinase *K* for up to several days (e.g., 2–4,6).

We set out to design and validate a simplified protocol that would:

- Completely remove exogenous material from the nail;
- Retain the quality of the DNA from the nail following removal of exogenous material;
- Retain the quality of the DNA from the exogenous material for subsequent analysis;
- Assure that the exogenously-derived DNA was not cross-contaminated with nail DNA following its removal.

In order that a large number of procedures and modifications could be efficiently tested, a simple PCR-based assay was developed that permitted exogenous DNA to be easily differentiated from nail-derived DNA. This assay allowed careful testing and comparison of hundreds of samples, including multiple replicates for each procedure, without time-consuming DNA sequencing or other analysis of each potential mixture. Through this, simple methods for discrete isolation and purification of nail DNA and DNA from exogenous sources were developed.

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Materials and Methods

The fingernails of Mary Sullivan were obtained during her exhumation in October of 2000. Sullivan was found murdered in her apartment in Boston on January 4th, 1964. Following discovery by roommates the body was autopsied, embalmed, prepared for viewing, and buried in the St. Francis Xavier Cemetery in Hyannis, Massachusetts. The cemetery is approximately 50 ft above sea level, and while the casket was partially collapsed, within was a complete skeleton consistent with 36 years of interment. Personal items buried with the body, as well as subsequent genetic analyses, confirmed the identity of the exhumed individual.

Upon exhumation the fingernails were easily removed using forceps and placed individually into envelopes. These were sent to The Department of Forensic Sciences at The George Washington University where they were transferred to individual petri plates for microscopic examination. The nails were in a very friable condition (Fig. 1) and contained remnants of red fingernail polish, as well as apparent fungal growth.

Endogenous DNA Extraction from Nail Material

Methods developed for DNA isolation from the Sullivan nails were compared with standard procedures including proteinase *K*/SDS-based organic (e.g., 2–4,6,7), and Chelex (6,7) extractions. Multiple test nails (clipped fragments of approximately 3–7 mg) were obtained from volunteers (over 15 individuals during a 1.5 year period, made up of approximately equal numbers of males and females) and stored at room temperature from one day to four months. No pretreatment of nails (e.g., cleaning) was conducted prior to experimentation. Directly solubilizing nails was attempted by incubating them for 2–24 h in strong denaturants, including sodium hydroxide (1N, 2N, and 5N) and guanidine-HCl (0.1M, 0.5M, 2M, and 5M). The damaging effect of these denaturants on DNA was examined by incubating 100 ng of intact (supercoiled) bacterial plasmid DNA, pM12 (8), in 200 μL of each for 24 h, purifying the DNA as described below for nails, and comparing it to untreated pM12 through electrophoresis on 1% agarose gels.

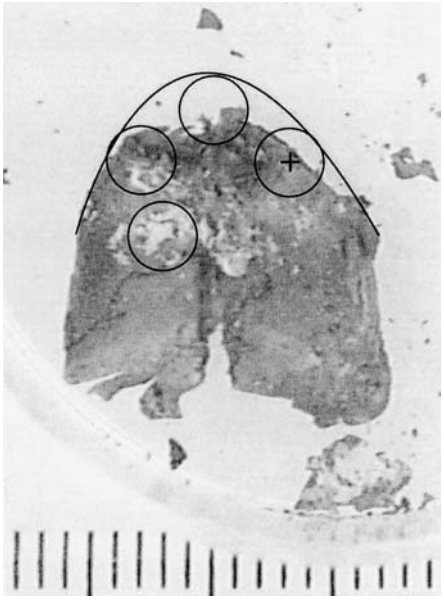

FIG. 1—*The underside of nail L3 from Mary Sullivan, showing its extreme friability. Areas processed for exogenous DNA are denoted by circles. Note the light colored material (likely fungal) particularly abundant under the lowest circle. The single area that produced a positive PCR result is denoted by +. Scale shows mm. The tip of the nail was later removed for processing, and has been drawn in.*

