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The Effect of Sample and Sample Matrix on DNA Processing: Mechanisms for the Detection and Management of Inhibition in Forensic Samples

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

THE EFFECT OF SAMPLE AND SAMPLE MATRIX ON DNA PROCESSING:
MECHANISMS FOR THE DETECTION AND MANAGEMENT OF INHIBITION IN
FORENSIC SAMPLES

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

Lilliana I. Moreno

2015

To: Dean Michael R. Heithaus
College of Arts and Sciences

This dissertation, written by Lilliana I. Moreno, and entitled The Effect of Sample and Sample Matrix on DNA Processing: Mechanisms for the Detection and Management of Inhibition in Forensic Samples, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: March 23, 2015

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Florida International University, 2015

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DEDICATION

She paused right before compromising and stood tall while watching the masses pass her by. After reflecting for a short while, she turned her back and with a smile took command of uncharted path.

I watched her closely and as approaching the daunting moment of having to decide I took a breath, a deep long breath to cast my fears aside.

It proved demanding the expedition through unexplored tracks

How to stay focused, how to look forward when it all seemed howling black.

Yet she continued to work through hardship so motivated by the reward

That without knowing, her every action impelled the strength that carried me by.

But soon the battle between fatigue and sacrifice ensued

Promptly reminding about the matters I'd given up to do

Yet as I crumbled her self-denial cleared out the blurry view

And as relinquishing became imminent she gave the stamina to push me through

The work was arduous and resources were spent

But looking back feels so fulfilling now that the road is paved

And all the memories, the lessons learned and knowledge acquired throughout the way

Make all the anguish and deprivations oh so worthwhile and not astray.

To you I dedicate this road. Thanks for lifting me when I fell, guiding me when I got lost and for always believing in me.

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“Feeling gratitude and not expressing it is like wrapping a present and not giving it” –

William A. Ward

As I close this chapter of my life, I look back and realize that it wouldn't have been possible without the resolute support of some individuals. I would like to thank my major professor Dr. Bruce McCord, for accepting the challenge of working with me. I am blessed and forever appreciative to have worked under such wonderful guidance. You pushed me beyond my boundaries, taught me to think ahead, believed in my abilities and helped me build up on my strengths. Your words ‘...I will not give up on you’ gave me a reason to not give up on myself. To the rest of my committee, Dr. Alexander Mebel, Dr. Kathleen Rein, Dr. George Duncan and Dr. DeEtta Mills, thanks for your constructive criticism and guidance through all of these years. Dr. D, thanks for keeping me in ‘check’ and Dee, thanks for teaching me strength, being my sounding board and such an amazing role model and inspiration.

A special thanks to Dr. James Robertson and Dr. Thomas Callaghan. Completing a doctoral degree away from home and while working full time would have been impossible without your constant caring and words of encouragement. To the ‘chemistry crew’, especially Mike Smith, Jay Clark, Eshwar Jagerdeo, Jeff Leibowitz and Lou Reda for your invaluable knowledge and technical assistance. A big thank you to my ‘DNA family’ for your patience and assistance, and to Cayman Taylor for her helpful technical support.

I owe my most deepest gratitude to my family and friends, especially my mom, siblings and partner for picking me up when I crumbled, pushing me when I stalled,

celebrating each little success and staying by my side despite my lack of reciprocity. Last but not least I would like to thank GOD for giving me the mental strength and blessing me with self-motivation to complete this journey.

A trip is better when accompanied by friends and I am honored to be surrounded by so many of you.

ABSTRACT OF THE DISSERTATION

THE EFFECT OF SAMPLE AND SAMPLE MATRIX ON DNA PROCESSING:
MECHANISMS FOR THE DETECTION AND MANAGEMENT OF INHIBITION IN
FORENSIC SAMPLES

by

Lilliana I. Moreno

Florida International University, 2015

Miami, Florida

Professor Bruce R. McCord, Major Professor

The presence of inhibitory substances in biological forensic samples has, and continues to affect the quality of the data generated following DNA typing processes. Although the chemistries used during the procedures have been enhanced to mitigate the effects of these deleterious compounds, some challenges remain. Inhibitors can be components of the samples, the substrate where samples were deposited or chemical(s) associated to the DNA purification step. Therefore, a thorough understanding of the extraction processes and their ability to handle the various types of inhibitory substances can help define the best analytical processing for any given sample. A series of experiments were conducted to establish the inhibition tolerance of quantification and amplification kits using common inhibitory substances in order to determine if current laboratory practices are optimal for identifying potential problems associated with inhibition. DART mass spectrometry was used to determine the amount of inhibitor carryover after sample purification, its correlation to the initial inhibitor input in the sample and the overall effect in the results. Finally, a novel alternative at gathering investigative leads from

samples that would otherwise be ineffective for DNA typing due to the large amounts of inhibitory substances and/or environmental degradation was tested. This included generating data associated with microbial peak signatures to identify locations of clandestine human graves. Results demonstrate that the current methods for assessing inhibition are not necessarily accurate, as samples that appear inhibited in the quantification process can yield full DNA profiles, while those that do not indicate inhibition may suffer from lowered amplification efficiency or PCR artifacts. The extraction methods tested were able to remove >90% of the inhibitors from all samples with the exception of phenol, which was present in variable amounts whenever the organic extraction approach was utilized. Although the results attained suggested that most inhibitors produce minimal effect on downstream applications, analysts should practice caution when selecting the best extraction method for particular samples, as casework DNA samples are often present in small quantities and can contain an overwhelming amount of inhibitory substances.

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“When you come to the end of your rope, tie a knot and hang on.” - Franklin D. Roosevelt

I. INTRODUCTION

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Bruce McCord, PhD

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The use of biological evidence to establish criminal leads and resolve criminal investigations became a major breakthrough in the forensic field in the 1980's. Since then, great strides have been made and major accomplishments have improved the science making it more reliable, sensitive and easier to execute. We now rely on state-of-the art instrumentation to carry out the majority of the sample processing and on improved chemistries that makes the interpretation easier and the artifacts less prominent. Developments such as magnetic bead based extraction methods, more sensitive quantification and amplification chemistries, and faster capillary electrophoresis instruments have all improved the efficiency of the process. New instruments based on next generation sequencing have been designed which are capable of performing autosomal, Y- and X-short tandem repeat [1] analysis as well as single nucleotide polymorphism (SNP) yielding a world of data with a minimal amount of material [2-4]. In addition, Y-chromosome STRs and autosomal STR amplification kits encompassing a higher number of loci have been introduced in the market to improve detection and discrimination [5, 6]. Nevertheless, there are still vulnerabilities in the process and areas that are poorly understood and open for improvement. Most of the improvement efforts have been focused in quantification and amplification chemistries as well as

downstream instrumentation and little attention has been given to the forensic samples themselves. These exemplars are collected from a wide variety of surfaces and background matrices some of which can pose analytical problems. It is the goal of this thesis to evaluate the impact of the samples/sample matrices on the analytical process, develop methods to better understand these effects and establish ways to minimize the impact on the subsequent sample result processing. This thesis provides an overview of the analysis process of forensically relevant biological samples, current methods of inhibition detection and their associated challenges, as well as a novel approach to detect sample contaminants co-purified with the samples. It is our goal to provide forensic analysts the necessary knowledge and tools to manage the processing of these exemplars without compromising the overall results.

DNA Collection and Purification

The DNA analysis process starts with the collection of a biological specimen. This process often includes collecting a portion of a substrate where the material was deposited and/or the collection of other substances that could have been deposited or mixed with the biological fluid. The cells within the biological material collected are then lysed in a mixture of detergents, salts and buffers to expose the DNA and any other chemicals within the cell. During this process cell debris, dyes and chemicals from substrates are also solubilized. This new mixture of buffers, detergents, nucleic acids and other substances is then purified in an attempt to isolate the DNA from all other mixture components. The most well-known DNA purification method is that which comprises a liquid-liquid extraction [7] involving phenol-chloroform-isoamyl alcohol reagents followed by ethanol precipitation. This method, also known as the ‘gold standard’ for

DNA extraction, relies on the solubility of the different components within the mixture into the organic and aqueous phases of the solution. DNA is a polar molecule which will dissolve in the polar aqueous layer of the solution whereas other cell products will ideally dissolve in the organic phase. However, proteins and other components are also soluble in the aqueous phase posing potential ‘contamination’ of the DNA. Nevertheless, the presence of phenol and chloroform reagents promotes the denaturation of proteins via hydrogen bonding interactions causing them to fold in a manner that favors their solubility in the organic phase of the mixture. In addition, the pH of the solution is kept slightly basic to prevent RNA from being solubilized in the aqueous phase, thus providing the most suitable environment to isolate the DNA from all other cell and substrate components. The aqueous phase is then removed and the DNA is ethanol precipitated or bound to a membrane from which it is later eluted with an aqueous buffer. While this method is known to yield a substantial amount of DNA, phenol is partly miscible in water and is often co-eluted with the DNA causing problems in downstream applications as it is known to be an inhibitor of the *Taq* Polymerase used during the quantification and amplification processes [8]. In addition, other polar mixture constituents can co-elute with the extracted DNA and potentially cause problems in downstream applications by interacting with the DNA or other reagents needed to complete these procedures. In the past 20 years, cell lysis processes and DNA purification methods have evolved into more reliable and advanced systems capable of isolating nucleic acids of superior quality. The field has started to transition from liquid-liquid extraction procedures to solid-phase extractions that yield not only ‘cleaner’ DNA but also allow for automation and high-throughput processes [9]. These technologies use

silica membranes or silica-coated magnetic beads and rely on the strong but reversible affinity of DNA to the silanol groups [9-11]. The presence of chaotropic salts such as guanidinium, urea or perchlorate for example, provide a high ionic strength, high entropy environment that promote the adsorption of DNA to silica coated surfaces by intermolecular hydrogen bond formations allowed through dehydration of the DNA and silica surfaces and shielded intermolecular electrostatic forces [12] (Figure 1).

Figure 1. DNA-silica bonding mechanism in the presence of chaotropic salts. Both DNA and silica become dehydrated in the presence of the salts which allow the interaction to take place. Once the salt concentration and pH are reversed, the interactions between the molecules can be undone.

Because of the selective nature of the process, the DNA molecules become adsorbed to the beads while impurities are washed away. Changes in salt concentration and pH of the solution then allow the DNA to become dissociated and eluted into the final extract solution [13]. Since the method provides the right conditions for DNA to be adsorbed to the beads, only inhibitors that are bound to the DNA or that have similar affinity for the silanol groups will be co-eluted with the nucleic acid. However, remnants of the chaotropic salts which can be deleterious to downstream applications can sometimes be found in the final extract [14]. Therefore, although this new mode of DNA purification reduces the likelihood of inhibitors to be co-extracted there is still the possibility of carryover of adverse substances into the final eluate. Because the extraction and purification of DNA is the first step of the analysis process, any inhibitory substance that is introduced or maintained after this procedure will likely be carried throughout the remainder of the DNA analysis causing problems with both analytical methods and interpretation. Although the presence of inhibitors is a well-known challenge in the field, there are still questions surrounding the most adequate solution. Most DNA inhibitor studies have focused on the mechanism of action of the inhibitory substances rather than on establishing how much of these substances is necessary to affect the recently enhanced quantification and amplification chemistries and determining what purification method is most reliable at consistently providing sufficiently 'clean' DNA extracts.

DNA Quantitation

Once the DNA is purified, it is important to establish how much nucleic acid is present to predict the optimum amount of sample for subsequent analysis. Too little DNA will yield amplification results plagued with stochastic effects that make data

interpretation a complicated process. In contrast, too much DNA will yield a high number of artifacts such as non-specific PCR, artificially elevated stutter, and off-scale peaks that can also pose problems in the final analysis of the data. The process of quantification is therefore essential to producing high quality results. There are a series of options available to provide assessments of DNA yields after extraction [15].

Spectrophotometry is a simple and accurate way to estimate the mass concentration of compounds in a given sample. It does so by measuring the absorbance of the sample at 260 nm to estimate the mass [16]. Nevertheless, this method lacks sensitivity and is not selective therefore any impurities contained in the sample that absorb in the UV spectrum will be detected [17] and the results of the nucleic acid of interest will be overestimated. Other methods such as measuring the amount of UV-induced fluorescence emitted by molecules such as ethidium bromide and Hoechst dyes when bound to the nucleic acid can yield more sensitive and specific results [18, 19]. With these methods, the amount of fluorescence emitted is proportional to the total amount of DNA and this measurement can be calibrated by comparing the fluorescence obtained from the sample to that of a series of standards. Yet although the aforementioned methods are more specific than spectrophotometry, they can still overestimate the amount of human genetic material as it is equally suitable to quantify molecules originating from other biological sources such as plants, animals or bacteria. Other spectrofluorometric methods have been developed using enzymatic techniques and hybridization assays which are more sensitive and are also specific to the human species. Of these human specific procedures, real time quantitative PCR is the most commonly used. The advantage of this technique is that it is easily automated, and because it is PCR based, it better models the results of the

subsequent PCR amplification of STRs. There are a variety of real time PCR methods which have been developed for forensic applications. One of the processes uses a target specific probe with a reporter dye and a quencher attached to opposite ends of the molecule. When the polymerase extends the primer to the location of the probe, its exonuclease activity cleaves the reporter dye, separating it from the quencher and allowing it to emit fluorescence [20](Figure 2).

Figure 2. Example of real-time quantitative PCR using a molecular probe. An increase in temperature denatures the molecule and enables the probe and primers to bind to their complimentary sequences. The polymerase extends the molecule and cleaves the probe thus separating the fluorescent tag from the quencher and allowing it to fluoresce. This fluorescence is detected and processed by a computer software.

The fluorescence generated will increase with every PCR cycle and will be proportional to the amount of target amplicon in the sample. Other chemistries involve the use of fluorescent intercalating dyes, scorpion probes and specially designed primers to similarly quantify the DNA [21-24]. For example, the Plexor HY [25] procedure

utilizes a fluorescently labeled modified nucleotide attached to the forward amplification primer whose fluorescence will be quenched upon the incorporation of the quencher modified nucleotide in the complementary strand [25]. With this method the decrease in fluorescence will be measured and will correlate to the amount of DNA in the sample. Real time PCR quantification methods use standard curves generated with known amounts of DNA to extrapolate the quantity of nucleic acid in a particular sample yielding good estimates of the amount of DNA recovered after the extraction procedure. In addition, these methods use internal positive controls (IPCs) or other techniques (melting curves, slope of amplification curves, etc.) to establish the presence of potential inhibitors and monitor the overall performance of the quantitative process. In real-time PCR, cycle threshold [4] measures the number of amplification cycles needed in order for the fluorescent signal of a sample to exceed the fluorescence generated by background noise. Therefore, the higher the Ct value the lower the amount of DNA in the sample. Internal positive controls are synthetic oligonucleotides that are added to the quantification reaction and are expected to amplify at with a pre-determined cycle threshold. Positive deviations from the cycle at which detection of the IPC is expected could be indicative of potential inhibition in the affected specimen. However, this assumption should be interpreted cautiously as it presumes that the effect that any given inhibitory substance has on the synthetic oligonucleotide will be the same as that attained when a real DNA fragment is subjected to the amplification process, assumption that has been challenged by some experts in the field [26, 27]. Some inhibitory substances have a high affinity for certain sequences within the DNA, in such cases the cycle threshold will increase if inhibitors are present. However, if the sequence affected by the inhibitor

is not present in the IPC, a misleading result may occur. Similarly if the inhibitor affects the polymerase at later stages of the amplification, the cycle threshold may not change sufficiently to be detected and lower amounts of product will be formed [28-30]. In these cases the synthetic oligonucleotide might fail to provide a good representation of the presence of inhibition. There are also inhibitors such as indigo dye which can interfere with the fluorescence detection by producing background fluorescence yielding results that do not properly represent the amount of DNA in the sample. Therefore, although there are quantification assays that are highly sensitive and specific to the human species, the estimation of the amount of genetic material recovered during extraction is highly dependent on the quality of the DNA recovered. As a consequence the estimation of DNA available after the extraction process can be over - or underestimated in every reaction affecting the decision making process for the following DNA analysis step. Studying the behavior of the IPC in samples spiked with various inhibitors at increasing concentrations could provide a better understanding of what might be occurring when unexpected results are observed during short tandem repeat amplification.

DNA Amplification

The process of amplification targets a series of pre-defined locations within the human genome. These target areas, referred to as short tandem repeats (STRs), are known to have a high degree of variability between individuals making them suitable candidates for person-to-person differentiation [31-34]. The nucleotide sequences and overall size of the STRs targeted in this process vary usually from 100 to 500 base pairs making the efficiency of amplification higher at certain locations as compared to others, especially as the DNA ages or gets exposed to the elements or certain inhibitory substances [35, 36].

