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THE USE OF MINISTRS AND MITOCHONDRIAL DNA TO IDENTIFY HANDLERS OF PIPE BOMBS

presented by

Stefanie Lee Kremer

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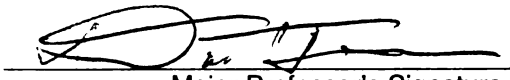

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THE USE OF MINISTRS AND MITOCHONDRIAL DNA TO IDENTIFY HANDLERS OF PIPE BOMBS

By

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Abstract

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By

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The deflagration of pipe bombs produces very high temperatures, which, in combination with the general nature of DNA from shed skin cells, means that only degraded DNA is likely to remain on the resultant bomb fragments. Further, because the bomb surface has only touch DNA, low copy number (LCN) techniques must be utilized during analysis. Previous research employed short tandem repeat (STR) (Esslinger et al., 2004) and mitochondrial DNA (mtDNA) (Gehring, 2004) analyses to identify the handlers of pipe bombs. The rate of obtaining an STR profile was very low, while increased success was garnered with mtDNA. The goal of the current research was to use miniSTRs to better identify individuals who handle or assemble pipe bombs. In this research, 17 volunteers were asked to touch two sets of pipe bomb components, one made of PVC and the other of steel, for a total of 34 bombs, which were then deflagrated. DNA was amplified using two sets of multiplexed miniSTR primers as well as mtDNA primers. MtDNA profiles were more likely to produce correct assignments than miniSTRs. Further, when data from both miniSTR and mtDNA profiles were combined, the number of correctly assigned bombs was even higher. These results indicate that both nuclear and mtDNA should be used in conjunction when DNA that is degraded and in low quantity is encountered on items of evidence.

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Introduction

Improvised explosive devices (IEDs) are used by many rogue organizations, militia groups, and individuals to cause destruction and panic. Between January 2001 and February 2006, over 18000 explosive incidents occurred in the United States, including almost 3400 bombing incidents that caused 409 injuries, 56 deaths, and over $25 million in damages (Bureau of Alcohol, Tobacco, Firearms, and Explosives, 2006). IEDs are also problematic on the world stage. Between July 2003 and April 2008, over 1600 American soldiers were killed by IEDs while in Iraq (iCasualties.org, April 2008). The increasing use of IEDs has created the need to identify the person or persons who handled, assembled, and deflagrated a device.

IEDs can be made from various materials and employ myriad configurations. Over 40% of IEDs worldwide are pipe bombs (Burke, 2007). High profile incidents involving pipe bombs include mail bombings by Theodore Kaczynski (the Unabomber), the Olympic Park Bombings by Eric Rudolph in 1996, and the Columbine High School massacre in 1999. Pipe bombs are hazardous because of the high-velocity fragments that are produced following deflagration; fragments from the container can reach speeds of 20000 feet per second (Lenz, 1965). They can be assembled from materials that are purchased without difficulty at hardware and sporting goods stores and typically consist of a length of pipe, end caps, explosive, and detonator mechanism. The explosive is usually black or smokeless powder sold to individuals who make their own cartridges and shotgun shells for hunting purposes. Smokeless powders can be subdivided into three groups: single base (containing nitrocellulose (NC)), double base (NC and nitroglycerine (NG)), and triple base (NC, NG, and nitroguanidine) (Beveridge, 1998). Single and

double base smokeless powders are commercially available and therefore encountered in IEDs. Pipe bombs may also contain projectiles, such as nails, bolts, or shot, to increase the amount of injury and damage they cause. The method of detonation can be as simple as a fuse or as complex as a switch or timing system. Several types of improvised switches that have been used to activate IEDs include mousetraps, aluminum foil, and clothespins (Thurman, 2006). Timing delays on pipe bombs allow the perpetrator to leave the scene before the bomb deflagrates. These too can be improvised from common objects including wind-up clocks and timers and items such as cell phones and pagers that can be remotely activated (Thurman, 2006). The relative ease of obtaining the materials as well as the general stability of the explosive means pipe bombs can be effortlessly transported with a low risk of accidental deflagration.

Metal is the container material used most often for making pipe bombs (National Research Council, 1998). Between 1992 and 1994, 485 bombing incidents occurred in the United States that utilized metal pipe containers and black or smokeless powder. During the same time period, there were 105 incidents in which plastic pipes were found (National Research Council, 1998). Both metal and plastic pipes are regularly employed for construction purposes and can be purchased without difficulty. Steel pipes are generally galvanized, i.e., coated with a thin layer of zinc (to prevent rust) that will peel off at temperatures above 200°C (American Galvanizers Association, 2000). Plastic pipe bomb containers are usually manufactured from polyvinyl chloride (PVC). Steel pipes and end caps typically have threaded fittings, whereas PVC pipes and end caps have smooth fittings that require an adhesive such as PVC cement or cyanoacrylate to glue them together.