Nails treated with guanidine-HCl were extracted with phenol/chloroform as described below. Nails solubilized in NaOH were neutralized (pH 7–8; measured on pH paper) by addition of Tris base (pH 7.5) and normal equivalents of concentrated (11.6N) HCl. This solution was quickly vortexed to prevent solubilized product from precipitating. An equal volume of 50:50 phenol-chloroform was added, the solution was vortexed, and centrifuged at 15,000 X g for 5 min. The top, aqueous layer was transferred to a clean tube, extracted again if it was discolored or had a large interface, and DNA precipitated by addition of 1/10 volume of 3M NaAcetate and 2 volumes of 95% ethanol. The DNA was incubated at −20°C for 1 h, centrifuged at 15,000 X g for 15 min, the supernatant removed, and the DNA pellet dried under vacuum. DNA samples were resuspended in 10 μL of TE/mg of nail.

In an alternate procedure the NaOH solution was neutralized as above and then purified using three 300 μL TE washes through a Microcon-100 filter column (Millipore, Bedford, MA). Final volumes were brought to 10 μL of TE/mg of nail.

Numerous comparisons among the NaOH-based method and standard SDS/Proteinase *K*/DTT and Chelex-based extractions were conducted to assess DNA yields and quality. An example included three 2–7 mg nail fragments collected from three volunteers. One nail set had been aged four months, one set for one month, and one was fresh. Exogenous material was first removed using the proteinase *K*/SDS treatment described below, then a nail from each volunteer was prepared using each method, with DNAs resuspended in 10 μL TE/mg nail, or remaining in 200 μL for Chelex. For subsequent experiments an appropriately larger volume of Chelex DNA was used to compensate for its more dilute nature.

Quantification of nuclear DNA yields was attempted using slot blot analysis of DNA representing 0.3 mg of nail, following the manufacture's protocol (Quantiblot, Applied Biosystems, Foster City, CA) or for mtDNA, by amplifying successive 10-fold serial dilutions of the DNA until no amplification was observed on an agarose gel (e.g., if 1 μL of a DNA sample amplified at 10, 100, and 1000-fold dilutions while another sample amplified only down to a 100-fold dilution, the former was assumed to have a higher concentration of starting material). Likewise, the quality of the DNA from each preparation was assayed by amplifying progressively larger fragments of mitochondrial and nuclear DNA until a negative result was obtained (see Genetic Analyses below).

Removal of Exogenous Material from Nails

Human test nails were heavily coated with mouse liver and allowed to dry from one to several days. The presence or absence of each species' DNA could then be directly confirmed through mtDNA amplification using PCR primer sites conserved in all mammals ((9) sequences below). These generate different sized products—612 bp for human and 500 bp for mouse—that are easily differentiated on a 2% agarose minigel.

Liver-contaminated nails were placed in 1.5 mL microcentrifuge tubes and incubated at room temperature for 1 h in 200 μL of one of the following: sterile water (or water with 25mM EDTA), 5% Terg-A-Zyme (Alconox Inc., White Plains, NY), 1% SDS, 1% SDS + 0.2 mg/mL proteinase *K*, 10% commercial bleach, 1N HCl, chloroform, or acetone. The liquid was transferred to a new microcentrifuge tube for exogenous DNA analysis (detailed below). The nail was next rinsed 5–10 times with

sterile (or 18.3 M-ohm) water to remove the soak solution and any residual material detached during the incubation. A final rinse was done with sterile water, the sample was spun briefly in a microcentrifuge, and the remaining water was removed with a pipet. Nail DNA and any remaining exogenous DNA was then isolated using the NaOH method.

The quality of DNA from the detached exogenous material and its contamination with nail DNA was examined by preparing DNA directly from the aqueous-based soak solutions (including NaOH neutralization of the 1N HCl soak) or by extracting the organic-based compounds (chloroform and acetone) with 200 μL TE. Each sample was brought to 1% SDS and 0.1mg/mL proteinase *K*, incubated overnight at 55°C, and purified by organic extraction as described above for nails.