Usually smaller loci have a higher amplification success as they are less likely to be affected by degradation and other factors that might trigger the fragmentation of the target DNA into smaller pieces [37, 38]. Amplification of DNA involves the production of thousands to millions of copies of the STRs so that they can be readily visualized using specialized instrumentation [39]. The process involves denaturing the DNA into single stranded molecules and allowing for primers to bind to the highly conserved sequences flanking the short tandem repeats of interest. A polymerase enzyme then extends these primers to make copies of the targeted regions. This process is repeated many times and with every repetition, the number of copies of the DNA theoretically doubles. [39]. In the early days of forensics, limitations in instrumentation and chemistry only allowed concomitant amplification of a small number of these target areas. In subsequent years the chemistries were improved and the technology was updated to allow the amplification of larger numbers of STR loci, increasing the discriminatory power [40]. Although results from just 13 specific locations is necessary in the United States to enter a DNA profile into the Combined DNA Index System (CODIS) [41], commercial kits now provide analysts the opportunity to recover information from up to 23 genetic markers in a single reaction [40]. Because each of these markers is individually inherited, the frequency of occurrence of the observed results at each locus for each individual tested can be multiplied thus yielding an exceptional level of discrimination between samples and a high degree of confidence in the results. Yet, the success in developing a high degree of multiplex amplification has also increased the number of DNA fragments to which inhibitory substances could potentially bind.

The method of PCR amplification is not perfect and in certain circumstances, unwanted artifacts and peak imbalances are produced, yielding results which can be difficult to interpret. Some of these artifacts can be caused by substances that are introduced to the process at the moment of collection and are carried through all the way into the amplification reaction. As the DNA amplification process progresses, these molecules can freely interact with the DNA. Some of these interactions are sequence specific [28] which means that the molecules will preferentially bind to certain regions of the DNA and can prevent the amplification of target locations. As previously stated, the success of the amplification process and the ease of interpretation of the results are highly dependent on the quantification estimate which is in turn reliant on the success of the purification process at removing unwanted substances. Though improvements in the amplification procedure have pushed the field forward by accommodating an extended number of markers in one reaction, and the manufacture of enhanced buffers have tamed the impact of inhibitors in the overall results, the general source of concern, the extraction process, has not been fully investigated. More information is needed on the level of inhibition that the amplification reaction can tolerate, and how much inhibitor is actually recovered after the extraction procedure is completed.

DNA Profile Generation

Capillary electrophoresis continues to be the tool of choice for DNA separation because of its ease of automation, minimal sample consumption, high-throughput capabilities and the fact that it has been widely tested by laboratories across the nation. The principle underlying capillary electrophoresis is size separation. A series of analytes are selectively sized using a sieving mechanism and an applied electric field. The ability

to apply high voltages to the silica capillaries [42] and the power to detect multiple wavelengths based on laser induced fluorescence [43] gave capillary electrophoresis an edge as compared to previously used slab gel techniques by allowing increased throughput and faster sample processing times.

The initial capillary electrophoresis method relied on cross-linked polyacrylamide gel filled capillaries [44] which allowed for good separation but suffered from short lifetimes due to the formation of voids in the gel during the electrophoretic process [45]. In addition, the use of this gel 'design' led to potential carryover between runs which is a problem when dealing with valuable evidence samples. To overcome these shortcomings, CE systems with entangled polymers were developed [46]. These allowed for similar separation efficiencies and offered the additional advantage of being able to re-fill and re-use the capillaries for up to an average of 100 times before replacement. Nonetheless shortly after the development of these polymers, it was observed that they could at times provide non-reproducible sizing due to the effect of the electroosmotic flow (EOF) on the migration time. These variations could have a huge impact on DNA analysis as it relies on the precise sizing of the DNA fragments for proper allele calling. Due to this issue, polydimethyl acrylamide (PDMA) became the polymer of choice in forensic applications due to its low viscosity and its ability to eliminate EOF [47, 48]. Buffer composition was also an important element in establishing accurate nucleic acid separation. For fragment length determination, a buffer capable of producing low, stable currents and suited to separate DNA under conditions where the formation of secondary structures is halted by denaturing agents such as heat, formamide or urea [49, 50] is the preferred option. For most genotyping applications buffers consisting of 100mM TAPS

at pH 8 with 5% pyrrolidinone and 8 M urea as denaturants are used [48]. EDTA is also present to minimize problems with metal contamination and DNA binding.

Polydimethyl acrylamide is added to help sieve the DNA and eliminate EOF. Uncoated fused-silica capillaries of a 50 μm in diameter are typically used because they provide a good compromise between resolution, sensitivity and resistance to clogging.

Electrokinetic sample injections are used for forensic applications because they provide good peak shape and intensity as a result of field amplified sample stacking. The stacking process results when the DNA sample is prepared in low ionic strength solutions. When an electric field is applied, the DNA accelerates to the sample/buffer interface where it becomes concentrated because of the drop in field strength at that location. Because this process depends on the ionic strength of the sample solution, it is important to maintain a low salt concentration in the sample. Since most PCR amplifications occur at high salt concentrations, the samples need to be diluted before injection as smaller buffer ions have higher electrophoretic mobility and interfere with the injection of the DNA. Sample injection is important because there is a direct relationship between the peak heights obtained and the sample concentration which helps the analyst assess the quality of the sample. Peak heights are also very important in deconvoluting mixtures as they help define the relative contribution of different individuals to this type of sample. Improper handling of the salt concentration in a sample and/or improper input levels of DNA in amplification can yield peak intensities that are not representative of sample quantity and therefore affect the aforementioned relationships. Usually a small volume of amplified samples is diluted in a higher volume of formamide.

After injection, samples are detected using laser induced fluorescence. This method is highly sensitive and offers multiplexing capabilities. Argon-ion lasers are used in combination with charged coupled devices (CCD) that collect the fluorescence emission produced by the various dyes bound to the DNA molecules. These dyes are incorporated into the samples using dye-labeled PCR primers or bases for genotyping and sequencing respectively [51, 52] and are designed to absorb at a single wavelength but emit at a variety of different wavelengths. This characteristic allows multiple loci to be concomitantly amplified by assigning a different dye to each set of primers. The different emission wavelengths combined with virtual filters and specialized calibration procedures allow the separation of the samples by correcting for dye overlap and interferences [51]. Current commercial systems are engineered to detect up to six dyes simultaneously on a single CE capillary with one of these dyes being assigned to the internal size standard. This standard consists of a number of peaks that span the size range of targeted loci. The analysis software uses these peaks to make precise estimations of size of the unknown fragments obtained from the sample in question. It should be noted that although very precise size estimates are obtained ($<2\text{bp}$) [53], these can be affected by factors such as temperature and sequence effects [54, 55] and that therefore careful monitoring of the temperature and denaturant concentration are important in order to maintain the sizing precision.

Capillary electrophoresis technology is widely used in forensics for a variety of applications all of which go through the same general process of separation and suffer from the same drawbacks. The detection of short tandem repeats, the most widely used application of capillary electrophoresis in the field of forensic sciences, suffers from the

potential presence of microvariant alleles which require single base pair resolution and high sizing precision for their accurate detection. Stochastic effects which are the result of amplification of concentrations lower than 100 pgs of DNA are also apparent in capillary electrophoresis [56]. These intensity fluctuations lead to peak imbalance and the occasional loss of signal at particular locations. The presence of mixed samples is also a phenomenon that can be present and affect data interpretation [57, 58]. The ability to produce clear and unambiguous electropherograms is of utmost importance in criminal casework since DNA evidence may be the only information connecting the suspect to the crime. Loss of peak intensity and the presence of artifacts in a sample can complicate data interpretation. When an inhibitor is carried through the process, it can also be the culprit for the generation of these hard to interpret artifacts in a DNA profile. In addition, inhibitory substances might have a downstream effect in the injection process which can in turn affect the migration and subsequent sizing of particular peaks. Understanding the behavior of these substances and the effects they might have in the final DNA profile can aid forensic analysts in the results interpretation and potentially provide information as to what steps to take to alleviate the effects of the inhibitor in a particular sample.

DNA Inhibitors

Unfortunately, DNA analysts have no control over what type of biological material or on what surface or substrate a sample gets deposited on during the commission of a criminal act. The type of evidentiary material submitted for analysis varies from case-to-case and sample-to-sample. Methods of DNA examination need to be flexible enough to accommodate the wide variety of sample types and matrices with which unknown samples are potentially contaminated. Inhibitors are substances that are present in the

substrate on which the sample has been deposited. On occasion, inhibitors can arise as a result of carryover from one of the analytical steps (i.e. phenol, guanidinium).

Regardless of the origin, these substances are often co-extracted with the biological material of interest. When present, they affect sample processing at various stages by interfering with fluorescence, preventing the polymerase from extending the DNA, binding to the DNA and blocking its extension or interfering with cofactors needed for appropriate amplification of the sample. An affected exemplar will exhibit sequence specific inhibition, allelic imbalance or complete allele drop-out, all of which will have a negative impact in the profile interpretation [59, 60] (Figure 3).

Figure 3.Example of effect of humic acid (HA) inhibitor on DNA profiles. HA is suspected of limiting the amount of DNA template. In this example, locus specific inhibition is apparent. (A) control sample, (B) 5 ng HA, (C) 10 ng HA, (D) complete drop-out attained with 15 ng spiked HA.

Some of the most common inhibitory substances found in biological samples and/or during the course of DNA analysis are hematin (blood), collagen (bone and connective tissue), calcium (bone) bile salts (feces), melanin (hair), tannic acid (soil), humic acid (soil), indigo (clothing), EDTA, guanidine and phenol (sample processing).

Blood is a common biological fluid analyzed during the course of criminal investigations.

This material is comprised of a series of molecules including hematin, a component of red blood cells [28, 61]. Hematin is a metal chelating compound which is known to form stable complexes with DNA polymerase and cause dissociation of the polymerase from the DNA during the extension step of the amplification reactions [62, 63]. Collagen is another common inhibitor encountered during DNA analysis of bone and/or tissue samples [64-66]. Although most studies suggest that DNA in bone is preserved due to its adsorption to hydroxyapatite – the inorganic component of bone [67-69] -other studies have shown that there is a direct correlation between the amount of DNA available for typing and the amount collagen present in the sample [70-73]. The same mechanism by which DNA becomes ‘protected’ by the presence of collagen in a sample is responsible for preventing this DNA from being extended during the process of amplification [29].

Calcium, a mineral also encountered in bones has also been identified as a potential DNA processing inhibitor and it is presumed, based on its size and charge, to interfere with the magnesium cofactor necessary to maintain the fidelity of the polymerase [74]. Melanin another known inhibitor, is a naturally occurring pigment present in hair and other areas of the body [75]. Previous studies have suggested that melanin binds to the DNA template present in a sample, preventing it from being amplified and detected [76-79].

Bile acids are synthesized in the liver and metabolized in the lower digestive track into

bile salts [80]. Therefore, they are commonly encountered in samples comprised of or containing fecal material and urine [81, 82]. These compounds are soluble in water but are responsible for aiding in the digestion of hydrophobic compounds by emulsifying fats. This physiological activity is possible due to their structural arrangement which allows them to act as surfactants [83-85]. This property is also used at times to disrupt cell membranes and aid in the extraction of cellular material [86]. Sodium cholate and sodium deoxycholate are anionic detergents similar to the sodium dodecyl sulfate (SDS) used in some lysis buffers. The usefulness of these surfactants as membrane disruptors should be exploited with care as there may be interactions between bile salts and DNA that can inhibit the analysis of the latter [29, 87].

Humic and tannic acids are inhibitors found in environmental samples and substrates derived from - or dyed with - plant material, such as leather [88-91]. These inhibitors are suspected of limiting the amount of DNA template available by interacting with the molecule or the polymerase enzyme. Humic acids have characteristics similar to DNA [92] which makes them likely to be co-extracted with the molecule. They can also covalently bind to nucleic acids or proteins, reducing their availability for amplification [28, 93]. Tannic acid and its derivatives have also been associated with DNA damage when in the presence of copper presumably due to chelation effects [94-97]. All of these interactions limit the availability of the DNA molecule and pose problems when dealing with samples that can potentially contain tannins [28]. Understanding the role of these inhibitors in PCR amplification is particularly important as they can interfere not only with human DNA typing but also with plant and/or microbial samples [98-102].

Indigo is a dye commonly used as a colorant for denim and other textiles [103, 104]. It is also a known DNA inhibitor which gets co-eluted with the nucleic acid material when processed via the organic extraction procedure. This compound, characterized by its blue color, is non-polar and highly soluble in chloroform [103] – one of the components of the phenol-chloroform purification reagents. Because of its dark coloration, studies attempted at studying the mechanism of interaction with DNA or its amplification components have proven difficult [28]. This very perception might be the reason for its inhibition. The dark coloration of this dye could be quenching or interfering with the detection of the dyes used in the quantification procedure, giving the impression that the DNA is directly affected by the presence of the compound. Ethylenediaminetetraacetic acid (EDTA) is a water soluble, metal chelating agent [105, 106] commonly used as a blood anticoagulant [107] and encountered in many foodstuffs and other industries due to its antioxidant and stabilizing properties [108, 109]. Interestingly, this molecule may hinder the DNA analysis process by binding to the magnesium ions necessary for the polymerase enzyme to operate with high fidelity [110, 111]. EDTA has also been found to affect the PCR process by interacting with certain sequences of the DNA [29, 112].

As mentioned previously, some chemicals or compounds inherent to the process of DNA analysis can also be inhibitory. An example of this type of compound is phenol. Phenol is an organic molecule used in the purification of nucleic acids. When carryover of this compound into the DNA eluate occurs, the molecule will be present in the downstream quantification and amplification procedures. Its presence will affect the efficiency of amplification, as phenol is a known protein denaturant and has been associated with *Taq* polymerase inhibition [8, 113]. Another example of this type of

compound is guanidinium. This is a protein denaturant that is commonly used in nucleic acid purification procedures [114]. During processing, it is likely that some amount of this compound will be transferred into the ‘purified’ DNA. When present in sufficient quantities, this molecule can affect DNA analysis by interfering with the *Taq* polymerase or preventing its binding to the DNA during the amplification process [115, 116].

In recent years, the surge in terrorist attacks have increased the amount of improvised explosive devices (IED) debris evidentiary material [117, 118]. These ‘home-made’ bombs are built from a variety of materials including certain types of metals found in wires and other sources. When attempting to identify the individual(s) responsible for the manufacture of these devices, metallic ions can become co-extracted with the DNA. Metals such as copper (Cu^{2+}), zinc (Zn^{2+}), cobalt (Co^{2+}) or nickel (Ni^{2+}) are known to chelate DNA, fragmenting it [119] and affecting downstream analysis by inducing conformational changes or aggregation [120].

Regardless of the mechanism of action, the presence of one or more of these inhibitory molecules in the ‘purified’ DNA sample can have a detrimental effect on the processing or analysis of the specimen. At worst this may mean stopping the analysis process prematurely based on misleading quantification results. At other times, the presence of an inhibited sample will be noticeable in the final result due to the presence of an uninterpretable or incomplete profile. Establishing the best method to process a sample suspected of inhibition may greatly reduce the incidences of cases in which this type of phenomenon is encountered. Throughout a series of studies, this thesis will attempt to establish the effects of inhibitory substances on the quantification and subsequent

amplification procedure for DNA evidence, as well as determine the optimal extraction methods for a wide variety of sample types.

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“The sky is not my limit, I am” - T. F. Hodge

II. DETERMINING THE EFFICACY OF QUANTIFILER® DUO IN ASSESSING SAMPLE INHIBITION WHEN USED IN COMBINATION WITH IDENTIFILER® PLUS AMPLIFICATION KIT

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Abstract

One of the major obstacles in DNA processing and analysis is the presence of inhibitors which can be co-extracted with the genetic material or are an inherent part of the sample and can interfere with downstream processing. Analysts continue to struggle with the processing and interpretation of such samples. While major forensic kit manufacturers have made great strides in improving the resistance of PCR kits to inhibition, challenges remain when it comes to identifying, and overcoming the effects of inhibitors in biological samples. The goal of this project is to assess the relationship between the quantification results obtained with Quantifiler® Duo and its downstream effect on amplification with the Identifiler® Plus STR kit by spiking samples with inhibitor concentrations that may result from carryover following sample extraction. While the Quantification kit is effective at detecting inhibition for certain compounds at the concentration levels examined, substances such as humic acid and phenol may not show strong inhibitory affects during real-time PCR assays yet can negatively influence downstream STR typing. Therefore analysts should practice discretion when using IPCs for inhibition detection as real-time PCR systems can differ in response depending on the type of PCR inhibitor present.

Keywords: DNA inhibitors, internal positive control (IPC), DNA quantification, DNA amplification

Background

PCR inhibitors are non-genetic substances that are part of the sample itself, the substrate where the sample was found or the chemicals used to treat the sample during processing.

Some of the most common inhibitory substances include collagen, humic and tannic acids, hematin, melanin, indigo dye, bile salts, phenol, guanidinium, and EDTA [1-9].

These are often co-extracted with the genetic material because like DNA they are soluble in the aqueous portion of an organic extraction or because they bind to the DNA with sufficient affinity to prevent them from being removed during the purification procedures. When present following extraction, these substances will prevent the *Taq* polymerase enzyme from processing the sample because they are bound to the DNA, to the *Taq* polymerase itself or to the co-factors needed for this enzyme to operate properly [10, 11]. Whichever the mechanism of action of the inhibitors, the outcome is usually the same: a partial or negative profile that may or may not be useful due to loss of alleles, the presence of peak imbalance or the detection of PCR artifacts [12]. Despite some of the recent advancements intended to address these common problems, little has been done to try to confidently identify the presence of inhibitors in a sample or understand their mechanism of action.