Pipe bombs as evidence

When pipe bomb evidence is gathered, certain class characteristics such as the type or brand of the explosive can be determined. Some manufacturers of smokeless powders add a specific unique chemical, or taggant, to the powder that can be detected following a deflagration. Further, the general morphology of the powder is a characteristic that allows for brand identification (Beveridge, 1998). These class characteristics may be helpful for identifying suspects; however, individualizing evidence provides a definitive association between the perpetrator and the crime scene. Elements left on a pipe bomb that could definitively identify the handler include fingerprints and DNA from the person or persons who assembled or handled the device; however, fingerprints are not likely to be found on deflagrated pipe bombs due to the intense heat produced during the deflagration (Beveridge, 1998). This makes the search for DNA on deflagrated pipe bombs that much more imperative.

DNA is present in every cell in the body, except mature red blood cells. While touching a surface, skin cells are sloughed off and can adhere to it. Van Oorshot and Jones (1997) demonstrated that not only could DNA be recovered from latent fingerprints, but also items that had been touched by individuals could be matched back to those individuals. This type of DNA analysis has become increasingly common and is often referred to as "touch DNA" or "trace DNA" analysis. Researchers have shown that DNA can be deposited on a variety of surfaces. Balogh et al. (2003) found that fingerprints deposited on paper contain both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Other surfaces such as glass, plastic, ceramic, and vinyl (van Oorshot and Jones, 1997) can also retain DNA after being touched. Therefore, it is possible that

DNA from skin cells can be transferred to both PVC and metal pipes during the assembly of a pipe bomb.

STRs and miniSTRs

The standard method for examining nDNA in forensic applications is short tandem repeat (STR) analysis. STRs consist of tandemly repeated segments of DNA, each of which is typically two to six bases long (Butler, 2005). Individuals contain varying numbers of the tandem repeats at many loci, and it is these repeat numbers that can be used to identify a person. The STR loci used for forensic identification are included in several commercial DNA-typing kits, including PowerPlex® 16 System by Promega and AmpFlSTR Identifiler® PCR Amplification Kit by Applied Biosystems. Both kits utilize the polymerase chain reaction (PCR) to make copies of the STR loci. All of the primers used to amplify the loci are contained within a single reaction mixture (multiplex), reducing the number of PCRs that need to be performed. In the PowerPlex 16 kit, the 16 loci range in size from 100 base pairs (bp) to 450 bp (Promega Corporation, 2007), while the range in the Identifiler kit is from 100 bp to 375 bp (Applied Biosystems, 2001). One primer used to amplify each locus has a chemical dye attached to it. A laser excites the dye during the detection stage and a detector captures the wavelength emitted. The dyes have different ranges of emission, and thus the detector can differentiate them. In order to distinguish the loci detected using a given dye, the loci are separated by size, which is influenced by both the size of the STR itself and the amount of flanking DNA amplified on either side of it. The latter can be increased by moving the primers away from the repeat region, which allows for loci using the same dye to occupy their own size range,

preventing them from overlapping in size. When high quality DNA is amplified during STR analysis, the increase in locus size does not usually affect the level of the profile produced. However, in DNA that is degraded, the larger loci may not amplify.

MiniSTRs were designed so that primers anneal closer to the repeat region and therefore yield profiles utilizing much smaller segments of DNA (Wiegand and Kleiber, 2001; Butler et al., 2003). They range from approximately 50 bp to 300 bp (Krenke, 2002), which is particularly beneficial when attempting to amplify degraded DNA. Multiple miniSTR primer sets have been combined to produce a "miniplex" in which three to six loci are amplified in the same PCR (Butler et al., 2003). Opel et al. (2006) showed that the "miniplexes" are more successful at producing a full STR profile with degraded DNA from human bone than using traditional STR analysis.

Low copy number DNA

Findley et al. (1997) showed that it is possible to obtain STR results from a single human cell. The optimum amount of DNA added to the STR PCR is around 1 ng (Promega Corporation, 2007; Applied Biosystems, 2001). Given that a normal diploid human cell contains approximately 6 pg of nDNA, around 167 cells are needed for an optimal reaction, however this amount may not be available. When the amount of genomic DNA present in a sample is less than 100 pg, it is commonly referred to as low copy number (LCN) DNA (Gill et al., 2000). After touching an object there may be only a few cells from which DNA can be obtained, thus rendering it LCN.