The Effects of Nail Polish

Nail polish (Revlon "super nails" (New York, NY)) was applied to nail fragments and allowed to dry from one day to several weeks. Endogenous DNA was extracted from polished nails using NaOH as described above, as well as following polish removal by soaking the nail for 10 min in acetone and rinsing with sterile water. Further, mouse tissue was applied over polished nails and allowed to dry. Acetone removal of polish on liver-contaminated nails was conducted at one of three stages: preceding a one-hour aqueous soak of the polished nail, following that step but preceding a 1% SDS/0.1mg/mL proteinase *K* soak of the nail, or following both steps and before the final NaOH digestion procedure. DNA was extracted from the soaks and from nails as described above.

Genetic Analyses

MtDNA amplification of fragments ranging form approximately 200 base pairs (bp) to over 1000 bp was conducted using standard control region primers for human identification (F15989, F16190, F15, R16410, R285, and R484; sequences available through the Armed Forces DNA Identification Laboratory's web site (3)). The conserved mammalian mtDNA primers (9) are located in the threonine tRNA locus flanking the control region (forward, 5′-TACACCAGTCTTGTAAACC-3′), and in the D-loop at position 16498 in the human sequence ((10) reverse, 5′-CCTGAAGTAGGAACCAGATG-3′). These primers produce amplicons of 612 bp for human DNA and 500 bp for mouse.

Amplifications were performed on Perkin-Elmer (Foster City, CA) 2400 and 9600 thermalcyclers. In general, 1 μL of template DNA was used for each 10 μL PCR reaction, with cycling parameters of 94°C for 3 min, 38 cycles of 94° for 30 sec, 55° for 30 sec, 72° for 45 sec, followed by a final extension at 72° for 7 min. PCR products were visualized through ethidium bromide staining of 1 to 3% agarose gels as appropriate.

MtDNA sequencing was performed on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA) using a BigDye sequencing kit (Perkin-Elmer) according to the manufacturer's protocol. The same oligonucleotides used to amplify the DNA were used to prime sequencing reactions. Nuclear STR loci were assayed on an ABI 310 using Promega's (Madison, WI) PowerPlex 1.1 system (D5S818, vWA, D13S317, TH01, D7S820, TPOX, D16S539, and CSF1PO, in increasing order of size). The manufacturer's protocol for the kit was followed. If negative results were obtained the number of PCR cycles was increased up to 10 cycles to determine whether results from the limited amount of nuclear DNA in nails could be augmented. Alleles matching the nail donors', at 100 relative fluorescence units (RFU) or higher, were sought.

DNA Extraction from the Nails of Mary Sullivan

In a UV irradiated PCR-setup hood, 5 μL of sterile water was gently pipetted up and down at single points on the underside tips of the nails, where exogenous material would most likely be found (Fig. 1). Several points along the tips of each nail were processed, as were areas well back in the nail bed that would not have come in contact with foreign material, and areas containing fungal growth. Next, small sections of the nail tip were removed and processed using the NaOH procedure to act as known controls. MtDNA was amplified and sequenced as described above. These sequences were verified through mtDNA analysis of organically extracted buccal samples from maternally-related ancestors of Mary Sullivan.

Standardized Protocol for the Isolation of DNA from Nail Material

Based on results obtained from all experiments, the following standardized protocol for exogenous and endogenous DNA extraction from nail material was developed. DNA isolation from exogenous material on the nail begins at step 1. DNA isolation from the nail itself begins at step 2.

Exogenous DNA Isolation—1a. Soak nail in 200 μL sterile 25mM EDTA (in H_2O) at room temperature for 1 h. Gently vortex periodically. Transfer liquid to new tube. 1b. To this solution add 20 μL 10% SDS and 1 μL 20 mg/mL proteinase *K*. Incubate at 50°–60°C overnight. Extract DNA using phenol/chloroform as described in step 6.