For years, forensic DNA analysts have relied on the internal positive control (IPC) included within quantitative PCR kits to establish the presence of inhibitors in a sample (16-20). These IPCs are synthetic oligonucleotides with an amplicon size similar to that of the target human DNA sequence. The IPC serves as an indicator of inhibition in any

given sample and helps the analyst distinguish it from a potential true negative (no DNA containing) sample. However, it has been recently noted by some case-working laboratories that a substantial amount of evidentiary samples that yield good quantitation results and have normal IPC cycle thresholds fail to amplify at all. These negative looking samples, when diluted and re-amplified usually yield complete DNA profiles that can be used for statistical comparisons. However, because the initial tests do not reveal the presence of inhibition, analysts normally have to process a sample multiple times to achieve a positive result which increases the turnaround time of the process. There is also the possibility that the analyst may be required to blindly explain the phenomenon observed. In addition, diluting a low concentration sample may result in the loss of precious evidence or prevent the generation of important leads in a particular criminal case. To confound the issue, at times the IPC results will indicate inhibition on samples that would have amplified without further manipulation, once again potentially compromising the quality of the end result due to unnecessary sample handling. Validation studies performed by the manufacturing companies usually only assess a handful of inhibitors and are not comprehensive. Furthermore as the chemistry of each kit improves, the quantitation process must also be improved or the quantitation and amplification steps will fall further out of sync [13-15].

The Quantifiler® Duo kit contains a minor groove binding (MGB) TaqMan probe and primers designed to bind specifically to the IPC during the quantification procedure. This probe, which contains a reporter dye and a quencher dye at opposite ends, will bind to a complementary sequence between the primer binding sites of the IPC and will emit no fluorescence unless the *Taq* polymerase cleaves the reporter away from the quencher dye

[16]. The latter only happens when all components are hybridized to their respective sequences and the forward primer begins to be extended [17]. If cleavage of the reporter dye is reduced due to ‘blockage’ of the complimentary sequence of the probe or forward primer, the detection of the IPC will be compromised and will be suggestive of the presence of an inhibitory substance in the sample as this internal sequence will normally amplify even if no external DNA target is present. The dilemma with this inhibition assessment approach is that there is the assumption that the IPC will react in a similar way to the DNA target for both quantitation and subsequent amplification procedure and therefore be a good predictor of the level of inhibition. It has been noted however, that such assumption might not be an accurate forecaster of inhibition [18, 19]. Other studies have explored the effect of inhibitors in melting temperature curves [20], on amplification efficiencies [21, 22] and on the sequence of the oligonucleotides representing the primers [23]. Unfortunately few metrics exist on practical aspects of detecting PCR inhibition with these commercially available quantitation kits due to the complexity of determining unknown inhibition in an operational crime laboratory. As a result, it becomes important for researchers to establish the basic limitations of the quantification kit when it comes to inhibition assessment and how to translate these results for forensic practitioners when using certain amplification kits. The goal of this project is to establish the inhibitor tolerance level of the Identifiler Plus (Life Technologies) amplification kit for each of the inhibitors examined and ascertain if the IPC results obtained with the Quantifiler Duo quantification kit (Life Technologies) are in fact an accurate representation of the presence of inhibitors in a sample.

Materials and Methods

Sample preparation

Blood samples from a consenting donor were extracted using the Prepfilr Express (Life Technologies, Foster City, CA) chemistry in combination with the Automate (Life Technologies) platform using manufacturer's recommendations. The extracts were quantified using Quantifiler® Duo following manufacturer's recommendations and subsequently diluted to ~1 ng/μl.

Inhibitor preparation

Stock solutions of inhibitors were prepared as follows: Calf skin collagen (Sigma Aldrich, St. Louis, MO), 1 mg/mL in 0.1 N acetic acid (Fisher Scientific, Waltham, MA); humic acid (AlfaAesar, Ward Hill, MA), 1 mg/mL in water; tannic acid (AlfaAesar), 1 mg/mL in water; melanin (MP Biomedicals, Solon, OH), 1 mg/mL in 0.5 N sodium hydroxide (Fisher); hematin (Sigma), 100 mM in 0.1 N sodium hydroxide (Fisher), indigo (MP Biomedicals), 1 mg/mL in water, bile salts (sodium cholate plus deoxycholate) (Fluka, St. Louis, MO), 100 mg/mL in water, phenol (Sigma), 100 mM in water, EDTA (Fluka), 0.5 M in water, guanidinium thiocyanate and guanidinium hydrochloride (Sigma), both 100 mg/mL in water. All subsequent dilutions of inhibitors were prepared in water and are listed in Table 1.

Real –Time Polymerase Chain Reaction

To assess the level of inhibitor needed to cause an adverse effect on the real-time quantification reaction when using Quantifiler Duo (Life Technologies) a master mix consisting of 11.5 μl of reaction mix, 10.5 μl of primer mix, 2 μl of previously diluted to 1 ng/μl DNA and 1 μl of inhibitor at any given concentration (Table 1) were subjected to

amplification following manufacturer's recommendations [24]. Inhibition was assessed based on the IPCs cycle threshold as compared to the uninhibited control samples. Normally, IPC fluorescence detection at 30 or more amplification cycles is an indication of inhibition. All quantifications were carried out in triplicate.

STR analysis

The DNA sample used in the quantification experiment was further diluted to 0.1 ng/μl before adding 10 μl of DNA to a mixture of 9 μl Identifiler Plus buffer mix, 5 μl Identifiler Plus primer mix and 1 μl of the corresponding inhibitor and associated concentration. Samples were amplified according to manufacturer's recommendations [25] but using 27 amplification cycles. All amplifications were performed in duplicate. This process would determine if the quantification results were truly indicative of the sample-inhibitor dynamics. The relative fluorescence units (RFUs) for each of the alleles corresponding to the various markers within the kit are expected to be proportional to the amount of DNA amplified for that particular sample. Therefore, since the amount of DNA was the same for every sample, each resulting signal was compared to that from the uninhibited control to determine if the presence of any given inhibitor in the spiked samples had a negative impact in the amplification process. These results were then cross-referenced with those obtained from the quantitation step to determine if there was a correlation amongst the results obtained between the two processes.

Results and Discussion

Quantitative real-time PCR is used in criminal laboratories to assess the quantity of DNA available after purification and to provide a partial quality assessment of the sample by predicting the level of inhibition, if any, present in a given exemplar. The process by

which inhibition is determined, although a good predictor in certain instances, might not be equally efficient at ascertaining the effects of these substances in downstream applications depending on the type of inhibitor present in the sample. The mechanism of action of some inhibitors has been established in previous studies [20] and can be used to explain some of the observations. However it is important to establish the range of sensitivities of the quantification kits to different types of PCR inhibitors in order to guide the analyst on how to establish best practices for overcoming inhibition when faced with certain types of samples. In the following sections, the relationship between the response by the Quantifiler Duo kit to different inhibitors and the amplification results attained with Identifiler Plus are discussed.

Collagen

Collagen is found in bone and connective tissue and can therefore pose a problem in cases involving these types of specimens [26]. The addition of 1 ng of this protein to the sample exhibited a 1.2 cycle shift in the IPC detection threshold as compared to the uninhibited sample, indicating reduced reaction efficiency that may be attributed to polymerase inhibition (Table 1). When quantities of 5 ng or greater were added to the reaction, the Quantifiler® Duo kit suggested full inhibition of the process (IPC Ct >30 cycles) (Table 1). Amplification of the samples using Identifiler Plus resulted in a slight decrease in relative fluorescent units (RFUs) as the concentration of inhibitor increased from zero to 1 ng and consistent with the quantification results, no data were obtained when concentrations of 5 ng or higher were used (Figure 1). Other investigators have demonstrated that collagen acts as a mixed mode inhibitor affecting not only the

Figure 1. (A) Control and (B) collagen spiked sample (5 ng). Electropherograms depict the effect of collagen in the amplification reaction. After the addition of at least 5 ng of collagen no results were attained.

availability of *Taq* polymerase, but also that of DNA by forming hydrogen bond interactions [27, 28]. The difference in electrostatic forces can cause the collagen to surround the DNA molecule and thus ‘block’ its availability for amplification. The lack of detection of the internal size standard in the amplification reaction is also consistent with the interaction of collagen with DNA.

Humic Acid

Humic acid is encountered in most environmental samples and therefore is commonly seen in evidence that has been exposed to the elements as it is one of the major components of soil [29].

Table 1. Quantification results, IPC cycle threshold data and associated standard deviations for the various inhibitors and concentrations examined

Inhibitor	Final concentration	RPPH1 ¹		IPC ²		Δ Ct as compared to uninhibited sample
		Ave. Quantity	SD	Ave. Ct	SD	
Collagen (ng)	0	0.706	0.038	28.44	0.163	---
	1	0.543	0.028	29.68	0.104	1.2
	5	---	---	≥ 40	---	---
	10	---	---	≥ 40	---	---
	15	---	---	≥ 40	---	---
	25	---	---	≥ 40	---	---
Humic Acid (ng)	0	0.640	0.050	28.46	0.552	---
	1	0.003	0.001	32.90	0.424	4.4
	5	---	---	≥ 40	---	---
	10	---	---	≥ 40	---	---
	15	---	---	≥ 40	---	---
	20	---	---	≥ 40	---	---
	25	---	---	≥ 40	---	---
Melanin (ng)	0	0.603	0.117	28.68	0.304	---
	10	0.677	0.068	27.84	1.683	-0.84
	20	0.596	0.007	28.82	0.255	0.14
	30	0.002	0.002	≥ 40	---	---
	40	---	---	≥ 40	---	---
Tannic Acid (ng)	0	0.631	0.091	28.93	0.014	---
	1	0.586	0.017	29.32	0.028	0.39
	5	0.015	0.013	30.47	0.042	1.54
	10	---	---	≥ 40	---	---
	15	---	---	≥ 40	---	---
	17.5	---	---	≥ 40	---	---
	20	---	---	≥ 40	---	---
Indigo (ng)	0	0.968	0.290	29.7	0.076	---
	0.2	2.077	0.107	29.8	0.044	0.05
	0.4	2.073	0.091	29.7	0.046	-0.06
	0.6	2.203	0.042	29.7	0.013	-0.07
	0.8	2.100	0.030	29.8	0.069	0.03
	1	2.100	0.020	29.6	0.212	-0.10
Hematin (μ M)	0	0.682	0.021	28.59	0	---
	5	0.554	0.072	27.62	0.537	-0.97
	10	0.012	0.008	31.39	0.544	2.8
	15	---	---	≥ 40	---	---
	20	---	---	≥ 40	---	---
	30	---	---	≥ 40	---	---
	40	---	---	≥ 40	---	---
Bile Salts (μ g)	0	0.587	0.126	28.37	0.877	---
	0.25	0.096	0.011	30.01	0.064	1.64
	0.50	0.002	0.001	33.41	0.467	5.04
	0.75	---	---	≥ 40	---	---
	1.00	---	---	≥ 40	---	---
	1.25	---	---	≥ 40	---	---
	1.50	---	---	≥ 40	---	---

		RPPH1 ¹		IPC ²		
Inhibitor	Final concentration	Ave. Quantity	SD	Ave. Ct	SD	Δ Ct as compared to uninhibited sample
EDTA (mM)	0	0.673	0.019	28.54	0.078	---
	0.5	0.839	0.003	28.49	0.488	-0.05
	1.0	0.701	0.008	29.09	0.021	0.55
	1.5	0.635	0.012	28.92	0.481	0.38
	2.0	0.537	0.026	29.38	0.113	0.84
	2.5	0.550	0.132	29.32	0.141	0.78
Phenol (mM)	0	0.962	0.010	29.59	0.040	--
	15	0.999	0.072	29.78	0.014	0.19
	30	0.937	0.044	29.69	0.003	0.10
	45	0.898	0.076	29.78	0.054	0.19
	60	0.947	0.052	29.85	0.031	0.26
	75	0.918	0.001	29.76	0.011	0.17
	90	0.886	0.054	29.64	0.066	0.05
	105	0.896	0.042	29.75	0.033	0.16
Guanidinium-Thiocyanate (μ g)	0	0.741	0.146	28.84	0.021	--
	1.5	0.618	0.030	29.03	0.233	0.19
	3.0	0.026	0.010	31.17	0.502	2.33
	4.5	---	---	37.42	0.438	8.58
	6.0	---	---	≥ 40	---	---
	7.5	---	---	≥ 40	---	---

¹RPPH1 – Human quantification target, ribonuclease P RNA component I; ²IPC – internal positive control

Previous studies have suggested that the presence of humic acid in DNA samples interferes with the processivity of the polymerase enzyme [30] while others attribute the inhibitory effects to the interactions of this compound with DNA [20]. In the present study, the addition of humic acid to the samples at concentrations as low as 5 ng, had a deleterious effect in the quantification process. The IPC had a substantial shift in cycle number (11 cycles) in comparison to the un-inhibited control DNA sample (Table 1, Figure 2). Higher concentrations of this compound completely inhibited the IPC, an indication that this sample would likely fail to produce a result unless diluted or further purified. Nevertheless when amplified, these samples exhibited little if any inhibition at all concentrations of humic acid (Figure 3). There was no indication of any loci affected significantly by the addition of this inhibitor to the samples. It should be noted that

variations in the composition of humic acids may also cause differences in the effects of this substance on the various DNA processing reactions. Although based on this data no mechanism of action can be confirmed, it is relevant to note that when working with environmental or environmentally exposed samples DNA analysts should use caution when interpreting quantification results if the quantitation kit in question is being used. Clearly there are differences in inhibition resistance between the quantification kit and the STR amplification kit. Dilution of such samples might be the approach of choice, however the analyst risks loss of signal if the sample is already present in low concentrations. Because there is no guidance as to how much input material should be added to the amplification reaction if no data is obtained during quantification, individual laboratories should establish their own protocols should they choose to address samples which produce no quantitative results.

Melanin

The presence of melanin in the samples during the quantification process suggested inhibition after the addition of 30 ng of the pigment. There was significant shift in the IPC cycle threshold at the aforementioned concentration and above suggesting strong inhibition of the sample (Figure 4, Table 1). No apparent decrease in RFUs was

Figure 2. Graph depicts the relationship between the fluorescence emissions of the internal positive control and the cycle number at which they started to be detected for samples spiked with humic acid. The control IPC was detected at ~28.5 cycles. Samples spiked with the inhibitor didn't cross the threshold before 40 cycles and are therefore not observed in the graph.

Figure 3. Average relative units after amplification of 1ng of DNA with Identifiler Plus and various final concentrations of humic acid. Contrary to the suggestion of the quantification results, the samples exhibited no obvious sign of inhibition. No significant differences were observed in the amplification of the samples regardless of the amount of inhibitor present in the reaction.

observed during amplification at the lower melanin concentrations. Nevertheless when samples were spiked with 40 ng of melanin, the highest concentration examined, a drop in peak intensity became apparent (Figure 5). Further inspection revealed that although slightly lower in intensity, no amplification peaks were detected below 50% of the control sample. The most affected loci were D18S51 and D13S317 with drops of 34 and 30 % relative fluorescent units respectively in comparison to the control sample.

Melanin is a natural pigment that is generated from the oxidation of tyrosine and is found primarily in skin and hair [31]. The presence of this substance in a DNA sample has been determined to interfere reversibly with polymerase enzyme activity [2]. Because the process is reversible, the activity of the enzyme can be restored on its own or can be overcome by increasing the amount of enzyme in the amplification reaction or by adding other proteins that could interact with the inhibitor, such as bovine serum albumin [32]. Newer STR kits contain buffer formulations that can help bind inhibitors and minimize their effect during amplification. The fact that higher concentrations of melanin had a slightly negative impact on the STR amplification results suggest that melanin might also be interacting with the DNA itself.

Tannic Acid

Tannins are polyphenolic compounds found in plant material and leather [33] that can be co-extracted with environmental or environmentally exposed samples. Increasing quantities of this compound in the samples caused increased Ct's of the IPC suggesting

Figure 4. Relationship between the fluorescence emissions of the internal positive control and the cycle number at which they started to be detected for samples spiked with melanin. Samples spiked with inhibitor didn't cross the threshold before 40 cycles.