Findley et al. (1997) identified several problems that occur in LCN analysis. Stochastic effects materialize when random loci or alleles are sampled more than others

leading to peak height imbalance or complete allelic drop-out. It has been suggested that the amplification and analysis of LCN DNA can be made more sensitive by increasing the number of PCR cycles (Wiegand and Kleiber, 1997; van Hoofstat et al., 1998); however, this could decrease the accuracy of the results due to over-amplification of exogenous DNA, which may even be amplified in place of the target DNA. Taberlet et al. (1996) proposed that alleles only be considered as part of a LCN profile if they appear in at least two PCR replicates. Budowle et al. (2001) expanded on this and suggested several approaches to increase sensitivity without increasing cycle number. They include:

1) reducing the PCR volume;
2) post-PCR filtration to remove ions that compete with DNA when being injected into the capillary;
3) use of formamide with low conductivity;
4) adding more amplified product to the formamide; and
5) increasing injection time.

Finally, it should be noted that STR profiles produced from LCN DNA should not be used to exclude individuals; because of the increased amount of allelic drop-in and drop-out, the accuracy of the results may be low.

DNA from pipe bombs

The deflagration of pipe bombs produces very high temperatures for a short period of time. It is not known what effect this heat has on DNA, however past research has explored DNA degradation following exposure to high temperatures. Threadgold and Brown (2003) showed that DNA from wheat seeds degrades at temperatures above

200°C. Wheat seeds were placed (*Triticum aestivum* L.) in an oven at temperatures ranging from 150°C to 250°C for different time periods (15 to 300 minutes). They found that at temperatures above 200°C and at times longer than 15 minutes, the DNA degraded to a point where a 246 bp portion of a nuclear gene and a 181 bp portion of a mitochondrial gene could not be amplified. Smokeless powder ignites at 315°C, causing a rapid change in temperature during deflagration of a pipe bomb (Lenz, 1965). Although the temperature produced during deflagration is not known and the length of time the bomb spends at that temperature is likely very short, it seems possible that the DNA deposited onto the surface of the bomb degrades.

Two groups of researchers have analyzed the DNA remaining on pipe bomb evidence following deflagration. Esslinger et al. (2004) isolated DNA from deflagrated pipe bombs and analyzed it using standard STRs. The study design included 20 pipe bombs that had been decontaminated using 10% bleach and UV irradiation and were then handled by subjects for approximately 30 seconds. The bombs were deflagrated in a hole in the ground that was covered with a large rock to contain the fragments. Resulting DNA was amplified at nine STR loci and the sex marker amelogenin. The difference between using unconcentrated DNA and concentrated DNA prior to amplification was also compared. No profiles contained all ten loci using the unconcentrated DNA, while concentration prior to PCR resulted in one full profile. One partial profile was recovered and six active profiles were found on the pipe bombs using unconcentrated DNA, and two partial and five active profiles were recovered using concentrated DNA. Further, the level of bomb fragmentation and fragment recovery influenced the ability to obtain a profile, with the more intact bombs yielding more complete profiles. Bombs that were

categorized as being highly fragmented with few recovered pieces yielded no profiles at all. The findings indicated that nDNA can survive the heat produced during a bomb deflagration; however most of the DNA is too degraded to be amplified using standard STR primers.

Gehring (2004) showed that mtDNA could be obtained from deflagrated pipe bombs. MtDNA is a circular genome found in mitochondria, which can number between 80 and 680 in a cell (Robin and Wong, 1988) in contrast to STRs that are present in only two copies per cell. This, in combination with its apparently protected location within the mitochondrion (Foran, 2006), may explain why mtDNA is more likely to amplify than nDNA in degraded materials. MtDNA is maternally inherited, thus a mtDNA haplotype is shared among siblings and other maternal relatives. That reduces its usefulness as an individualizing characteristic, although it provides for greater accessibility to reference samples if a suspect or victim is not available. Gehring (2004) prepared 36 pipe bombs in a manner similar to Esslinger et al. (2004). Following deflagration in an enclosed brick room and subsequent DNA extraction, the hypervariable regions of the mtDNA genome were sequenced. Bombs were assigned blindly to study participants. Eighteen of 36 bombs were correctly assigned to the individual who handled the bomb, and seven more were assigned to a subset of three individuals who shared the same mtDNA haplotype, thus there was a 69% success rate in correctly assigning the donor to a profile from a pipe bomb. Further, the research showed a trend between the level of fragmentation of the deflagrated pipe bomb and the quantity of DNA obtained, with higher levels of fragmentation resulting in lower amounts of recovered DNA.