Nail Preparation and DNA Isolation—2. To the nail add 200 μL 1% SDS/25mM EDTA and 1 μL 20mg/mL proteinase *K*. Vortex and incubate for one hour at room temperature. 3. Pipet or pour off liquid, and rinse nail 5–10 times with high quality (18.3 M-ohm) or sterile water. If pouring off water, work over a clean petri plate or similar. Following a final rinse with sterile water, centrifuge nail briefly and pipet off all remaining liquid. 4. Add 200 μL 2N NaOH to nail. Incubate overnight at room temperature. Vortex periodically if desired. 5. Following incubation, vortex nail to ensure it is completely solubilized. Neutralize solution (to pH 6–8, checked by spotting 1 μL onto pH paper), by adding 100 μL of 200 mM Tris (pH 7–8) and 34.5 μL concentrated HCl. Vortex immediately. If the pH is too low a precipitate will form. Adjust pH with dilute NaOH as needed to re-dissolve precipitate. (Note: the 34.5 μL of concentrated HCl (11.6N) is equal-normal with the 200 μL of 2N NaOH. If the HCl is more dilute the volume added should be increased accordingly. Old HCl may work poorly.) Proceed to step 6 or 7.

Organic Extraction—6a. Add an equal volume of phenol/chloroform (or PCI) to neutralized sample, vortex, spin at high speed for 5 min in a microfuge, and transfer aqueous (top) layer to a clean tube. Repeat this extraction if the aqueous layer is not clear. 6b. Precipitate DNA by adding 1/10 volume 3M sodium acetate and 2 volumes of 95% ethanol. Incubate at −20°C for one hour or longer. 6c. Centrifuge at high speed for 15–30 min. Note location of DNA pellet, which may or may not be visible. Carefully pipet off all liquid. Dry pellet and resuspend in TE (10 mM Tris/1 mM EDTA) at 10 μL/mg nail.

Microcon-100 Purification—7a. Add an equal volume of TE to the neutralized sample and place on column. Centrifuge at 500 X g for approximately 20 min, or until liquid is pushed through. Dis-

card flow-through. 7b. Add 200 μL TE to top portion of column and centrifuge at 500 X g as in 7a. Repeat step 7b once. 7c. Collect retentate containing DNA (generally 10–20 μL remaining in top of column; add TE if needed to 10 μL/mg nail).

Removal of Nail Polish—Following step 1 above add 100 μL acetone to nail, vortex and soak at room temperature for 10 min. Draw off acetone, discard, and repeat. Allow nail to dry and continue to step 2.

Results and Discussion

The fingernails of Mary Sullivan presented to our laboratory for examination represented unique and complicated evidence. Obtaining DNA from nail material in general is straightforward and well-established (e.g., 4,11), and as has been previously observed, nail material represents a simple and reliable source of genetic information from exhumed bodies (12). On the other hand, testing the nails for cellular material that may have originated from her murderer, our primary goal, was more problematic. Even for fresh nails, obtaining DNA from exogenous material is difficult, with the nails themselves a constant source of contamination (e.g., 6,7). In our case, the nails were extremely friable and any attempt to swab them, or even soak them, would result in cross-contamination with more-abundant nail DNA. This was resolved only after extensive experimentation on test nails.

Secondary aims of these experiments were to design a faster and more efficient method for extracting DNA from human nails, and to perfect methods for completely removing any contaminating cellular material while maintaining the ability to isolate typable DNA from this exogenous material if desired. In preliminary experiments, guanidine-HCl did not dissolve test nails, nor did low concentrations of NaOH. At 2N and above however, NaOH, which has been used for DNA isolation from forensic stains (13), produced complete breakdown of the nail in far less time and with less manipulation than other protocols. When the purified plasmid DNA, pM12, was incubated in 2N or 5N NaOH overnight, neutralized, and allowed to reanneal, it showed no decrease in form I, II, or III DNA (data not shown), indicating that solubilizing nails under these conditions was not likely to have a detrimental effect on their DNA quantity or quality. Overnight treatment in 200 μL of 2N NaOH thus became the method of choice for subsequent experiments.