Figure 5. STR amplification results summary observed with Identifiler Plus kit after spiking the samples with various melanin concentrations. Despite the shift in cycle threshold observed in quantification there was no significant impact detected on the amplification results.

limitation of the *Taq* polymerase and thus reduced amplification efficiency. At a concentration of 5 ng, there was a reduction in the expected quantification results and a 1 cycle shift in the IPC cycle threshold. At concentrations above 5 ng, the cycle threshold was too high to be detected (Table 1, Figure 6). These results suggest that the presence of this inhibitor in the samples at concentrations higher than 5 ng should have a negative impact in the amplification process. Nevertheless, STR amplification results were consistent with those expected from an un-inhibited sample (Figure 7) indicating that the quantification kit is not necessarily a good predictor of inhibition when this compound is present or suspected in a sample. There was no substantial loss of intensity in any of the loci amplified using the Identifiler Plus kit after the addition of up to 17.5 ng of tannic acid to the sample. The percent decrease in intensity when tannic acid was present ranged from 0 to 10 % and although some variability in intensity was observed within the markers at the various inhibitor concentrations, none of them were of significance. Previous studies conducted with other commercially available quantification kit attained similar results [28]. It has been suggested that the abundance of electronegative groups in this molecule could interfere with cofactors necessary for *Taq* polymerase processivity [8], however the concentration of inhibitor needed to cause this effect appears to be substantially high. The amplification chemistry used in this study contains some amount of the bovine serum albumin (BSA) protein. This protein, commonly used to placate inhibition has been reported to have a very high affinity to tannic acid [34, 35]. These strong interactions could be responsible for the absence of inhibitor indicators in the process. The high availability of electronegative groups suspected of interacting with the

Figure 6. Effect of tannic acid on Quantifiler Duo IPC cycle threshold. Concentrations of 5 ng and greater in the reaction cause a substantial shift in the cycle threshold of the internal positive control suggesting strong inhibition of the sample.

Figure 7. STR amplification results summary data for samples spiked with various concentrations of tannic acid. No negative effect was observed with increasing concentrations of the inhibitor. These observations contrast the results expected based on quantification data.

Taq polymerase is also responsible for the high affinity of the molecule to BSA thus limiting the availability of the inhibitor to interact with the polymerase.

Indigo

Indigo is a blue colored organic compound that is routinely used to dye denim and other fabric materials [7] therefore it could be co-extracted with evidentiary samples deposited on clothing items. Because of its color, it was presumed that the presence of indigo in the quantification procedure would interfere with the fluorescence emission and yield unreliable results. Although it appears that there was no positive shift on the cycle threshold of the IPC it was noticed that the quantification results were overestimated (doubled) when indigo was present in the reaction (Table 1). For this experiment, the input concentration for amplification was maintained at 1ng/μl regardless of the quantification results. When the samples were amplified there was some loss of intensity at the highest inhibitor concentration in the following locations: TH01, D18S51, D2S1338 and FGA (37, 37, 35 and 34 % respectively) (Figure 8). Whilst it might not be a problem when dealing with samples for which enough material is obtained, basing the DNA input on the quantification results might lead to over-dilution of a sample and affect the interpretation of the amplification results. It is difficult to attribute indigo ‘inhibition’ to any particular mechanism due to the challenges encountered because of the compounds’ ability to interfere with fluorescent detection. Thus the absorbance or quenching effects of the dye itself rather than any true inhibitory mechanism might be the culprit behind the results obtained when this molecule is present in a sample.

Figure 8. Identifiler Plus data summary for samples spiked with increasing concentrations of indigo dye. Although less than 50 %, there was some drop in relative fluorescent units as the concentration of the dye increased.

Hematin

The addition of hematin to the samples yielded a 3 cycle shift on the IPC cycle threshold, and thus partial inhibition when 10 μ M of the compound were added to the sample.

Quantities greater than 10 μ M suggested complete inhibition and an IPC cycle threshold of greater than 40 cycles (Table 1, Figure 9). When amplified with Identifiler Plus, no significant adverse effect was detected (Figure 10). Steady relative fluorescent intensities were observed across all loci with variations amongst loci ranging from 0 to 18 %. These observations indicate that the quantification kit is again not necessarily accurate at determining the level of inhibition in a sample suspected of containing hematin and the appropriate safeguards should be employed when dealing with low template samples to avoid the over-dilution of an exemplar which might complicate the interpretation of the

Figure 9. Effect of hematin on the IPC cycle threshold. The inhibitor has a negative effect on the detection of the IPC signal when present at concentrations at or above 10 μM .

Figure 10. Summary amplification data from samples spiked with hematin. No significant differences were noted in allele intensities for samples spiked with up to 40 μM of the inhibitor.

results when no dilution is needed. Hematin is a chelating molecule found in red blood cells and can therefore be co-extracted with any blood containing samples. While some studies suggest that the presence of hematin in a DNA sample interferes with the amplification process by establishing associations with Mg^{2+} [3]; other studies suggest that it is more likely that the inhibitory effects are caused by interaction with the DNA molecule itself [20]. Although not noted in these results, hematin could pose a problem similar to that encountered with indigo when very concentrated amounts of the compound are co-extracted such that the dark coloration can interfere with the accurate depiction of the interaction of the molecules within a sample tube.

Bile Salts

Bile is a compound substance produced by the liver and used for digestion of fatty acids [36]. As such, this compound is commonly encountered in fecal material in addition to the liver, gallbladder and intestinal tissues. The major components of bile are bile salts and these have been known to cause PCR inhibition by limiting the activity of *Taq* polymerase [37]. These salts can also sometimes be found in DNA lysis buffers where they are added as detergents to aid in the extraction of DNA from cells. Increasing concentrations of bile salts caused gradual shifts on the IPC cycle threshold indicating partial inhibition when 0.25 μg were spiked in the sample and full inhibition with a Ct shift of 5 cycles when spiked with 0.50 μg or more (Table 1). The expected quantification results started to decrease as the IPC Ct increased in the samples. When amplified using the Identifiler Plus kit, the samples exhibited some loss in intensity with the greatest loss being 27% at D16S539 (Figure 11). Although some impact on the

Figure 11.Effect of various concentrations of bile salts after addition to amplification reactions. No significant effect was observed at any particular locus.

amplification was observed as the inhibitor concentration increased, the overall effect on the results was minimal. These results once again suggest that the quantification kit might not be a good indicator of inhibition when used as a precursor to the Identifier Plus kit as it might trigger unnecessary manipulation of a sample prior to STR amplification, potentially compromising the results.

EDTA

Ethylenediaminetetraacetic acid (EDTA) is a powerful chelating agent generally used to remove di – and trivalent metal ions [38, 39]. The addition of this compound to DNA up to 3 mM yielded minimal shifts in IPC cycle threshold results and no overall effect on the expected quantitation data (Table 1) unlike what has been reported in studies conducted with different quantitation chemistries [28]. When the same amount of DNA was added

Figure 12. Results of amplification with Identifiler Plus after the addition of various EDTA concentrations to the samples. EDTA had a significant effect on the amplification reactions causing a substantial reduction in RFUs after the addition of 0.5 or 1 mM and complete inhibition at higher concentrations. These observations can be attributed to the chelating nature of EDTA and the reduction in available magnesium ions in the reaction necessary for polymerase activity.

to an STR amplification reaction with Identifiler Plus, a gradual decrease in amplicon intensity (>50 %) was observed at all locations after the addition of 1 mM and concentrations above 1.5 mM completely inhibited the amplification reaction (Figure 12). These results suggest that in addition to the expected chelation of the magnesium ions in the amplification reaction, there might some additional binding to the DNA template.

Phenol

The presence of phenol in DNA samples is usually attributed to carry over from phenol-chloroform-isoamyl DNA purification procedures. When present in high enough amounts, this organic compound is known to have a negative impact in the amplification procedure by inactivating the *Taq* polymerase [9]. The addition of phenol to the

quantification reaction had no negative impact on the results and only exhibited slight shifts in the IPC cycle threshold of the reactions (Table 1, Figure 13). Nevertheless, when the same amount of the compound was added to the amplification reaction the negative effects were very noticeable and became progressively worse as the amount of phenol increased. These observations contrast what was expected based on the quantification data, suggesting that this kit is not a good predictor of inhibition when phenol is present in the samples. The effect on the intensity of the alleles during amplification was indicative of substantial inhibitory effects especially at the highest concentrations (Figure 14). At concentrations of 75 μ M and above, reductions in intensities of $\sim$ 50% as compared to the control started to be observed for some of the loci. As the concentration of phenol increased, these reductions in RFUs became more evident. At a 105 μ M phenol concentration most of the profile was strongly inhibited. Although with a much lower affinity than tannic acid, phenol also likes to interact with BSA [34, 40], therefore the differences observed between quantification and amplification kits could be explained by the differing concentrations of both BSA and polymerase between them which allow for different levels of interaction even when the same concentration of DNA and inhibitor are present in the sample.

Guanidinium

The thiocyanate and hydrochloride salts of guanidinium are commonly used in silica based nucleic acid isolation procedures and can therefore be carried over into the 'pure' DNA eluate [41]. The addition of these salts to the quantification reaction showed a shift of 2 cycles in the IPC cycle threshold when 3 μ g of guanidinium were added to the reaction, suggesting partial inhibition. At concentrations higher than this, quantification

Figure 13. IPC cycle threshold results for samples spiked with increasing concentrations of phenol. Data suggests no inhibition when phenol is present in the samples at up to a concentration of 105 mM.

Figure 14. Overall representation of phenol effect on amplification results. Increasing concentrations of phenol in the sample had a significant impact on the amplification results causing a reduction in amplification efficiency and a decrease in relative allele intensities of greater than 50 % at concentrations of 75 mM and above.

Figure 15. Effect of guanidinium salts on the Identifiler Plus amplification reaction. The presence of guanidinium hydrochloride or thiocyanate yielded similar results.

results were indicative of strong/full inhibition (Table 2). The addition of the compound to the amplification process exhibited results similar to those attained during quantification. A reduction in intensity was observed when the quantities of guanidinium of 6 µg or greater were included in the sample. Concentrations lower than this yielded no significant differences in the amplification efficiency contrary to what was expected based on the quantification results. TH01, D8S1179, D16S539 and FGA all exhibited decreases in intensity of ~50 % (Figure 15).

Conclusions

While real-time quantification assays can provide good estimates of human DNA quantity, these estimates are commonly only reliable if a relatively uncontaminated DNA sample is subjected to the process. The presence of exogenous compounds especially those with known inhibitory nature, can interfere with the PCR and yield data indicative

of the need for additional sample manipulation when unnecessary and vice-versa. The assessment of the internal positive control as a measure of inhibition can be confounding as the results are not always indicative of what will be observed in the subsequent STR amplification step. To further complicate the assessment, the results obtained will vary when different quantification and/or amplification kits are used since the formulations and concentrations of the individual components are expected to be different. In addition, the sequence of the internal positive control contained in the amplification kits of different manufacturers will likely be different, which can also affect the level of inhibition detected, especially when the compounds have a preference to bind the nucleic acid.

Since it is up to individual laboratories to choose their combination of quantification and amplification kits, it is important to note and study these differences so that more informed decisions can be made when possible and so that explanations for the instances in which the problems cannot be prevented are available. The presence and effects of inhibitors are deleterious to the successful analysis of precious DNA samples that usually come in already small quantities. Additional markers and more sensitive instrumentation serve no purpose if there is no consistent and reliable way of monitoring, identifying and handling the presence of these detrimental to the process substances. Analysts cannot control the reality of inhibitors but if there are ways to study, understand and control their effects, these should be used to enable the successful processing of a given DNA sample and minimize the time and money required to do so.

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“The best way out is always through” - Robert Frost

III. THE USE OF DIRECT ANALYSIS IN REAL-TIME TO ASSESS THE LEVELS OF INHIBITORS CO-EXTRACTED WITH DNA AFTER PURIFICATION WITH VARIOUS METHODS AND THE ASSOCIATED IMPACT IN QUANTIFICATION AND AMPLIFICATION RESULTS

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Abstract

The measure of quality in DNA sample processing starts with an effective nucleic acid isolation procedure. Most problems with DNA sample typing can be attributed to low quantity DNA and/or to the presence of inhibitors in the sample. Therefore, establishing which isolation method is best at removing potential inhibitors may help overcome some of the problems analysts encounter by providing useful information when trying to determine the best analysis approach for any given sample. The use of Direct Analysis in Real Time (DART) mass spectrometry was used in this investigation to correlate the amount of inhibitor carryover to that of the amount of inhibitor spiked in a sample following DNA extraction using the phenol-chloroform, Prepfilier and EZ1 chemistries. Samples were next quantified and amplified to determine the extent of the effect that inhibitors co-eluted with the nucleic acid would have in the overall results. Data suggest that organic extraction methods would likely always include varying detrimental amounts of phenol carryover while the automated methods could co-elute bile salts or other chemicals that are drawn to the silica beads. Both of these can have a direct impact on the overall DNA typing results.

Keywords: DNA inhibitors, direct analysis in real-time (DART), DNA purification

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Background

Previous studies have established that the effect of inhibition can vary depending on the amount and type of inhibitory substance present in the sample during quantification and amplification procedures. Therefore it is important to determine the efficiency of different extraction methods at removing various inhibitory substances during the process of DNA purification. The phenol-chloroform-isoamyl alcohol or ‘organic’ procedure has been the gold standard for DNA purification in crime laboratories for years[1, 2]. However, despite the well-known high recovery attained when utilizing this isolation method, scientists often struggle with the quality of the genetic material obtained. The underlying cause for the compromised results is usually the presence of DNA inhibitors from the samples themselves, from the substrates where they were deposited or from phenol carryover as a result of the reagents used. In an effort to reduce the use of potentially harmful chemicals as well as improve efficiency, forensic manufacturers have developed semi-automated, solid-phase extraction methods to replace organic purification methods [3, 4]. These new methods utilize a solid phase extraction in which the DNA is bound to magnetic beads through chaotropic or ion exchange mechanisms. The adsorption and desorption of the DNA can then be controlled by pH, buffer composition, and ionic strength. Typically the DNA is loaded under conditions in

which it binds strongly to the magnetic particles and then can be eluted by increasing the pH, or varying the buffer composition [5, 6]. Binding the DNA to magnetic beads also permits vigorous washing of the nucleic acid thus yielding a purer extract [7]. However, these new methodologies for the isolation of DNA tend to yield lower amounts of purified material in comparison to the organic extraction, and are not exempt from reagent carryover. Thus certain compromises must take place when using automated solid phase extraction. Although the automated methods are more efficient and produce cleaner extracts, the lower DNA yields may result in an incomplete STR profile that is just as problematic as a profile that results from PCR inhibition. Furthermore, despite manufacturer's claims of cleaner extracts, few if any studies have been performed to establish a quantitative estimate of the relative quality of the genetic material with respect to the percent inhibitors removed following processing. In addition, since the mechanism of operation of various extraction methods can differ greatly, it is important to develop an understanding of which purification process performs best when confronted with different classes of PCR inhibitors.

Chemical analysis methods can be used to establish the relative presence or absence of these inhibitory compounds as well as provide quantitative estimates of their relative recovery in the DNA eluate following the extraction process. While various chromatographic methods could be used for this type of analysis, most of these procedures require a significant time for sample preparation and data evaluation, and the range of compounds detected is sometimes limited based on size and chemical structure as well as the type of method (GC or LC) and stationary phase utilized (for example, ion exchange or reversed phase). Instead, we propose to utilize a recently developed method

known as direct analysis in real-time (DART) mass spectrometry. This procedure permits instantaneous ionization of samples in liquid, solid and gas forms at atmospheric pressure with minimal or no sample preparation [8, 9]. Because of its simplicity, ease of use, and its capability to provide qualitative as well as quantitative information, this technique has gained popularity in the analytical chemistry field and has been recently used to study analytes in various scientific disciplines [10, 11] including forensic sciences [12, 13]. In this study, DART-TOF was used to determine the presence or lack thereof of indigo, phenol, EDTA, bile salts, melanin and tannic acid in the DNA eluate following the extraction of spiked blood samples. Various purification methods were examined to determine which procedures yielded the cleanest extracts. The relationship between the relative amount of inhibitor present in the original samples and the resulting extracts was also determined in order to characterize the efficiency of each extraction method for various classes of inhibitors. The overall goal of the study was to provide forensic analysts with useful information on how to process various sample types, especially when know inhibitors are present.

Materials and Methods

Standards and Sample preparation

Stock solutions of the known inhibitors were prepared by diluting them into various concentrations (Table 1). To prepare the stock solutions, melanin was diluted in 0.5N sodium hydroxide (NaOH) and all other inhibitor stock solutions were prepared with water. Subsequent dilutions were all prepared in water.

Mixtures of liquid blood and inhibitors were prepared at the following volume ratios: 1:1, 1:5, 1:10 and 1:20 at the concentrations listed in Table 1. The final amount of inhibitor

per microliter of blood is also included on the table. Ten microliters of each of the sample mixtures were aliquoted into three different tubes for extraction using Qiagen's EZ1 (Madison, WI), Life Technologies' Automate platform (Foster City, CA) and phenol/chloroform (organic) extraction methods. Ten microliters of neat blood per inhibitor were also extracted with each of the platforms to be used as 'negative' inhibitor controls.