The studies by Gehring (2004) and Esslinger et al. (2004) together revealed important points. First, it was possible to recover DNA from a deflagrated pipe bomb. This indicated that the heat produced during the deflagration was not high enough to completely destroy or remove DNA deposited on the pipe by the handler. Second, the DNA that remained following the deflagration was of a sufficient quality to yield DNA profiles. Third, sequencing of mtDNA was more successful in identifying the handler of a pipe bomb than were standard STR analyses. Finally, both studies established the connection between the level of fragmentation of a bomb and the success of obtaining positive results from the fragments.

Research goals

The objective of the research detailed here was to determine, using both nDNA and mtDNA, the identity of persons who assemble pipe bombs. Distinguishing individuals by mtDNA analysis is promising, however, STRs are currently the most widely used method of DNA identification in crime laboratories and mtDNA is not individualizing. MiniSTRs allow degraded evidence to be analyzed with many of the same loci as traditional STR analysis. Further, the reagents and instrumentation required for miniSTR analysis are the same as those for analyzing standard STRs, permitting integration of the miniSTR profiles into existing DNA databases. Therefore, the first goal was to investigate the efficacy of miniSTR analysis in the identification of assemblers of pipe bomb devices. Also explored was the relationship between the size of the loci analyzed and the success rate in obtaining alleles from each. It was hypothesized

that alleles from smaller loci would be more likely to amplify than alleles from larger loci.

The second goal was to compare the success rate of obtaining a correct mtDNA profile to the success rate of obtaining a correct miniSTR profile. Also, the usefulness of each kind of profile in identifying the individual who handled the pipe prior to deflagration was examined. Further, both methods were used in conjunction to determine the overall success of assigning a pipe bomb to an individual.

The final goal was to investigate the profiles produced from metal and PVC pipes. Esslinger et al. (2004) did not find a correlation between the container material and the ability to obtain a STR profile. The current study however used both mtDNA and nDNA to develop profiles; therefore more information was available to determine if a difference exists between the two types of containers.

Materials and Methods

Obtaining and decontaminating pipe bomb materials

IEDs were assembled from 1-foot pieces of PVC or galvanized steel pipe (1 inch diameter) and PVC or steel end caps, purchased at local hardware stores. A 1/4 inch hole was drilled in the center of one end cap for each device. A PVC end cap was affixed to one end of a PVC pipe using PVC cement and allowed to dry. Pipe pieces were soaked for 1 hour in 10% household bleach, rinsed with distilled water, UV irradiated for 5 minutes, turned, and irradiated for an additional 5 minutes. Surfaces of the container were then wiped with ELIMINase® (Decon Laboratories, Inc., Bryn Mawr, PA), according to the manufacturer's instructions and rinsed twice with sterile water to remove residual ELIMINase®. The pieces were dried in a laminar flow hood and stored in paper bags. Eighteen devices of each type were assembled.

Handling of containers by subjects

The use of human subjects as handlers of experimental bombs was approved by the Michigan State University Committee on Research Involving Human Subjects (IRB# 06-601). Subjects signed a consent form prior to participation. They randomly selected one PVC and one metal pipe assembly, removed them from the paper bag, handled each for 30 seconds, and then placed them back in the bag. Buccal swabs as DNA reference samples were also provided. Random identification numbers were assigned to each pipe bomb as well as the reference samples, and the rest of the experiments used blind scoring for analysis. One unhandled PVC and steel pipe were controls.

Deflagration

Pipe bombs were transported to the Lansing Fire Fighting Facility in Lansing, MI. There, a member of the Michigan State Police Bomb Squad filled the bombs with Green Dot Smokeless Shotshell Powder (Alliant Powder Co., Radford, VA), placed an approximately 1.5 foot long safety fuse into the hole in the end cap, and attached the second end cap to the device; PVC pieces were glued together using PVC cement and steel pieces were assembled using the threaded ends. Facemasks and latex gloves were worn when handling the bomb components. The bomb was placed in the brick room, the fuse lit, and the door closed. The length of fuse took approximately 30 to 45 seconds to burn. Following deflagration, two other investigators gathered the pieces and placed them in a new paper bag. Between deflagrations, the room was swept to remove any debris. One of the investigators who gathered the pipe bomb pieces performed the rest of the analyses in this study.