Solubilizing Nails Versus Standard DNA Preparations

There were several differences between the NaOH-based DNA preparation and standard SDS/Proteinase *K*/DTT or Chelex procedures. The Chelex method was quick, but it did not disrupt nails in any visible way. The SDS/Proteinase *K*/DTT method was extremely tedious, requiring large amounts of handling including dicing nails into small fragments and multiple additions of proteinase *K* and DTT over a period of 3–5 days to break down the material. Even after this period small fragments of nail could often be observed. In contrast, soaking nails in 2N NaOH reliably and completely solubilized them overnight, and often in a far shorter time (a few hours). If any nail material was visible in the morning, it quickly dissolved with brief vortexing.

Not surprisingly, DNA yields from each procedure also differed. The nuclear quantification assay using Quantiblot was negative (below the 0.5 ng standard) when yields from 0.3 mg of nail were assayed. Once PCR-based methods were used however, DNA yields could be compared. Small fragments of mtDNA (approximately 200–500 bp) were successfully amplified from 1 μL of DNA using any of the preparation procedures; however, serial dilutions showed that in each case the DNA yields from NaOH preparations were highest, followed by the SDS/Proteinase *K*/DTT procedure and finally Chelex extractions. MtDNA from some Chelex preparations was successfully amplified following 10-fold dilution while in others it was not. MtDNA from SDS/Proteinase *K*/DTT purification could be amplified at 100-fold dilution, but generally not further. The NaOH prepared mtDNA could be amplified routinely at 1000-fold dilution.

DNA quality (based on successful mtDNA amplification) was similar for all procedures at smaller mtDNA sizes (approximately 200–500 bp), while only the NaOH and SDS/Proteinase *K*/DTT DNA could be amplified to over 800 bp. The largest fragments amplified, using mitochondrial primers 15989–484 (1063 bp), were only generated from NaOH preparations. For nuclear (STR) loci, the best results were also from the NaOH prepared samples, where alleles approaching 300 bp (generally half or more of the PowerPlex 1.1 loci) could be assayed. As expected, larger products showed decreased peak heights, and the largest STR loci (over 300 bp), while often observable, were below 100 RFU.

Nails with polish were tested using the NaOH extraction in parallel with unpolished samples. When polish was not removed amplification of endogenous DNA was inhibited. However, when polished nails were first soaked for 10-min in acetone and the material rinsed with water, mtDNA amplification was successful.

Removal of Exogenous Material

Removal of exogenous cellular material was assayed by simultaneous amplification of human and mouse mtDNA using the conserved mammalian primers. Had human test nails been contaminated with other human tissue (e.g., blood), studying its removal would have been far more laborious and inexact, as the identification of two human mitochondrial haplotypes in a sample requires sequencing. Moreover, if one haplotype exists at levels substantially below the other, its presence may be missed completely. In contrast, contaminating nails with mouse tissue permitted quick and accurate differentiation of endogenous and exogenous PCR products owing to the 112 bp difference in amplicon size (Fig. 2), allowing hundreds of experiments to be conducted on test nails in a reasonable amount of time. Because the commingling of samples is often a concern in the forensic sciences, this two species assay, which makes recognizing mixtures easier and thus developing protocols to eliminate them simpler, should be of widespread interest.

A "successful" removal of exogenous material from a nail was one in which no contaminating PCR product was generated following a one hour soak and subsequent rinse of the sample. Anything less than the most thorough cleaning resulted in an exogenous signal due to the smaller size of the mouse amplicon and the far greater amount of starting DNA in the fresh tissue. The organic solvents tested did not remove substantial amounts of exogenous tissue, i.e., the predominant PCR product was from mouse. Ten percent bleach and 1N HCl gave inconsistent results, wherein treated nails sometimes showed no exogenous material while other times it was the major PCR product. Further, these chemicals act to directly degrade nucleic acids and would not only destroy exogenous DNA, but may damage nail DNA as well. Water alone and the two detergents tested, 1% SDS and 5% Terg-A-Zyme, removed a substantial portion of the exogenous material, but in no instance was it removed completely. The one treatment that did remove all mouse tissue was a 1-hour SDS/Proteinase *K* soak. This treatment