Table 1. List of inhibitors used and associated starting concentrations and quantities

Inhibitor	Source	Concentration	Amount per μl of blood (mg)			
			1:1	1:5	1:10	1:20
Indigo	MP Biomedicals, Solon, OH	1mg/mL	0.001	0.005	0.010	0.020
Phenol	Sigma, St. Louis, MO	100mM	0.009	0.045	0.090	0.180
EDTA	Fluka, St. Louis, MO	0.5 M	0.186	0.930	1.860	3.720
Bile salts	Fluka	100mg/mL	0.100	0.500	1.000	2.000
Melanin	Sigma	1mg/mL	0.001	0.005	0.010	0.020
Tannic Acid	Sigma	1mg/mL	0.001	0.005	0.010	0.020

DNA purification

Extractions were conducted following manufacturer's recommendations for both EZ1's DNA Investigator and Prepfilr Automate procedures [14, 15]. The organic extraction was conducted by adding 450 μl of a mixture of stain extraction buffer (TRIS-HCl, NaCl, EDTA, 2% SDS, DTT) and Proteinase K (Amresco, Solon, OH) to each sample before incubating them at 56 °C for two hours. After the incubation period, an equal volume of phenol-chloroform-isoamyl alcohol reagent (Invitrogen/Life Technologies, Foster City, CA) was added to the sample before removing the organic fraction and filtering the aqueous phase to elute the DNA in 25 μl of water.

DART-TOF Analysis

The DART ion source was coupled to a JEOL AccuTOFTM mass spectrometer (JEOL USA, Peabody, MA) and calibrated using Polyethylene glycol (PEG) 600 (Thermo Fisher, Waltham, MA). Mass CenterTM Main software (JEOL USA) was used to control the collection of the data. The closed end of borosilicate glass capillaries was used to introduce the sample, standard, or control into the DART gas stream after dipping them into the respective solutions. Each solution was sampled in triplicate and an average, centroided spectrum was produced after subtracting the background noise. Data corresponding to individual inhibitors were collected within a single data file so that it could all be normalized against the same standard. The following parameters were used to collect the data: ring lens voltage – 5V, orifice 1 voltage – 30V, orifice 2 voltage – 5V, detector voltage – 1950V and peaks voltage – 800V. Data were collected with helium as the carrier gas when the instrument was run in positive mode or nitrogen as the carrier gas when negative ion mode was used. The distinction was made in order to promote proton transfer reactions after the ionization of water, or to maximize the efficiency of Penning ionization. Data corresponding to bile salts and melanin were collected in negative ion mode at 500 °C, all other data were gathered in positive ion mode at 400 °C. The parent ion and isotopes for each of the inhibitors were determined using their corresponding chemical formula/molar mass and the Mass Mountaneer software isotope identification capability. This software was also utilized to determine the composition of possible fragments generated through on-site fragmentation or hydrolysis of the compounds being examined and to establish which fragment ions

originated from the inhibitor in question by either molecular composition, mathematical inference or both. All data collections were performed in triplicate.

Quantification and STR analysis

To establish the relationship between the amount of inhibitor remaining in the extract detected by DART and the actual inhibitory effect on the quantification and/or STR amplification procedures, each sample was quantified using Quantifiler Duo (Life Technologies) and amplified with Identifiler Plus (Life Technologies) after extraction. Both processes were performed following manufacturer's recommendations [16, 17] with a slight modification involving the use of 27 amplification cycles in comparison to the recommended 28. The data generated were used to determine any negative correlation between inhibitor carryover in the sample extract and the observed downstream results.

Results and Discussion

Indigo

Indigo dye has a molar mass of 262.07 g/mol and produces a protonated molecular ion at 263 amu. Figure 1a illustrates the spectrum obtained from a control blood sample spiked with indigo. The results from the 3 different extraction processes demonstrate a large difference in the level of carryover of this inhibitor into the final extract (Figure 1b and Table 2). Data showed the largest amount of carryover was produced by the EZ1 chemistry followed by organic extraction. The organic procedure was able to completely remove the inhibitor when one microliter of blood was spiked with up to .005 mg of indigo but carry-over increased as the initial quantity of the inhibitor increased (Figure 1b). Indigo is an organic compound used to dye jeans and other textiles that has been

known to inhibit DNA amplification[18] by potentially interacting with the polymerase or simply affecting the fluorescent detection in the process due to its dark coloration. Because of its relatively poor solubility in water, this dye is mainly solubilized in the organic phase. However, either due to slight solubility in the aqueous phase or incomplete phase separation some indigo is carried over onto the next step and co-elutes with the DNA. The polar structural components of the indigo dye may also permit the molecule to bind to the magnetic beads and/or the DNA in the solid-phase extraction processes [19, 20]. These specific processes result in indigo molecules appearing in the DNA extracts following manual or automated processing.

Figure 1. (A) Indigo mass spectrum signature peaks. (B) Relative amount of indigo carryover in samples after extraction procedures. The average carryover of indigo after purification of DNA with the EZ1 Investigator chemistry was $2 \pm 1.7\%$ of the input amount which corresponds to ~ 0.001 mg/ml in the extract. Organic and Automate Prepfilers exhibited some remnant of the inhibitor after extraction but were both much lower in comparison to the first method, $\sim 0.2 \pm 0.1\%$. The arrow indicates the presence of very low intensity of the characteristic peak at that particular sample-to-inhibitor ratio.

Table 2.Relative percent inhibitor removed by each of the extraction methods assessed

		Organic		Automate		EZ1	
	Initial amount (mg)	% removed	St. Dev (± %)	% removed	St. Dev (± %)	% removed	St. Dev (± %)
Indigo	0	---	---	---	---	---	---
	.001	100	---	100	---	100	---
	.005	100	---	99.9	5	97.5	5
	.01	99.6	3	99.9	5	95.9	6
	.02	99.5	4	99.9	6	98.2	12
Phenol	0	*		---	---	---	---
	.009	69.7	2	100	---	100	---
	.045	67.5	8	100	---	100	---
	.09	48.5	7	100	---	100	---
	.18	48.3	4	100	---	100	---
Bile Salts(391 amu)	0	100	---	*		*	
	.1	100	---	76.7	15	99.8	1
	.5	99.9	2	94.7	2	99.9	3
	1	100	---	97.1	2	100	---
	2	99.9	3	99.3	18	100	---
Melanin	0	---	---	---	---	---	---
	.001	100	---	100	---	100	---
	.005	100	---	100	---	91.4	6
	.01	100	---	100	---	95.6	10
	.02	100	---	100	---	95.6	22

Table 2. Continued

		Organic		Automate		EZ1	
	Initial amount (mg)	% removed	St. Dev (± %)	% removed	St. Dev (± %)	% removed	St. Dev (± %)
Tannic acid (Gallic acid)	0	100	---	100	---	100	---
	.001	100	---	100	---	100	---
	.005	99.9	.0001	100	---	100	---
	.01	100	---	99.8	.1	99.9	.1
	.02	100	---	100	---	100	---
EDTA (205 amu)	0	98.5 [†]	2	96.9 [†]	8	96.5 [†]	3
	.186	99.2	3	99.9	6	99.7	4
	.93	99.9	4	99.8	3	99.8	4
	1.86	99.8	4	99.9	2	99.9	3
	3.72	99.8	4	99.9	2	99.8	4

*Inhibitor was detected after extraction of control sample suggesting the presence of the substances in the corresponding extraction chemistries.

†Blood used for experiments was collected in EDTA treated tube, therefore some contamination with this inhibitor was detected in the control sample.

Phenol

Because phenol is a component of the organic extraction procedure, its presence was expected to be observed in the neat blood (control) sample when this method of purification was used. Phenol is a chemical utilized in the organic extraction procedure and will generally be present at high concentrations due to laboratory processing or exposure to certain consumer products and medicines. The peak corresponding to this inhibitor is expected to be observed at 95.05 amu after protonation (Figure 2a). No phenol was detected in any of the samples extracted with the EZ1 Investigator, or the Prepfilr Express kits (Figure 2b). Interestingly, the relative amount of phenol obtained

from the organically extracted blood control sample was on average 1.7 times higher than that of the spiked samples (Figure 2c). The co-elution of phenol with DNA is perhaps the most frustrating aspect of the organic purification method. This organic molecule has been reported to have deleterious effects in DNA typing by interacting with *Taq* polymerase and causing denaturation of the enzyme [21]. To exacerbate the issue, it is impossible to predict how much phenol will be carried over due to variation amongst analysts and also from one extraction procedure to another. As illustrated by the results (Figure 2d, Table 2), the amount of phenol detected after the organic procedure was abundant and variable throughout all samples. Fortunately, phenol was not detected in any of the eluates obtained from either automated platform which suggests that if prior contamination with phenolic substances occurs; either of these methods would be suitable for the processing of the sample. These semi-automated procedures should also prove valuable for reprocessing samples contaminated by phenol through the organic extraction.

Bile Salts

The presence of bile salts in spiked samples was determined by assessing the incidence of two separate peaks corresponding to cholic and chenodeoxycholic acids with molecular masses corresponding to 408.57 and 392.57 amu respectively. These are the two main bile salts produced by the liver in humans and because of their micelle forming capability are also sometimes used as components in lysis buffers. Thus in certain circumstances, these salts may be present in extracted biological samples as a result of carryover from the lysis procedure. When analyzed using negative ion mode, the molecular ions were produced at 407 and 391 amu $[M-H]^-$ (Figure 3a). Initial results demonstrated substantial

amounts of bile salt carry over into the eluate of the un-spiked (extracted blood) sample, especially chenodeoxycholate, when the Prepfilier chemistry was used (Figure 3b and c). Although cholic acid was also observed in the samples extracted using the Prepfilier chemistry, the results were less obvious than those observed with chenodeoxycholic acid possibly due to a lower concentration of one in relation to the other in the lysis buffer.

Figure 2. DART spectrum corresponding to mixtures of blood:phenol after extraction with (A) organic, (B) Prepfil and (C) EZ1 investigator methods. (D) Represents the relative abundance of phenol in the samples after extraction. It can be noted that there is no phenol detected in the samples obtained from the automated platforms. However phenol was detected in all organically extracted samples both before and after the purification process.

It should be noted that the bile salts used to spike the samples were a mixture for which there was no available component ratios, therefore the mixture itself could have been comprised of an abundance of one bile salt over the other impacting the relative abundance of the individual salts. The data show no significance difference between the carryover of the deoxycholic acid at the lower blood:inhibitor volume ratios. However, the highest concentration exhibited a decreased relative abundance unlike what was expected. This observation was also true for the cholic acid for which there was a steady decrease in the detection of the salt as the blood:inhibitor volume ratio increased.

Although unexpected, these observations could be the result of fragmentation of the larger salt. The loss of a hydroxyl group could generate the fragment present at 389 amu which is also present in the spectrum. As the amount of the salt increases, so does the potential rate for fragmentation. The organic and EZ1 procedures exhibited sporadic, minimal carryover with no obvious pattern associated with amount of inhibitor spiked (Figure 3c).

Figure 3. (A) Bile salt control spectrum; (B) MS spectrum corresponding to neat sample after being extracted using the Automate Prepfil procedure. The presence of bile salts is apparent even in this un-spiked sample. (C) Comparison of relative amounts of bile salts detected after extraction with the three examined methods. Although traces of these compounds were detected using both the EZ1 investigator and organic chemistries, larger amounts were co-eluted with when the Prepfil chemistry was used.

Bile is a common inhibitor found in fecal material [22]; nevertheless it is also a naturally occurring detergent used in the digestion of fats [23]. Because of this amphipathic nature it is capable of forming micelles to disperse water insoluble compounds into aqueous media. As suggested by the results, this non-denaturing detergent appears to be used as part of the Prepfilr Automate Express chemistry and thus it is easily carried over throughout the procedure and eluted with the otherwise purified DNA. The presence of these salts has been associated with oxidative stress damage to DNA in bacteria and humans [24, 25] as they are known to generate reactive oxygen species [26, 27]. Oxidative stress has been linked to reduced amplification efficiency during the DNA processing due to breakages in the DNA chain [28], therefore the presence of these salts in the purified DNA can cause serious problems in downstream DNA typing processes.

EDTA

Although the molar mass of ethylenediaminetetraacetic acid (EDTA) is 292.24 g/mol, the molecule has been reported to generate fragments at 132 and 160 amu due to hydrolysis[29, 30]. Also, when subjected to temperatures higher than ~200 °C, EDTA is known to decarboxylate and generate similar fragments [31]. Major fragment ions produced by the DART process include peaks at 205 and 217 amu.

Figure 4. Potential EDTA rearrangements corresponding to the 217 m/z and 205 m/z peaks.

For this particular compound, a large number of fragment ions appear (Figure 5a). In examining the data, the 205 amu fragment appeared to be most representative of the overall relative abundance of the compound in the sample. It is apparent from the results (Figure 5b, Table 2) that all three extraction methods assessed were able to remove the inhibitor with approximately the same efficiency. Although some carryover was observed, on average 99 % of the EDTA added to the sample was removed. It should be noted that because the blood used for the experiment was collected in EDTA coated

Figure 5.(A) EDTA control mass spectrum,(B) represents the summary EDTA carryover results obtained from each of the samples as they pertain to each of the extraction methods examined. No obvious differences in inhibitor removal efficiency were observed between the extraction methods used.

tubes, remnants of this compound can be observed in the neat blood sample prior to extraction (Table 2). EDTA is a chelating agent [32] that blocks the activity of the magnesium cofactor necessary for proper functionality of the polymerase enzyme, therefore its presence in a sample can have deleterious effects in both quantification and amplification of DNA molecules.

Tannic acid

Tannic acid is normally encountered in leather substrates or in environmental samples as this compound is commonly present in plant material. Due to its large molar mass and multiple charges it was originally thought that the detection of this compound using DART would not be possible. However, this compound produced a series of diagnostic fragments during the DART process. Although the parent ion was not observed in the controls or samples examined, a protonated fragment at 171.02 amu corresponding to gallic acid ($C_7H_6O_5$), was detected and confirmed using previous results in the literature as well as through the presence of corresponding isotopic peaks (Figure 6a) [33]. The presence of a second abundant peak at 455.35 amu (Figure 6a) was also monitored in the samples. This peak is presumed to belong to oleanolic acid, a triterpenoid also present in plant material and that could have been present as part of the purchased tannic acid control reagent. Other peaks present in high abundance in the spectrum have been characterized as those of potential contaminants and were not used in the interpretation of the data [34]. A summary of results is depicted in Figures 6b and 6c corresponding to the relative abundance of the 171 amu and 455 amu peaks. It can be noted that after extraction with any of the methods, the presence of tannic acid derivatives was very low regardless of the extraction method used. The results for oleanolic acid are more variable,

presumably due to the low abundance of the MS fragment. Due to the phenolic nature of the tannins, it is believed that the presence of this compound or its derivatives in any particular sample would have an effect similar to that of phenol in the *Taq* polymerase.

Figure 6. (A) Spectra generated from tannic acid. The major building block, gallic acid, is represented by the 171 amu peak. (B) Relative abundance of gallic acid after extraction using different methods. The presence of this molecule is minimal after purification with any of the methods examined and not expected to affect the subsequent analytical steps. (C) Relative abundance of oleanolic acid after DNA purification with various methods. Although most of the molecule was removed when present at high concentrations, some remnants were observed. It is suspected that the lingering amounts will have no effect on subsequent steps.

Melanin

Naturally occurring and synthetic melanins have been regarded as having considerably different chemical compositions. In fact, it has been established that the chemical composition of eumelanins and pheomelanins, responsible for dark and light coloration respectively, have a widespread range of molecular masses due to their varied origins. Because of this, an examination of their structural composition is somewhat challenging. Nevertheless, some studies have been performed in which thermal decomposition products of interest corresponding to certain pheomelanins have been characterized [35, 36]. Two of these pheomelanin markers were used to establish the presence of melanin

in our samples after the DNA extraction procedure - 4-hydroxybenzothiazole (151 amu) and 7-ethyl-2,3-dihydro-5H-1,4-benzothiazin-5-one (193 amu), Figures 7a and 7b. A third representative peak corresponding to 2,3-dihydro-5H-1,4-benzothiazin-5-one (165 amu) was also observed but the data were not recorded since its behavior mimicked that of 4-hydroxybenzothiazole. Product ions corresponding to fragments of the aforementioned compounds were also observed and used to confirm the presence of the pheomelanin markers. The results suggest that of the three extraction methods, the EZ1 procedure consistently exhibited carryover (~4 - 9 %) of pheomelanin derivatives when the samples were spiked with at least 0.005 mg of the compound. The organic extraction method co-extracted the largest amount (~0.5 – 1 %) of the melanin associated 2,3-dihydro-5H-1,4-benzothiazin-5-one compound whereas the Automate procedure exhibited no carryover of either compound (Figure 7c).

Figure 7. Mass spectrum depicting the presence of (A) 4-hydroxybenzothiazole and (B) 7-ethyl-2,3-dihydro-5H-1,4-benzothiazin-5-one with their corresponding product ions. These molecules are well known pheomelanin markers. (C) Relative abundance of the pheomelanin markers after DNA purification with each of the three extraction chemistries investigated. Some carryover is observed when the EZ1 or organic chemistries are used; however, the levels obtained shouldn't be adverse to the process of DNA typing.