DNA isolation and extraction

Bombs were processed individually to avoid cross-contamination. Bomb fragments were removed from the paper bag under a laminar flow hood. A double-swab technique (Sweet et al., 1997) was used to recover DNA from the fragments. A cotton swab was moistened with 100 µL digestion buffer (20 mM Tris, 50 mM EDTA, 0.1% SDS, pH 7.5), swiped over the outside surface of all fragments and placed in a 1.5 mL sterile tube. A dry swab was then swiped over the surfaces to collect remaining moisture and placed in the same tube. For the metal bombs, only those fragments that retained the galvanized coating were swabbed. Four hundred microliters digestion buffer and 6 µL

proteinase K (20 mg/mL) were added to the tube. The contents were vortexed and incubated overnight at 55°C. The swabs were placed in a spin basket in a new 1.5 mL tube and centrifuged for 1 minute at 14000 revolutions per minute (rpm) to remove the liquid, which was then combined with the liquid remaining in the first tube. An equal volume of phenol was added, vortexed, and centrifuged at 14000 rpm for 15 minutes. The aqueous layer was transferred to a new tube and an equal volume of chloroform was added. The tube was vortexed and centrifuged at 14000 rpm for 15 minutes. The aqueous layer was placed on a Microcon® YM-30 spin column (Millipore Corporation, Billerica, MA) and centrifuged at 14000 x g for 15 minutes. The DNA on the column was washed twice with 100 µL TE (10 mM Tris, 1 mM EDTA, pH 7.5) and centrifuged after each wash at 14000 x g for 5 minutes. The DNA was resuspended in 20 µL TE and stored at -20°C. Reference buccal swabs were processed in the same manner.

Characterization of bomb DNA and reference samples using miniSTRs

DNA obtained from each bomb and the reference samples was amplified using two sets of miniplexed primers: miniSGM (http://www.cstl.nist.gov/div831/strbase /miniSTR.htm) and miniNC01 (Coble and Butler, 2005). MiniSGM PCR reactions included 1 µL Gold ST*R 10X Buffer (Promega, Madison, WI), 2.2 µL miniSGM primers (1 µM, NIST), 1.5 µL BSA (10 mg/µL), 0.25 µL AmpliTaq Gold® DNA Polymerase (5 U/µL, Applied Biosystems, Foster City, CA), 3.5 µL water, and 2 µL 1:10 diluted DNA template. Thermocycle conditions included an initial denaturation step at 95°C for 10 minutes, followed by 42 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. A final extension step at 60°C for 45 minutes was then performed.

Reference samples underwent the same PCR procedure except that 1 μL 1:10 diluted template DNA was added and 32 cycles of the denaturation, annealing, and extension steps were performed. Reference and bomb samples were amplified at different times to avoid cross-contamination. Amplification using miniNC01 primers was in reactions containing 2 μL Gold ST*R 10X Buffer, 2 μL miniNC01 primers (NIST), 1.5 μL BSA, 0.2 μL AmpliTaq Gold® DNA Polymerase, 2 μL 1:10 DNA template, and 2.5 μL water. Reference sample reactions contained the same except 1 μL 1:10 diluted DNA template was added. Cycling parameters were the same as the miniSGM reactions. All sets of reactions included a positive and negative control, as well as a reagent blank. Pipe bomb sample PCRs were performed in triplicate, while reference samples were amplified once.

The PCR product was added to a Montage® PCR Unit (Millipore Corporation) along with 300 μL TE and the unit was centrifuged for 15 minutes at 1000 x g. The DNA was washed twice with 100 μL TE and centrifuged at 1000 x g for 5 minutes. The resultant DNA was resuspended in 10 μL TE. During the data collection phase, an unknown error occurred which resulted in some samples producing unreliable data. Those samples and all remaining ones were purified using an UltraClean™ PCR Clean-Up Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to manufacturer's instructions. Following the extra clean-up step, the DNA was concentrated to a volume of 10 μL by adding 3.3 μL 3M NaOAc and 100 μL 95% ethanol and centrifuged for 5 minutes at 13200 rpm. The ethanol was removed and the resultant DNA was vacuumed dry and resuspended in 10 μL TE. Appendix A indicates the method used to clean each PCR.

Two microliters of the PCR product from the bombs and 0.5 μL GeneScan™ 500 LIZ® Size Standard (Applied Biosystems) were added to 23 μL deionized formamide. Reference samples were prepared by mixing 1 μL PCR product, 0.5 μL GeneScan™ 500 LIZ® Size Standard, and 24 μL formamide. The allelic ladder samples contained 23 μL formamide, 0.5 μL GeneScan™ 500 LIZ® Size Standard, and 2 μL of either miniSGM or miniNC01 allelic ladders (NIST). DNAs were analyzed using capillary electrophoresis on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). Samples were electrophoresed without denaturation using the following parameters: 5 seconds injection at 15 kV, 28 minutes run time at 15 kV, and a run temperature of 60°C through a 47 cm x 50 μm capillary. A 1X Running Buffer (Applied Biosystems) and POP-4™ Polymer for the 310 Genetic Analyzer (Applied Biosystems) were utilized. The resulting data were analyzed using GeneMapperID Software v. 3.2 (Applied Biosystems). The analysis method for the miniSGM loci was Microsatellite Default (Applied Biosystems) and for the miniNC01 loci was Microsatellite NC, created by the primary investigator. The allele panels for miniSGM and miniNC01 were mini-SGM and NC01 respectively, both created by the primary investigator based on panels provided at http://www.cstl.nist.gov/div831/strbase/miniSTR.htm. The miniplexed samples employed the CE_G5_HID_GS500 size standard setting and the D-33 Matrix Standard file (Applied Biosystems).