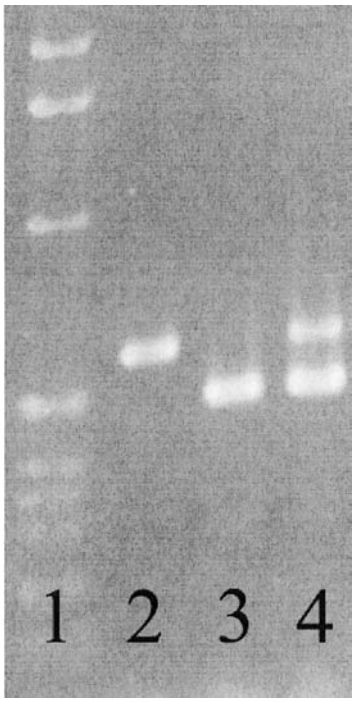

FIG. 2—*Two percent agarose gel showing the differentiation of human and mouse amplicons using the conserved mtDNA primers. Lane 1 is a size standard; Lane two is the 612 bp human mtDNA amplicon following cleaning of exogenous material (see Materials and Methods); Lane 3 is the 500 bp mouse mtDNA amplicon resulting from a water soak of the nail (see Materials and Methods); Lane 4 is the combination of mtDNA types resulting from an uncleaned nail.*

was tested further and used in subsequent experiments as the final step for removing exogenous material.

DNA Isolation from Exogenous Material

An important requirement of these experiments was successful amplification of DNA from exogenous cells, ideally without the presence of a contaminating nail component, which again was made much easier through the two species approach. It was noted that mtDNA amplification of the soak solution most effective at removing all exogenous material, SDS/Proteinase *K*, also generated human product, indicating that nail breakdown was occurring. A similar though weaker result was seen with both SDS or Terg-A-Zyme soaks. In contrast, a 1-hour H_2O/EDTA soak of a contaminated nail released only exogenous DNA, and thus was incorporated into the standard protocol for isolation of DNA from exogenous material.

It was not obvious at which step exogenous material should be collected from polished nails for subsequent analysis—before or after polish removal. The strongest mtDNA PCR signals were obtained from aqueous soaks preceding the addition of acetone; however, exogenous PCR product was also generated if the water treatment was done after acetone. Given this, if obtaining exogenous DNA from polished nails is a priority, aqueous soaks at both points may be called for.

Analysis of the Mary Sullivan Nails

The extremely friable nature of the Sullivan nails meant that a gentle aqueous pipetting extraction was required for isolation of exogenous material so as to avoid cross contamination with nail DNA. Further, extracting typable DNA from the exhumed nails was not as simple as from fresh nails, as they were substantially "dirtier" than normal; the samples required both organic extraction and Microcon filtration for successful amplification.

The areas of fungal growth on the nails were negative in all assays. In addition, exogenous extractions from the nail tips and internal regions of the nail beds did not produce nuclear amplification products, and most were negative for mtDNA. In a few instances however, exogenous extractions from the tips generated positive mtDNA PCR results (e.g., Fig. 1), as did the nails themselves when endogenous DNA was tested. In these cases, a 270 bp segment of HVII was the largest amplicon produced. When these were analyzed the sequences matched those of maternal relatives, and therefore Mary Sullivan; in no instance was a mtDNA sequence from another individual obtained. It is not possible to determine if the positive results from the nail tips came from nail contamination or from exogenous cells under the fingernails. We do know that Mary Sullivan was strangled, and it seems plausible that she would have struggled to remove the hands or ligatures placed around her neck, perhaps resulting in the scraping of her own skin and subsequent isolation of her DNA from the undersides of her nails.

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