Quantification and STR data

Real time PCR analysis performed by Quantifiler Duo (Life Technologies) produced the expected logarithmic curves. Samples were sequentially diluted with an increasing volume of inhibitor; therefore a reduction in DNA quantity results as the ratio of inhibitor to sample increased was observed (Figure 8). Interestingly, the indigo spiked samples extracted with the Prepfilier chemistry had a significant drop in the amount of quantifiable DNA even though the DART results indicated that the EZ1 extracted samples produced the highest indigo carry-over. Although the interaction of indigo with DNA could

Figure 8. Graphical representation of the averaged quantification results for each of the controls and inhibitor spiked samples.

prevent the detection of free indigo during mass spectrometry analysis, the effects would have been expected to occur with the EZ1 extraction as well. Unlike the EZ1 chemistry, Prepfiler appears to contain a large amount of bile salts, complexation of these salts with the indigo would change the properties of the dye making them undetectable with the DART and giving the appearance that they were removed during the purification process. Because a total input of 1 ng of genomic DNA was targeted for all amplification reactions, uniform intensities were expected in the resulting electropherograms regardless of the original volume of inhibitor in the sample. Nevertheless, the target 1 ng input could

not be reached at times due to the low quantification results. For example, Prepfilers extracts spiked with melanin at 0.010 mg and 0.020 mg per microliter of blood and tannic acid at 0.020 mg per microliter of blood did not produce sufficient results for quantification. In these cases, the maximum volume of DNA allowed in the amplification reaction (10 μ l) was used in the process in order to provide the best possible environment for a successful amplification. It should be noted that the low quantification and subsequent amplification results for the aforementioned samples was likely caused by poor extraction efficiency, since little or no inhibitor carryover of those inhibitors was detected with the Prepfilers method. Figures 9a-9e depict the variability in amplification results obtained for each of the inhibitors and extraction methods as they relate to relative fluorescent units. An example of an affected amplification is shown in Figure 10 where the presence of bile salts in the 1:1 mixture of sample and inhibitor yielded a compromised amplification following Prepfilers extraction due to excessive carry-over of the inhibitory compound. In the case of organic extractions, the presence of phenol in all samples and inconsistencies in the amount of carryover from extraction to extraction was most likely the cause of the variation in the intensities observed. Phenol is a known *Taq* polymerase inhibitor, thus the presence of this chemical in the sample, even at very low concentrations is deleterious to the results. Although the organic method was successful at removing bile salts, for example, the variability observed in the amplification results of samples spiked with this inhibitor and extracted using the phenol-chloroform extraction process can be attributed to phenol carryover. The same can be inferred for those samples spiked with melanin. With regards to other inhibitors, the problem is confounded as the lower intensities observed can be a combination of the effects of the

Figure 9. Graphs represent the overall average relative fluorescent units (RFUs) attained for each sample after amplification with Identifiler Plus: (a) Bile salts, (b) EDTA, (c) phenol, (d) indigo, (e) melanin and (f) tannic acid. All amplifications targeted a 1 ng input. A negative trend can be observed in the Prepfil results of the melanin spiked samples but these can be attributed to loss of DNA that resulted from inefficient extraction. Variability in the results obtained from the phenol-chloroform extractions is likely the result of differences in the amount of phenol in the reaction mixtures.

Figure 10. Effect of the presence of bile salts in the sample. Electropherograms represent amplification results of samples spiked with an equal volume of inhibitor (1:1 mixtures) – (A) EZ1 Investigator chemistry, (B) Prepfil Express and (C) Organic extraction. All electropherograms are adjusted to the same relative fluorescent units scale and all samples were amplified at a target of 1ng total DNA.

spiked inhibitor in combination with phenol carryover. All other methods show relatively consistent results and when compared to each other, the trend follows what was observed with the DART system in terms of inhibitor carryover.

Conclusion

The increasing use of automated platforms with solid phase extraction for DNA purification results in improved consistency of results, and increased throughput. Furthermore, downstream amplifications following these solid phase extraction have fewer problems with PCR inhibition, in spite of the fact that lower levels of DNA may be recovered. Unlike these modern solid phase extraction methods, the standard phenol-chloroform extraction procedure relies on a liquid-liquid purification technique. Therefore any molecule that is soluble in the aqueous phase of the extraction will be co-

eluted with the DNA and carried through the downstream real time quantification and STR amplification procedures. In addition, due to difficulties in phase separation and its appreciable solubility in water, variable amounts of phenol can get transferred into the aqueous phase. Phenol is a known *Taq* polymerase inhibitor; therefore its introduction into the DNA typing procedure is likely to be deleterious to the results. The presence of this molecule in the samples purified using liquid-liquid extraction is likely to have generated the disparities observed during sample amplification regardless of the amount of inhibitor originally spiked in the samples.

Both automated procedures examined in this project rely on a solid-phase extraction method which, unlike the organic procedure binds the DNA to magnetic beads and allows for the removal of inhibitors even when they are soluble in the aqueous phase by physically eliminating unbound molecules during the wash steps. Nevertheless, the use of these methods also creates the potential for transfer of inhibitory molecules that are utilized in the extraction process. For example, the Prepfilr extraction procedure appears to use bile salts as a detergent. These compounds can be carried through the process and co-eluted with the DNA. This may be an issue if the system is also challenged with additional, exogenous bile salts. There are also instances in which inhibitory molecules that bind to DNA or are sufficiently similar to the nucleic acid in structure or polarity may be captured by the beads. This appears to be the case with indigo for both the EZ1 Investigator and Prepfilr chemistries.

The results of this study indicate that it is important to understand the chemistry of the extraction process. In particular, the use of inhibitory chemicals in the extraction process such a phenol or bile salts can have a deleterious effect on downstream analysis,

particularly if these materials are present in the sample as well. In addition, inhibitory materials in the sample can also compromise amplification if they bind DNA or interact with the solid phase matrix in a similar fashion to DNA.

With only a few exceptions, these extractions produced clean and efficient amplifications, in spite of small amounts of inhibitor carryover. However, it is important to note that these results were produced with optimal quantities of DNA. Lower level samples may be more sensitive to these effects and as demonstrated, higher quantities of inhibitors will clearly affect amplification. In forensic scenarios it is often difficult to assess the quantity of inhibitors present in any given sample. Thus we believe that the DART process should prove to be a useful adjunct for laboratories and manufacturers interested in developing optimal DNA extraction processes. In addition, the procedure should permit experts in the field to have a better sense of what compounds are present in their extracts and which extraction process might best be used to remove them.

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“Perseverance is stubbornness with a purpose” – Josh Shipp

IV. UNDERSTANDING METAL INHIBITION: THE EFFECT OF COPPER (Cu^{2+}) ON DNA CONTAINING SAMPLES AND A PROPOSED SOLUTION

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Abstract

Copper wires are a common component of improvised explosive devices (IEDs) and other forensic evidence. When processed for DNA, ions from the metals can be co-extracted with the genetic material and potentially cause complications during the analytical procedure. It has been proposed that copper ions can have adverse effects on DNA processing by either interacting directly with the nucleic acid or affecting the electrophoretic mobility of the molecule. To explore this hypothesis, a series of experiments were performed to characterize the effects of copper on recovery of forensic DNA and its subsequent STR amplification. The data suggest that high concentrations of copper form a complex with the DNA that alters its physical properties and affects the electrophoretic separation of the nucleic acid. It was also found that these negative effects could be ameliorated by utilizing a solid-phase purification method capable of reducing the amount of copper carry-over into the sample. In addition, the data show no significant improvement of the results when enhanced chemistries are used during the amplification of the samples.

Keywords: metal-DNA complex (M-DNA), DNA inhibitors, solid-phase extraction, copper, divalent cations

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Background

The presence of metallic ions in forensic evidence while possible, is infrequent in biological samples as it is most likely to be associated with water or soil heavy metal contamination [1, 2]. Thus this type of contamination is most commonly found in cases involving skeletal remains and ammunition [3]. Nevertheless, in recent years the submission of ‘routine’ metallic evidentiary samples to criminal laboratories has increased due to the ongoing threats and acts associated with terrorism and theft [4-6]. Improvised explosive devices (IEDs) are commonly equipped with copper wires to provide electrical connections between its components (timers, detonators, etc.). The recovery of DNA from the devices themselves can provide critical leads to criminal casework. However, the process of collecting potential touch DNA samples from these types of exemplars can result in cross contamination with metallic salts and ions. Similarly, such contaminants may be expected in other types of casework including thefts of metallic objects and ammunition.

Copper is an essential co-factor for the normal function of certain metabolic processes but when present in high concentrations the levels are known to cause DNA damage [7-9]. Divalent metal ions are known to form complexes with DNA (M-DNA) by replacing hydrogen ions within the base pairing of the various nucleotides at pH values around 8.0 [10]. These interactions can normally be reversed by lowering the pH of the solution and/or the addition of chelating agents that will bind the metal ions. Nevertheless, on occasion the binding of these metal ions will change the conformation

of the DNA by replacing imino protons of certain nucleotides, creating difficulty in the recovery and amplification of DNA [11, 12]. The release of protons and irreversible binding of metals alter the nucleic acid conductivity and may promote cross-linking and aggregation of DNA strands [13, 14] making separation via capillary electrophoresis nearly impossible due to loss of resolution and/or distorted electrophoretic mobility. Studies have been performed to establish the consequences of the presence of some divalent metal cations on DNA and suggestions as to how to overcome the negative effects have been established [15]. However, the importance of mitigating the effect of copper in DNA samples and its downstream effects on capillary electrophoresis needs further investigation. Such studies are important as this metal has been increasingly present in forensic evidentiary samples, and solutions to overcome its negative effects need to be developed. For instance, if copper ions are suspected in a given exemplar and it is known that their presence will affect electrophoresis; a method to minimize the amount of these cations before the sample reaches that particular process should be employed. Identifying the DNA purification method most appropriate to curtail the presence of this metal could alleviate the difficulties associated with the results interpretation of the affected samples.

Materials and Methods

Effect of copper on DNA

To determine the effects of copper on DNA processing when working in an '*in vitro*' environment, copper chloride (Sigma, Aldrich, St. Louis, MO) was diluted to 0.1, 0.05, 0.025, 0.01, 0.005 and 0.001M and added to an equal volume of 0.5ng/μl 9947A control DNA. Each mixture was allowed to interact for a period of one hour before amplification

using the Profiler Plus kit (Applied Biosystems, Foster City, CA) following manufacturer's recommendations. The amplified product was then loaded onto a 3130xL capillary electrophoresis instrument (Applied Biosystems) for separation. Affected samples were incubated in formamide for 1 – 4 hours or overnight prior to analysis via capillary electrophoresis [15].

Establishing effect of copper on electrophoresis

Previously amplified samples were prepared for electrophoretic injection by adding 1 µl of amplified DNA to a mixture of highly deionized formamide (Life Technologies) and the appropriate internal lane standard (LIZ GS500) (Life Technologies). The prepared samples were then spiked with 1 µl of 0.01, 0.005 and 0.001M copper chloride and injected on a 3130xL capillary electrophoresis instrument (Life Technologies) following the manufacturer's recommendations. Control samples were included to assess the differences.

Role of pH as it relates to copper's effect on DNA

Ten microliters of blood were spotted on two sterile swabs and extracted using the standard phenol/chloroform/isoamyl alcohol extraction method [16, 17]. In short, samples were incubated in a mixture of 450 µl stain extraction buffer (10 mM TRIS-HCl, 100 mM NaCl, 10mM EDTA, 2% SDS, 39 mM DTT) and Proteinase K (Amresco, Solon, OH). The samples were then incubated at 56 °C for two hours. Following incubation, an equal volume of phenol-chloroform-isoamyl alcohol reagent (Invitrogen/Life Technologies, Foster City, CA) was added to the sample. The sample was vortexed and centrifuged to separate the phases before removing and filtering the aqueous top layer. Finally, the samples were eluted in 25 µl of water.

Immediately after the addition of the extraction solvent and centrifugation, each sample was split into two equal halves, with the first half subjected to washes and elution using a 10 mM Tris-HCl, 0.1 mM EDTA (TE) buffer at a pH of 8.3 while the other half was either washed with the above referenced buffer at pH 8.3 and eluted with 10 mM Tris-HCl, 0.1 mM EDTA buffer at pH 7 or both washed and eluted with the TE buffer at a pH of 7. Samples were quantified using the Quantifiler Duo real-time PCR kit (Life Technologies) to determine if the change in pH had a negative effect on the efficiency of extraction. Additional blood samples were spiked with copper at concentrations ranging from 0.001M to 0.1M and extracted using the previously described phenol/chloroform/isoamyl alcohol method with the washes and elution steps carried out using TE at a lowered pH of 7 to determine if the previously observed effect of this metal on DNA could be avoided with the change in pH.

The effect of increased copper salts on extraction and amplification

A sample of fresh saliva was extracted and quantified using the aforementioned phenol/chloroform/isoamyl alcohol extraction and a buffer at pH 8.3. Based on the results obtained from qPCR using the Quantifiler Duo kit, the samples were diluted 1:16 with water to obtain a DNA concentration of approximately 0.05 ng/μl during additional extractions. One mL of the diluted sample was next aliquoted into each of five tubes. The following amounts of copper salt were added to each of the saliva aliquots: 0.02, 0.05, 0.1, 0.15, and 0.2 grams. These amounts correspond to ~0.15, 0.40, 0.75, 1.1 and 1.5M copper salts, respectively. The samples were allowed to interact with the copper for 1 hour. Ten microliters of each of the mixtures were next spotted onto each of six pre-sliced (halved) swabs and allowed to dry. One half swab for each copper

concentration, and a positive (diluted saliva) and negative control were processed with each variable examined. Three swabs inoculated with each of the prepared saliva/copper mixtures were extracted using Qiagen's EZ1 Investigator chemistry and an EZ1 extraction robot (Qiagen, Madison, WI). A mixture of 475 µl of buffer G2 (proprietary formulation) and 25 µl Proteinase K were added to each sample prior to incubation at 56 °C for an hour. The substrate was then transferred to a spin basket and spun down to collect all of the lysis solvent in the tube prior to placement in the extraction instrument. Prompts for the 'large volume' protocol were followed. The other half of each swab was extracted using conventional organic (phenol/chloroform/isoamyl alcohol) extraction procedures as described in the previous section with buffer at a pH of 8.3. The resulting extracts were quantified using Quantifiler Duo and amplified with both Profiler Plus and Identifier Plus amplification chemistries (Life Technologies) to establish if differences between the two kits would affect the overall assessment of the DNA results.

Results and Discussion

The addition of copper to control DNA appears to have a detrimental effect on the DNA typing process

Control DNA samples spiked with as little as 0.001M of copper hydrochloride exhibited no DNA results (Figure 1a-b). This observation is consistent with either complete inhibition of the *Taq* polymerase during amplification, copper induced nuclease activity on the DNA or binding of this cation to the DNA molecule preventing the amplification and/or separation to proceed.

Figure 1. (A) Profile generated after injecting 0.5 ng 9947A control DNA in a capillary electrophoresis instrument. (B) Same control sample spiked with an equal volume of 0.001M copper hydrochloride.

Polymerase inhibition due to the presence of metal ions [18, 19] in a sample has been previously suggested. Studies have demonstrated that the presence of copper in a ready to amplify sample yields no results but when the samples are subjected to clean-up prior to the process, the expected product is attained [20]. These results, while suggestive of *Taq* inhibition are not necessarily the only explanation for the observation. Given the right conditions and co-factors (pH, reducing agents) copper has also been associated with forming complexes that could cause DNA scission [21-23]. Breaking the molecule into smaller pieces decreases the availability of the correct size target for amplification and therefore no results are observed. Moreover, the ion can bind and intercalate into the DNA molecule preventing the polymerase from extending the target fragments during amplification by altering the structure of the DNA through perturbation of hydrogen bonding between the bases or affect the separation process by altering the conductivity of the molecule [24-27]. Some researchers have been successful at reversing the effects of other metal ions when the sample is allowed to ‘incubate’ in formamide for a period of time. Presumably the improved results are a consequence of diluting the samples and allowing the metal ions to dissociate from the nucleic acid and equilibrate in solution [15]. When DNA samples containing copper in this study were allowed to sit in formamide for up to 24 hours the results didn’t change. The fact that copper has been reported to form stronger interactions with the DNA nucleotides than those observed with other divalent cations [28] can serve as an explanation of the difference in results attained between studies/metals.

Negative effect of copper is not caused by interaction with *Taq* polymerase

Based on the previous results, it is difficult to establish if the adverse effect of copper is caused by *Taq* polymerase inhibition, direct interactions with the nucleic acid, both of which could prevent amplification, or due to its potential effect on the electrophoretic process. To assess this, samples were spiked with copper cations after amplification and just prior to injection into a capillary electrophoresis instrument. Upon completion of electrophoresis it was noted that DNA was not detected on any of the copper spiked samples. In addition it was noted that unlike in the previous experiment, where the samples were spiked prior to amplification, there was no indication of the internal lane standard (ILS). When fluorescent genotyping is performed by capillary electrophoresis, an ILS, a mixture of DNA fragments of known length, is added to the sample prior to injection into the capillary in order to calibrate the system for determination of allele size (Figure 2).

When evaluated together, the data obtained when samples were spiked before amplification and just prior to injection in the capillary electrophoresis instrument suggest that copper is binding to the DNA and affecting the conformation so as to prevent amplification and/or electrophoresis, altering the conductivity of the electrophoresis itself or producing cross-linking of DNA strands and aggregation. These observations rule out the *Taq* polymerase inhibition theory since both before and after amplification the lack of DNA data observations remained consistent.