Characterization of bomb and reference samples using mtDNA

Three regions of the mitochondrial genome from each bomb sample were PCR amplified: the first half of hyper-variable region 1 (HV1-1), the second half of hyper-

variable region 1 (HV1-2), and hyper-variable region 2 (HV2). Primers are shown in Table 1; F82 was developed at the Forensic Biology Laboratory at Michigan State University and the remainder was developed at AFDIL (Edson et al., 2004).

Table 1. Primer pairs used to amplify mtDNA from pipe bombs.

HV1-1	
Non-nested Primer Pair	
F15989 5' CCCAAAGCTAAGATTCTAAT	R16322 5' TGGCTTTATGTACTATGTAC
Semi-nested Primer Pair	
F15989 5' CCCAAAGCTAAGATTCTAAT	R16251 5' GGAGTTGCAGTTGATGT
HV1-2	
Non-nested Primer Pair	
F16144 5' TGACCACCTGTAGTACATAA	R16410 5' GAGGATGGTGGTCAAGGGAC
Semi-nested Primer Pair	
F16190 5' CCCCATGCTTACAAGCAAGT	R16410 5' GAGGATGGTGGTCAAGGGAC
HV2	
Non-nested Primer Pair	
F82 5' ATAGCATTGCGAGACGCTGG	R484 5' TGAGATTAGTAGTATGGGAG
Semi-nested Primer Pair	
F155 5' TATTTATCGCACCTACGTTC	R484 5' TGAGATTAGTAGTATGGGAG

Reactions were performed in 20 μL volumes and included 1 μL 1:10 diluted DNA template, 2 μM forward and reverse primer, 1.5 μL BSA (10 mg/μL), 0.2 μL AmpliTaq Gold® DNA Polymerase, 200 μM dNTPs, 2 μL GeneAmp 10X PCR Buffer II (Applied Biosystems), and 2.5 mM $MgCl_2$. PCR conditions included a 12-minute 94°C denaturing step, followed by 38 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute, with a final extension step of 72°C for 5 minutes. Five microliters of the PCR product was electrophoresed on a 1.5% agarose gel. If a band was seen but contained

insufficient DNA for sequencing, the product was re-amplified with the non-nested primer pair for an additional 10 to 20 cycles. If bands were absent, the sample was amplified for 24 cycles with the corresponding semi-nested primer pair. Reference samples were amplified using primers F15989 and R569 for 32 cycles and the same PCR conditions as above. PCR products were purified with a Montage® PCR Filter Unit as described previously.

PCR products were sequenced utilizing a CEQ DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions in a 10 μL reaction volume. Sequencing reactions of bomb samples included the same primers that were used to amplify the DNA. Reference samples were sequenced with primers F15989, R16410, F15, and either R484 or R589. Sequencing reactions were purified according to manufacturer's instructions and analyzed with a CEQ 8000 Genetic Analysis System (Beckman Coulter) and a separation time of 60 minutes. Sequences were aligned with the software program Geneious Pro 3.0.6 (Drummond et al., 2007).

Determination of genetic profiles and assignations

Pipe bomb STR profiles were established by including alleles found in at least two of the three PCR replicates. Further, three peak height threshold values (50 RFU, 100 RFU, and 1000 RFU) were applied and a profile was developed for every bomb. Bomb profiles were compared to those of the subjects, and an individual (or individuals) was chosen as the most likely to have contributed the profile based on the number of loci with the same alleles. The bomb assignations were then compared to the true handler identity to determine correctness.

In a separate process using mtDNA, bomb samples were assigned to individuals based upon matching haplotypes. To assess the power of mtDNA and miniSTR analyses to determine the handler, the results were used in combination to make assignations according to the following rules:

1. If a single individual was determined using mtDNA, that pipe bomb was assigned to that individual.
2. If mtDNA assigned the bomb to more than one individual, the list of possible handlers determined from the miniSTR data was consulted, and the individual(s) found to overlap these two lists was chosen. If no overlap existed, the two lists were merged, thereby assigning the bomb to the group created from both lists.
3. If the mtDNA failed to assign a bomb, the list of possible handlers that had been determined from the miniSTRs was used.

Results

Pipe bomb deflagration and DNA isolation

Fragmentation levels of the deflagrated bombs varied from low to complete. Steel bombs tended to produce larger, more intact fragments whereas PVC bombs yielded fragments that were much smaller (Figure 1). Eight of the steel bombs resulted in a large fragment that consisted of about 90% of the original length of the pipe. The other larger fragments of the remaining bombs were between 25% and 75% of the original size of the bomb (exemplified in Figure 1A). All PVC bombs but one were highly fragmented, consisted of mostly small pieces (exemplified in Figure 1B), and produced some fragments that were too small to swab (less than one cm), which were not collected. One PVC bomb, 17P, did not fragment; only the end cap was blown off.

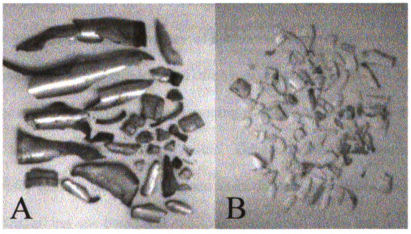

Figure 1. Post-blast debris. (A) represents the typical pieces collected from a steel pipe bomb, while (B) shows the remains of a PVC pipe bomb.

The swabs used to collect DNA from the bomb surfaces also collected residue from the burned powder, and from the steel pipes, flakes of the galvanizing layer, thus most of the swabs were discolored. The organic DNA extraction from PVC pipes removed most, if not all, of the discoloration. This was usually not the case with the steel

pipes however; many of the organic extractions resulted in a pink colored aqueous layer, which was not completely removed following filtration on Microcon® YM-30 columns. It was hypothesized that the color resulted from a reaction between metal particles and the phenol used in the organic extraction. Preliminary experiments indicated that the DNA from steel pipes amplified as successfully as DNA from PVC pipes, so no further action was taken to either identify or remove the discoloration.

MiniSTR profiles and bomb assignation

STR profiles were generated utilizing three peak height threshold values: 50, 100, and 1000 RFU. Every bomb had at least one allele that amplified two or more times in the triplicate reactions at 50 and 100 RFU. At a threshold value of 1000 RFU, no alleles amplified more than once from three of the bombs (3P, 4P, and 13S). There were no complete STR profiles produced from any of the bombs at any of the threshold values; each profile had some alleles missing, and often loci did not amplify. All STR profiles exhibited some extraneous alleles (not originating from the handler) that were seen in duplicate reactions, and thus were reproducible. The source of these alleles could not be determined; many of them did not correspond to the investigators participating in the study. However, all but one bomb that exhibited extraneous alleles had at least one allele in common with an investigator, and therefore the investigators could not be excluded as a source of contamination.

The bombs, individuals they were assigned to, and the individuals who actually handled them are shown in Table 2. Using a threshold of 50 RFU, 16 (47.1%) of the bombs were correctly assigned (Figure 2 and Table 3), half of which (23.5%) were assigned to either single individuals or a set of individuals, the latter encompassing

Table 2. Bomb assignations using miniSTRs. The bombs (P = PVC; S = steel) and study participants ("Indiv.") are listed along with the individual or sets of individuals to whom each bomb was assigned. Individuals that were correctly associated with a bomb are indicated in bold.

Bomb	Indiv.	50 RFU	100 RFU	1000 RFU
1P	313	**313**, 920	920	**313**, 485
2P	124	009, 177, 211, 875, 920	009, 177, 211, 875, 920	211, 875
3P	009			
4P	307	406		
5P	211	**211**, 875	124, **211**, 215, 313, 398, 485, 522, 622, 875, 920	124, **211**, 215, 313, 398, 485, 522, 622, 875, 920
6P	398	**398**	**398**	**398**, 622
7P	522	124, 211, 215, 313, 398, 485, **522**, 622, 875, 920	124, 211, 215, 313, 398, 485, **522**, 622, 875, 920	124, 211, 215, 313, 398, 485, **522**, 622, 875, 920
8P	209	920	920	**209**, 920
9P	736	177, 209, 398, 406, **736**	177, 209, 398, 406, **736**	177, 406, 446, **736**
10P	215	211, 485	211, 485	211
11P	622	485, 875	485	009, 211
12P	875	009, 211, 398, 485	009, 124, 177, 211, 313, 398, 446, 485, 522, **875**, 920	398, 920
13P	920	622, 875	875	398, 622
14P	446	875	875	209, **446**, 485, 875
15P	485	406, **485**	406, **485**	211, 875
16P	177	**177**	**177**	**177**, 485
17P	406	**406**	**406**	211
1S	313	**313**	**313**	211, 485
2S	124	177, 211, 209	177, 211, 215	177, 211, 313, 522
3S	009	485, 736	211, 485, 736	211
4S	307	**307**	**307**	009, **307**, 485
5S	211	**211**	**211**	**211**
6S	398	485	485	177, **398**, 485
7S	522	875	875	**522**
8S	209	622	622	**209**, 622
9S	736	215, 307, 398, 875	215, 307, 398, **736**, 875	211, 215, 307, 313, 398, 209
10S	215	177, 209, 211, 522, 622, 875, 920	209, 211, 622, 875, 920	209, 211, 622, 875, 920
11S	622	485	485	209
12S	875	211, 406, **875**	211, **875**	211, **875**
13S	920			
14S	446	398, 406, **446**, 485	398, 406, **446**, 485	406, **446**
15S	485	**485**	**485**	**485**
16S	177	**177**	**177**	**177**, 211, 875
17S	406	177, 211, 398, **406**, 736, 875	177, 211, 398, **406**, 736, 875	177, 211, 398, **406**, 736, 875