The relative acidity/basicity level of the solution does not appear to affect the overall results

Transition metals are necessary as cofactors for important roles within the body; they are involved in oxygen transport, muscle contraction and nucleic acid stabilization among others [28-30]. When in the body, they are active but not harmful at physiological conditions -- a relatively neutral and narrow pH range, ~7.3-7.4. Metal DNA complexes are the result of non-covalent interactions between transition metals and the DNA (Figure 3). Some studies have established that metal-DNA complexes (M-DNA) are favored when the pH of the solution is slightly basic [10, 13, 15] such as that used normally in the DNA extraction process. These interactions will change the physical properties of the molecule and thus also affect the normal physiological processes.

Figure 2. Electropherograms obtained from (A) control sample and samples spiked with 1 μ l of (B) 0.001M, (C) 0.005M and (D) 0.01M copper chloride. Samples spiked with copper showed no indication of DNA.

While it is true that pH has a role in the interaction of metals with nucleic acids, previous investigations have determined that structural perturbations at basic pHs are consistent with certain cations such as Cd^{2+} , Ni^{2+} and Zn^{2+} , while the presence of others like Mg^{2+} and Ca^{2+} have no apparent effect [31]. This is due to the variation in interaction between transition metals which can share electron density and thus promote the formation of coordinate covalent bonds and cations which can form much weaker interactions [31-33]. In addition, the same studies have suggested that the relative concentration of these metals also play a role on the structural changes to the nucleic acids, specifically that higher concentrations of certain species are needed to attain the M-DNA complex formation even at basic pH. To determine the role of pH on the association of copper to DNA, we first processed a control saliva sample using the phenol/chloroform/isoamyl alcohol procedure with the TE buffer at both pH 8.3 (control) and 7. This change did not appear to have an adverse effect on the results. Next copper spiked saliva samples were extracted using the phenol/chloroform/isoamyl alcohol with buffers at pH of both 8.3, which was suspected of promoting metal complexation and pH 7 which was used as a control. The results obtained from both extracts offered the same result, suggesting that observations obtained in the experiments conducted in the previous section, where copper had a substantial negative impact on the processing of the samples, were not caused by the basic nature of the extraction buffer. Nevertheless, since the concentration of the metal ion can be crucial for the generation of the M-DNA complex, the lack of reproducibility of the results might have been due to an insufficient ratio of metal to biological sample.

Figure 3. Metal-DNA interaction complex

Effect of increased copper concentration and extraction method

Evidentiary samples don't naturally occur as exposed genetic material; therefore it became important to assess the effect of the metal on cellular material as normally processed in a crime laboratory. Due to the possibility that high concentrations of copper are needed in order for it to complex with DNA, a diluted biological exemplar was used for this experiment in attempts to recreate the negative copper observations. Copper spiked saliva samples were extracted using both the phenol/chloroform/isoamyl alcohol organic procedure and the EZ1 Investigator chemistry (Qiagen) which is a silica based solid-phase extraction method. Both processes were carried on as explained in previous sections. After processing with the organic method, a DNA profile was obtained from the samples treated with 0.15 M copper concentration or less. As the amount of copper increased this method yielded no results regardless of the amplification method used (Figure 4). In contrast when the samples were extracted using the EZ1 Investigator solid-phase extraction chemistry and procedure, full profiles were attained for samples spiked with up to 0.75 M copper chloride. Samples spiked with amounts between 1 and 1.5 M

yielded partial or low intensity profiles that could be used for exclusionary purposes (Figure 4). It was also noted that a thick film was formed at the interface between the aqueous and organic phases of the phenol-chloroform procedure when 0.15 M or higher concentrations of copper were present. Free DNA could have been trapped in the film thus reducing its availability in the aqueous phase and decreasing the effectiveness of the purification process.

The organic method relies on the proper handling of a liquid-liquid interface for removal of substances that are not soluble in water and requires additional manipulation such as ethanol precipitation and/or filtration to free the eluate from substances that become solubilized with the DNA in the aqueous phase. Because no filtration of the aqueous phase was performed after sample purification, it is possible that the copper salts were co-eluted with the DNA and remained in solution for the downstream applications. The EZ1 method use silica coated magnetic beads to bind the DNA and applies a magnet to capture the beads during washes, therefore allowing this cell debris and extraneous substances such as unbound copper ions to be removed and thus generating a cleaner extract. Nevertheless, as the concentration of copper increases, there is an increased opportunity for the copper ions to interact with DNA making them more difficult to remove. The binding of these ions to the molecule could interfere with amplification and/or the electrophoresis and could explain why the success of amplification decreased with increasing concentrations of copper for extracts processed with this chemistry.

Figure 4. Relative amplification efficiency of the copper spiked samples after extraction with organic and EZ1 procedures and amplifications with Profiler Plus or Identifier Plus at 27 or 28 cycles.

Finally, although true for other inhibitors, the incorporation of alternate and enhanced buffer formulations within amplification chemistries such as Profiler Plus and Identifier Plus did not provide an advantage when dealing with copper in the sample. It can be inferred from Figure 4 that neither amplification kits produced a significant difference in amplification efficiency.

Conclusion

Copper ions strongly interact with DNA during processing binding the molecule and affecting conformation, aggregation and electrical mobility of the nucleic acid during capillary electrophoresis. Experiments with exposed DNA require only a very low

concentration of copper to exert a negative effect on the results. The presence of copper is best overcome by solid phase extraction chemistries. Nevertheless, when the collection of biological evidentiary material involves the concomitant and inevitable compilation of copper salts, the concentrations encountered can be sufficiently high to inhibit even solid phase extraction. It is in these cases where the analyst should understand the effects of the cation on the nucleic acid and the limitations of certain procedures and take the appropriate measures to minimize the risk of losing valuable evidentiary material.

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“Success doesn’t come to you; you go to it” – T. Scott Mclead

V. THE APPLICATION OF AMPLICON LENGTH HETEROGENEITY PCR (LH-PCR) FOR MONITORING THE DYNAMICS OF SOIL MICROBIAL COMMUNITIES ASSOCIATED WITH CADAVER DECOMPOSITION

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Abstract

The placement of cadavers in shallow, clandestine graves may alter the microbial and geochemical composition of the underlying and adjacent soils. Using amplicon length heterogeneity-PCR (LH-PCR) the microbial community changes in these soils can be assessed. In this investigation, nine different grave sites were examined over a period of 16 weeks. The results indicated that measureable changes occurred in the soil bacterial community during the decomposition process. In this study, amplicons corresponding to anaerobic bacteria not indigenous to the soil, were shown to produce differences between grave sites and control soils. Among the bacteria linked to these amplicons are those that are most often part of the commensal flora of the intestines, mouth and skin. In addition, over the 16 week sampling interval, the level of indicator organisms (i.e., nitrogen fixing bacteria) dropped as the body decomposed and after four weeks of environmental

exposure they began to increase again; thus differences in the abundance of nitrogen fixing bacteria were also found to contribute to the variation between controls and grave soils. These results were verified using primers that specifically targeted the *nifH* gene coding for nitrogenase reductase. LH-PCR provides a fast, robust and reproducible method to measure microbial changes in soil and could be used to determine potential cadaveric contact in a given area. The results obtained with this method could ultimately provide leads to investigators in criminal or missing person scenarios and allow for further analysis using human specific DNA assays to establish the identity of the buried body.

Keywords: Soil microbial profiling, amplicon length heterogeneity, clandestine graves, forensic sciences, capillary electrophoresis

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Disclaimer

Points of view in this document are those of the authors and do not necessarily represent the official view of the US Department of Justice.

Background

Soils are a complex matrix formed by both organic and inorganic compounds, plant debris, microbes and other living organisms. It has been established that soil composition varies with induced stresses including weather conditions and anthropogenic additions [1, 2], and it has been proven that these variations can be detected and measured [1, 2].

When cadavers are placed in such locations, the products of decomposition seep into the underlying and adjacent soils, temporarily changing the chemistry, the biota and the intrinsic functional biogeochemical cycles within the soil [3].

The use of microbial community variations of underlying grave soils has not been thoroughly studied for the determination of time and placement of a body [4]. If changes in the microbial community composition and functions can be associated with cadaveric decomposition, profiles obtained from suspected clandestine graves can support or exclude the possibility of cadaveric contact, burial or removal at a specific location providing potential leads to investigators. It has been established, that during the first stage of decomposition (autolysis) aerobic organisms predominate and thus deplete the body of oxygen [5], setting up the conditions for anaerobic organisms to prevail during the second stage (putrefaction). It is during this stage that the majority of the body's microbes are expected to become part of the soil as the tissues of the body break open and the fatty acids and protein by-products are transferred to the Earth. During the last stage of decomposition (decay), there is little or no transfer of microbes to the underlying soil as the cadaver has been skeletonized or mummified. However, the microbial activity has been known to remain active for months after the end of the putrefaction stage due to

the increased amount of carbon in the soil. An increase in pH and nutrient concentration has also been observed during this period [6].

Microbial profiling techniques such as Amplicon Length Heterogeneity (ALH) and Terminal Restriction Fragment Length Polymorphism (TRFLP) have previously been used [7-9] to differentiate soil types based on their microbial composition. These molecular techniques use similar chemistries, require only a small sample volume and can be automated. The LH-PCR method exploits the sequence length hyper-variability in different domains that exist within the 16S rRNA genes or inter-genic spacer regions, both of which are ubiquitous to bacteria. The metagenomic profile of a particular soil can thus be obtained, compared and contrasted with other samples. Microbial community dynamics will flux rapidly with any particular stage of decomposition because of the increased nutrient input from the cadaver and, thus, microbial data may help determine the previous placement of a body at a particular location. The identification of increased/decreased levels of particular microorganisms in suspected clandestine graves could serve as an additional forensic tool to strengthen other non-biometric evidence in a criminal scenario. The objective of this study was to determine changes in eubacterial metagenomic composition of the soil on which bodies had been placed with respect to 'pristine' soils using a fast and reliable molecular method commonly used in the ecological field and applying it to potentially identify burial sites.

Materials and Methods

Soils were collected within the Anthropology Research Facility, the outdoor laboratory of the Forensic Anthropology Center of the University of Tennessee in Knoxville, Tennessee. Because of the very limited number of 'new' cadavers available for study,

nine sites were sampled and soil was collected in triplicate from underneath the bodies at a maximum depth of 2.5 cm at 1, 2, 4, 8, and 16 weeks post-placement. The donated/available cadavers used were placed on the soil between late April and early November of 2007. In addition, four control site soil samples (non-grave sites) were collected during May and November of 2007 and February of 2008. The control sites were located at each of four cardinal points within the same enclosed wooded area. The control date collection was longer than that of the length of the study for any given grave site because not all bodies were placed on site on the same date. The three control soil collection dates were chosen at random and span the dates of the study as a whole. It should be noted that throughout this paper the term grave site or grave soil refers to the location or the soil under the location where the body was placed. In these experiments, the bodies were placed on the soil surface (not necessarily underneath) and were covered with a black plastic. All sampling sites were cleared of leaf litter and vegetation before placement of the bodies. Control sites were also cleared of visible debris. Soil collections were performed by personnel at the Forensic Anthropology Center over the time specified and the soils (one quart zip lock bag/sample) were shipped frozen to Florida International University for DNA extraction and microbial analyses.

The soil unit for the areas sampled is Corryton Loam [10], which has a 75-90 % Corryton soil composition, with the upper 15 cm being loam (sand, silt, clay in an ~40:40:20 %). These soils could be used for pasture and crops as they have high water capacity (0.15-0.24 in./in.), a saturated hydraulic conductivity of 4.23-14.11 $\mu\text{m/s}$, and a moist bulk density of 1.30-1.50 g/cm^3 , which prevents them from flooding. They also have a linear extensibility of 1-2.9 % which is characteristic of a low shrink-swell

potential. These soils are known to have a pH that ranges between 4.5 and 6 and the top layer of the soil (0-15 cm) has a cation-exchange capacity of 2.9-9.8 meq/100 g. No calcium carbonate or gypsum is measurable in this type of soil and the salinity and sodium adsorption ratio are negligible [11].

Soil extraction

Each sample was sieved and homogenized in its entirety before the extraction procedure to remove soil debris (nematode, insects, branches, leaves, etc.). Extractions were done in duplicate using the UltraClean™ Soil DNA Kit (MO BIO Labs, Solana Beach, CA) and 500 mg of soil following manufacturer's specifications. Extraction success was determined by loading a 1 % agarose gel with the extract and visualizing the bands with ethidium bromide staining. DNA concentration was estimated based on readings from a biophotometer (Eppendorf, Westbury, NY) and diluted to a concentration of 10 ng/μl).

DNA amplification

Two of the nine hypervariable domains of the 16S rRNA genes were amplified using universal eubacterial primers. Primers for the two domains, V1 and V3, were multiplexed using fluorescently labeled primers, P1F-6FAM (5'-6FAM -GCG GCG TGC CTA ATA CAT GC-3') and 338F-6FAM (5'-6FAM -ACT CCT ACG GGA GGC AGC AG-3') and their corresponding reverse primers, P2R (5'-TTC CCC ACG CGT TAC TCA CC-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3'), respectively [10]. The non-overlapping domains assayed were chosen based on the results from a previous study [9] that indicated these two domains in combination provided discrete information that could discriminate between soil microbial communities.

A separate amplification reaction that targeted the nitrogenase reductase (*nifH*) gene was also performed. For this reaction, forward primer *nifH*-F (5'-6FAM –AAA GGY GGW ATC GGY AAR TCC ACC AC-3') and reverse primer *nifH*-R (5'-TGS GCY TTG TCY TCR CGG ATB GGC AT-3') were used [12]. All amplifications were done in duplicate.

Both PCR reactions had a final volume of 20 µl distributed as follows: 1X PCR buffer, 2.5 mM MgCl₂, 250 µM dNTPs, 0.5 µM of each of the two forward and reverse primers, 0.5 U AmpliTaq Gold DNA PolymeraseTM (Applied Biosystems, Foster City, CA), 1.0% bovine serum albumin, fraction V (BSA), 10 ng/µl DNA and DEPC water to volume. The reactions were performed on a 9700 thermal cycler (Applied Biosystems) with an initial denaturation at 95 °C for 11 min, 25 cycles of denaturation at 94 °C, annealing at 55 °C (for the 16S rRNA primers) or 62 °C (for the *nifH* primers) and extension at 72 °C for 1 min each, and a final elongation at 72 °C for 10 min.

Determination of grave soil microbial composition

Amplified products were loaded onto an ABI 310 Genetic Analyzer (Applied Biosystems) by mixing 0.5 µl of the amplified product with 9.5 µl of a 96:1 mixture of Hi-DiTM formamide and GeneScanTM 500 ROXTM size standard (Applied Biosystems). Samples were separated using POP-4 polymer (Applied Biosystems), module DS31 (6FAM_VIC_NED_ROX) and D filter and separated for 28 minutes.

The data were analyzed using GeneMapperTM v. 3.7 using the Local Southern Method. The minimum threshold was set to 50 RFUs [13, 14]. A marker range covering the 50-290 base pair (bp) span of the amplicons was created for the V1_V3 domain

screening and a one base pair difference between peaks was established to cover all peaks present in a sample. Bins corresponding to the range of interest were created to ensure consistent base pair calls between samples, and four replicate PCR profiles were used to ensure reproducibility (two amplifications from each of the two soil extractions). A single peak at the 397 bp location was expected in the *nifH* data and this set was analyzed solely on the presence/absence of the particular peak.

Statistical analyses

The 16S rRNA data were exported into Excel (Microsoft, Redmond, WA) to be interleaved, filtered and normalized. The first filtering criterion was that each peak observed in a particular sample had to be present in three out of the four replicate DNA analyses for it to be scored as a true peak and not a PCR artifact or background noise; the second, that the relative peak ratio had to exceed 1 % for peaks to be retained for further analysis [13]. PRIMER 6 Software (Primer E Ltd., Plymouth Marine Laboratory, Plymouth, UK) was used to perform statistical analysis on the normalized data. Relative intensities were averaged and the results were subjected to a square root transformation before applying the Bray-Curtis similarity index function [15]. Analysis of similarity (ANOSIM) was performed on data based on site, collection time period (week) and average daily temperature. Comparisons between the various samples were made using analysis of similarity (ANOSIM) measurements. Samples were described as similar if their similarity score was between 100 and 60 %. They were considered dissimilar if a 0-40 % similarity score was obtained. These percentages of similarity/dissimilarity were established using our control samples as reference standards. The results obtained for all sample replicates and samples collected within the same site during the same time period

were greater than 90 % similar, therefore these intra-site data are not included. Previous work have also demonstrated the reproducibility of this method [9, 16-18].

Cluster and SIMPER [19] analyses were also performed and a non-metric multidimensional scaling (MDS) analysis [20] was used to compare data. The average of the relative abundances of the nitrogenase reductase gene amplicons corresponding to the same collection week samples was normalized to the averaged intensities of the reductase amplicons for the control soil sites.

Results

To test our hypothesis we first probed global changes in bacterial profiles. To do this we compared microbial profiles based on site, collection time period (week) and average daily temperature. We then applied a more targeted approach based on the fact that the wide range of bacteria present in the soil appeared to mask the more specific effects we were looking for.

Comparison of global microbial profiles between different grave sites

The initial data analysis indicated that with the exception of the control sites, every grave site was on average more than 50 % dissimilar with itself over the weeks they were sampled. The lowest similarity observed was with the weekly samples of site 31 (27.29 %) and the highest similarity was at site 21 (47.53 %) (Table 1). This can be contrasted with the control samples which showed within site seasonal similarities ranging between 68 and 79 % (Table 1). In addition, control sites showed minimal dissimilarity percentages when compared between themselves, 21.30 % to 37.34 % range (Table 2), suggesting that before the bodies were placed on the ground, the soils were similar in microbial composition. Figure 1 depicts the relative distances within and

between the different sites in a non-metric multidimensional scaling map in which groups whose dissimilarities are less than 40 % are highlighted with a circle. These data indicated that while there was clearly an effect on the microbial population caused by the placement of a body, it was not possible to define specific trends in the bacterial profile as a wide and time dependent range of microorganisms were impacting the soil community. The control samples were not affected in this manner, which indicates that potential geochemical variations were not impacting the microbial composition of the soil.

Table 1. Average similarity percentage within sites for the various collection periods

Site	Similarity (%)	Control	Similarity (%)
14	35		
15	47		
17	47	East	72
20	32	North	75
21	48	West	79
25	30	South	69
27	40		
31	27		
38	37		

Effect of decomposition time on global microbial soil composition

Samples from each grave site were collected at weeks 1, 2, 4, 8 and 16 to test if the microbial signature within the underlying soil was altered with increasing cadaver exposure time. It was found that the average dissimilarity within each of the different sampling weeks was above 50 % indicating significant microbial variation during the same collection periods (Figure 2). In contrast, the average obtained within the collection periods for the control sites was all under 30 % indicating a higher similarity for the control soil microbial fingerprint during the seasonal sampling intervals at each of the

four undisturbed control sites (Figure 2). The smallest observed variation (other than those observed with the control sites) was between collection week 1 and the November control group (58.31 %), whereas the largest variation was determined to be between week 16 and the May control group (71.24 %) (Table 3). A graphical representation of these results is depicted in Figure 2. These data again suggested that the broad range of bacteria present due to decomposition events masked any temporal correlation of the microbial composition in the soils.

Table 2. Average dissimilarity (%) between sites based on SIMPER analysis and pairwise similarity as established by ANOSIM.

Site ^c	14	15	17	20	21	25	27	31	38	Control N	Control S	Control E
15	58 ^b											
17	66	68										
20	68	69	74									
21	60	56	70	78								
25	67	69	75	66 ^b	76							
27	64 ^b	62 ^b	75	70	66	68 ^b						
31	65 ^b	66	69 ^b	67 ^b	69	66 ^b	68 ^b					
38	65	66	73	67 ^b	70	65 ^b	67 ^b	66 ^b				
Control N	68	64	72	61 ^b	79	63	64 ^b	65 ^b	65			
Control S	69	67	70	62 ^b	82	63 ^b	66	65 ^b	66	25 ^a		
Control E	67	63	65	63	76	64	67	67 ^b	65	31 ^a	37 ^a	
Control W	65	63	66	60 ^b	77	62 ^b	63 ^b	62 ^b	62	21 ^a	24 ^a	28 ^a

^aRepresent samples that had dissimilarity of less than 50 %.

^bRepresent sites that were more similar to their counterpart than to themselves as determined by a negative ANOSIM R value when calculated with a p<0.01.

^cThe soil sites are labeled according to the submission/donation number and not in order from 1 to 9.

Figure 1. Non-metric multidimensional scaling map showing the similarities/dissimilarities between and within site samples based on Euclidean distance estimates – site comparisons. The 60 percent similarity grouping is based on CLUSTER analysis of nearest neighbor. Within site comparisons exhibited similarities of less than 50 % with the exception of control sites which yielded within site comparison similarity percentages of at least 68 %. When between site comparison were made, the maximum similarity percentage was ~ 42 %, whereas control sites remained similar between themselves with a similarity percentages of at least 68 %.

Determination of bacterial community differences based on temperature averages during collection time

Because climatic variations can enhance or halt bacterial activity and because not all bodies were placed in the ground on the same date, the daily average temperature from the National Oceanic and Atmospheric Administration [21] for this location was downloaded and used to perform a comparison of samples using temperature as the grouping variable. For these data, low similarities were observed within groups with the same temperature (<53 %) (data not shown). However, it was noticed that greater

Figure 2. Non-metric multidimensional scaling map depicting the similarities between and within sample collection time periods based on Euclidean distances – time comparisons. The orange circles illustrate samples that were at least 60 % similar to each other as determined by CLUSTER analysis based on closest neighbor. Samples collected within the same sample period (week) were more than 50 % dissimilar from each other, whereas those collected within the same control sampling period were less than 30 % dissimilar. Between collection-period comparisons yielded dissimilarity percentages of up to 72 % whereas control samples remained fairly constant when compared between collection periods (Nov, Feb, May) with a maximum dissimilarity of 32 %.

variation in microbial communities, although not always significant, occurred between warmer temperatures as compared to the cooler ones (~70 %) (data not shown).

Determination of difference drivers between grave soils and control soils

Overall, neither of the examined variables (site, decomposition time or temperature) was able to group the tested grave soil sites; nor was the overall microbial profile useful in separating the different groups from one another. The results do show,

however, that the control soil sites consistently clustered together and were significantly different from the soil samples obtained in close proximity to a grave site.

Table 3. Pairwise average (%) of microbial fingerprint dissimilarities based on decomposition time

Week	1	2	4	8	16	C May^a	C Nov^a
2	63						
4	68	68					
8	69	70	69				
16	65	67	68	67			
C May^a	63	64	69	68	71		
C Nov^a	58	62	68	65	68	27	
C Feb^a	59	62	68	66	70	32	25

^aC represents control soils for the collection month listed

Given this fact, it became important to determine which variables were responsible for the marked difference (as given by a $p < 0.01$) between soils obtained from control sites and those collected near the grave sites (Figures 1 and 2, Table 2). It was proposed that the bacterial markers producing the observed segregation could be used to pinpoint a set of amplicons that would permit grave site identification based on the soil profile. To obtain this information, SIMPER [22] analysis using the combined grave soil sites as the first variable and the combined control sites as the second variable was performed. Our results indicated that the amplicons with the highest contribution to the variability were 82, 80 and 79 bp (Table 4) which provided an average dissimilarity between the groups of 5.42, 5.30 and 4.22 % respectively (Table 4). Although these amplicons cannot be attributed to a single type of bacterial because different microorganisms may share specific amplicon lengths, we examined a previously developed bacterial ribosomal analysis database [4, 23, 24] that can be virtually queried for the 16S rRNA domains used in this investigation. It was our intent that this information might provide a presumptive identification of bacterial species that could

produce these amplicons. The query results indicated that anaerobic bacterial species not normally encountered in soils in high concentrations, such as *Streptococcus* sp. and *Helicobacter* sp. commonly produced the three dominant amplicons determined to be contributing to the variation between the samples. These bacteria are part of the commensal flora of the intestines, mouth and skin and can also be the cause of certain human diseases. *Helicobacter* sp. is known to thrive in acidic environments which are characteristic of the human stomach and intestinal habitat and could survive and possibly contribute to the process of decomposition for a short period of time due to the anaerobic environment created within the corpse [25].

Table 4. Average dissimilarity provided by the different amplicons in relation to control and grave soil sites.

Amplicon size (bp)	Average Dissimilarity (%)
82	5.4 ± 1.5
80	5.3 ± 1.8
79	4.2 ± 1.0
175	3.3 ± 3.3
170	3.1 ± 2.3
71	3.0 ± 1.1
81	2.8 ± 0.7
89	2.5 ± 1.2
70	1.9 ± 0.9
86	1.9 ± 1.3
195	1.7 ± 1.2
73	1.7 ± 1.3
174	1.6 ± 1.2
84	1.6 ± 0.7
72	1.5 ± 0.4

Because it is known that the process of decomposition encompasses the break-down of amino acids and since other studies have reported the presence of excess nitrogen in areas

of suspected cadaver placement using ninhydrin [25, 26], we explored the possibility that the nitrogen fixing bacterial populations might also be contributing to some of the differences observed between grave soils and the controls. To do this, we re-analyzed the samples using primers that would specifically target the *nifH* gene. This gene codes for nitrogenase reductase, the enzyme responsible for reducing atmospheric nitrogen (N_2) into ammonia (NH_3). The relative differences in abundance of this targeted gene between control and grave soils established that nitrogen fixing bacteria had significant weight in discriminating between the two types of soil environments (Figure 3).

Figure 3. Relative abundance of *nifH* (nitrogenase reductase) gene amplicons during the various sample collection weeks as compared to the control soils. Results obtained for soils from the different grave sites were averaged and plotted according to the collection week. When assessed by site, the gravesites provided similar results. Differences in the gene presence were only detected based on time. The abundance percent of the amplicon was also determined. Data suggest that during the first 16 weeks of exposure, the soil surrounding or underneath a cadaver has less than 50 % *nifH* presence than what is encountered in un-exposed soils.

Discussion

Human decomposition studies have demonstrated that microbial activity, especially that of microbes present in the skin and intestines, plays an important role in decomposition [27]. These studies have shown that different individuals house distinct suites of commensal microbial flora in their guts [28-30]. While certain bacteria are common to all individuals [31-34], a great number of them differ, with this variability being attributed to diet selection and/or the health of the individual [4, 6]. In addition, the manner or cause of death of an individual might contribute to an increased decomposition rate. As depicted in Table 1, the percent dissimilarity between the various collection periods within a site is significantly high, thus Bray-Curtis similarity indices did not permit the distinction between grave sites in which different cadavers were placed as was observed in the multidimensional map presented in Figure 1.

If external factors that might influence the rate of decomposition of the different cadavers used in this study (environmental temperature, injuries, etc.) are ignored (since they could not be controlled for in the study), it can be argued that the lack of grouping of the individual sites was due in large part to the variability in the human microbiome. This assertion can be substantiated with the fact that amplicons corresponding to anaerobic bacteria not commonly indigenous to the soil but rather to human tissue were observed in the results. The fact that the control sites remained fairly similar tends to support the aforementioned observation. When microbial diversity was compared over the total sampling period at all sites, we observed extensive variation in the community profiles, especially within sites. This variation can be attributed to successional changes in the microorganism communities throughout the time period in which the samples were

collected. Such a result could be an expected consequence of the overall breakdown of tissues as body fluids and associated bacteria leach into the soil.

In addition to the leaching of bacteria present in the body, during autolysis (tissue breakdown), ammonia and other nitrogen related compounds, including free nitrogen are released [35]. Nitrogen and nitrogenous compounds are present in the body in discrete quantities and are by-products of the breakdown of essential molecules, like proteins and nucleic acids. In a metabolically active organism, ammonia is produced by the breakdown of proteins. This ammonia is then converted to urea and excreted by the kidneys into the urine and out of the body. These waste products accumulate when the organism stops its normal function. In the case of a deceased body, ammonia and other nitrogenous products are expelled through the nose and mouth onto the environment. After some time, the production decreases or ceases because the body's microorganisms have depleted the proteins available. Putrescine and cadaverine, molecules characteristic of decomposition, are also nitrogen-based and volatilize and diffuse out of the body [25].

During decomposition it can be expected that a corpse will excrete ammonia and nitrogenous components into the environment, including the underlying soil. This fact has been used to detect clandestine grave sites through the use of ninhydrin which binds to nitrogenous compounds to produce a visible color change [36, 37]. Because of a relative increase in ammonia and other nitrogenous products present in grave soil, it is reasonable to expect a concomitant reduction in nitrogen fixing bacteria or competitive suppression of the process in the soil areas underlying the corpses. This observation is consistent with our results (Figure 3). As decomposition continues, elimination of nitrogen precursors from the body occurs thus decreasing the *de novo* synthesis of

ammonia precursors by the nitrogen-fixing bacterial populations. Although we cannot establish with certainty the exact time of the reversal of events, it appears that after four weeks of exposure, nitrogen fixation started to increase (Figure 3); this was presumably due to a decrease of nitrogenous materials leached from the bodies into the soil and the increased demand of the microbial community for ammonia substrates. In this study we were not able to extrapolate the time at which this process reaches a plateau given that at the time of the last collection period (16 weeks) the abundance of these bacteria appears to still be on the rise.

Overall, microorganisms with amplicon lengths of 79, 80 and 82 bp were observed to be the significant drivers of the grave site differences with respect to the controls (Table 4). A database query indicated that a variety of bacteria, many of which are anaerobes associated with human tissue and not often in soil, were strong representative candidates for all three amplicons. Further tests determined that a significant proportion of the variation seen in the soils when compared to control samples was correlated with the flux of nitrogen fixation.

Conclusions

The results indicated that measureable changes occurred in the soil bacterial community during the decomposition process for all corpses. The differences between grave sites and control soils demonstrated that amplicons corresponding to anaerobic bacterial not necessarily indigenous to the soil were partially responsible for the differences between the grave sites and control soils. Each grave site profile was unique due to the decomposing cadaver but also unique from the background microbial community profiles; thus, such information can pinpoint a clandestine grave site and give

important data to a criminal or missing person's investigation. It may even be possible to subsequently test that specific soil/site for trace human DNA and perhaps give some information as to what individual corpse had lain there. The decomposition-related changes in key nitrogen cycling genes within the soil community (due to the high influx of nitrogenous compounds from the body) impacted and/or suppressed critical processes in the intrinsic soil nitrogen cycle. Thus, this profiling method does provide a potential application for identifying a site where a body had previously been laid. Future work should involve identification of specific indicator bacteria involved in decomposition as well as investigation of long-term changes in microbial composition and functional status within grave soils.

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“There is only one thing that makes a dream impossible to achieve: the fear of failure” - P. Coehlo

VI. CONCLUSIONS

Biological evidence, when available, is perhaps the most reliable type of proof for criminal cases. This attribute emanates from the fact that the genetic material is unique for every living organism and there are very robust technologies to enable criminal analysts to substantiate that. Nevertheless, these techniques rely on the proper acquisition of the evidence, their quality and quantity. Improvements throughout the years have granted analysts the leisure of being able to obtain successful results from picogram amounts of DNA. However, challenging the system with suboptimal samples in terms of quality can have its downfalls, especially if there are already minute quantities of material available for typing. Samples that have been exposed to the elements for long periods of time can exhibit signs of degradation that decrease the amount of valuable information attainable from a sample and increases the instances of artifacts. Both of these complicate the interpretation process and lessen the strength of the results. Although not ideal, there is not much that can be done to repair the DNA that has been damaged or recover nucleic acid material that has been destroyed.

An additional challenge is present in certain types of biological samples that contain materials that interfere with the DNA typing process. These substances, known as inhibitors, can completely alter the analytical results by yielding no data even when a substantial amount of biological material is available. This study also revealed that sometimes the current inhibition detection methods fail to indicate sample inhibition when it is actually present. Failure to detect DNA or a belief that there is a problem

where there is none can lead an analyst to decisions that can compromise the final results. Unlike the irreversible effects caused by exposure to the environment, there are ways to manage the presence of inhibitory substances in order to attain the greatest amount of information from any given exemplar. As the community grows aware of these substances, enhancements to the DNA typing process chemistries have been made to ameliorate the potential negative consequences that they produce. In spite of these advancements, problems remain. Despite current safeguards, the failure to detect inhibitors in a sample or to accurately assess the amount of an inhibitor or its mechanism of action can compromise the results. With this study, we were able to evaluate the current methods for inhibitor detection, and after establishing that there were some gaps, suggest better practices for the generation of higher quality samples. In order to do this, we combined tools from the forensic biology and chemistry fields and used relatively simple techniques to determine the amount inhibitor carryover into the DNA extract following various extraction procedures. The data clarified the viability of these methods to wash away the contaminants. The results from the remainder of the DNA analysis process enabled us to make associations and predict the outcomes for the various samples. Although no purification method performed better than another in terms of amount and quality of data recovered when samples were spiked with the various inhibitory substances, this was likely the result of using high quality samples for the study. Therefore, the results still provide great knowledge and useful information for the application to casework type samples where it is expected that the combination of low sample template and presence of inhibitors could negatively affect the results if not properly handled.

Throughout the course of the investigation, we also suggested a novel method to provide leads on cases in which the amount of inhibitors would likely impede the recovery of any useful nucleic acid material. As with human DNA, a unique DNA fingerprint can be obtained for certain microbiological organisms. Suspected clandestine grave sites are locations where any available human DNA is likely to not only be plagued with environmental inhibitors but also suffer from the effects of being exposed to the elements, thus nearly eliminating the chances of recovering useful human genetic material. However, certain microbiological species thrive in the presence of decomposing matter and can provide insight into the stage of decomposition of a body during its stay at that particular location yielding valuable investigative leads when no human DNA is available.

In conclusion, useful information can be obtained from samples that would otherwise be deemed non-optimal for DNA typing. In this study we have shown that analysts will be able to make more educated decisions on how handle particular samples and to establish educated expectations with respect to the data by understanding the mechanism of action of the various inhibitory substances responsible for affecting the DNA results and the potential of the methods available to process them.

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