Table 3. Summery of bomb assignations using miniSTRs. The number of pipe bomb assignations, as well as the percentage is indicated for each category and RFU value.

	50 RFU	100 RFU	1000 RFU
Correctly assigned to a single person	8 (23.5%)	8 (23.5%)	3 (8.8%)
Correctly assigned to a subset of people	8 (23.5%)	9 (26.5%)	15 (44.1%)
Incorrectly assigned to a single person	7 (20.6%)	9 (26.5%)	4 (11.8%)
Incorrectly assigned to a subset of people	9 (26.5%)	5 (14.7%)	9 (26.5%)
Not assigned	2 (5.9%)	3 (8.8%)	3 (8.8%)

2 – 10 people. Sixteen bombs (47.1%) were incorrectly assigned, with seven (20.6%) assigned to a single individual and nine (26.5%) assigned to a subset (2 – 7) of the study participants. Two (5.9%) could not be assigned due to the lack of amplified alleles.

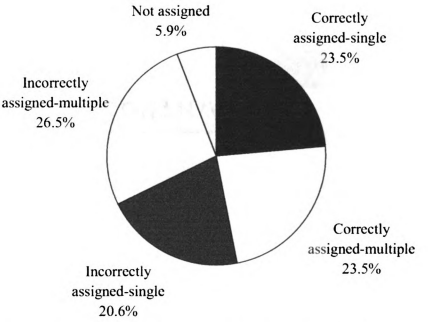

Figure 2. Correctly and incorrectly assigned pipe bombs at 50 RFU using miniSTRs. Segments of the chart represent the percentage of bombs that were assigned to single or multiple individuals.

When the peak height threshold value was raised to 100 RFU, 17 bombs (50%) were correctly assigned, including eight (23.5%) to a single individual and nine (26.5%)

to a set of 2 – 11 individuals (Figure 3 and Table 3). Fourteen (41.2%) bombs were incorrectly assigned, with nine (26.5%) to single individuals and five (14.7%) to a group of 2 – 5 individuals. Three (8.8%) bombs could not be assigned. Twenty-three bombs had the same classification at both 50 and 100 RFU, whereas six had different classifications. Two bombs, 12P and 9S, were incorrectly assigned to a set of donors at a threshold of 50 RFU, but were correctly assigned to a set of donors using the 100 RFU threshold.

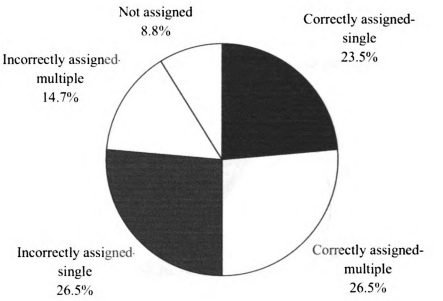

Figure 3. Correctly and incorrectly assigned pipe bombs at 100 RFU using miniSTRs. Segments of the chart represent the percentage of bombs that were assigned to single or multiple individuals.

Using a threshold value of 1000 RFU, 18 (52.9%) pipe bombs were correctly assigned, with three (8.8%) assigned to a single individual and 15 (44.1%) assigned to sets of 2 – 10 individuals (Figure 4 and Table 3). Thirteen (38.2%) bombs were incorrectly assigned—four (11.8%) to a single individual and nine (26.5%) to a group of

2 – 6 individuals. Three (8.8%) bombs could not be assigned. With the increase in threshold value from 100 to 1000 RFU, 15 bombs stayed in the same classification whereas 19 changed. Five went from being incorrectly assigned to a single individual to being correctly assigned to a set of individuals ranging in size from 2 – 4. One bomb was incorrectly assigned to a single individual at 100 RFU but correctly assigned to a single individual at 1000 RFU. The remainder of the bombs that changed classifications either went from being correctly assigned at 100 RFU to being incorrectly assigned at 1000 RFU, or the number of individuals to which they were assigned changed and the accuracy stayed the same.

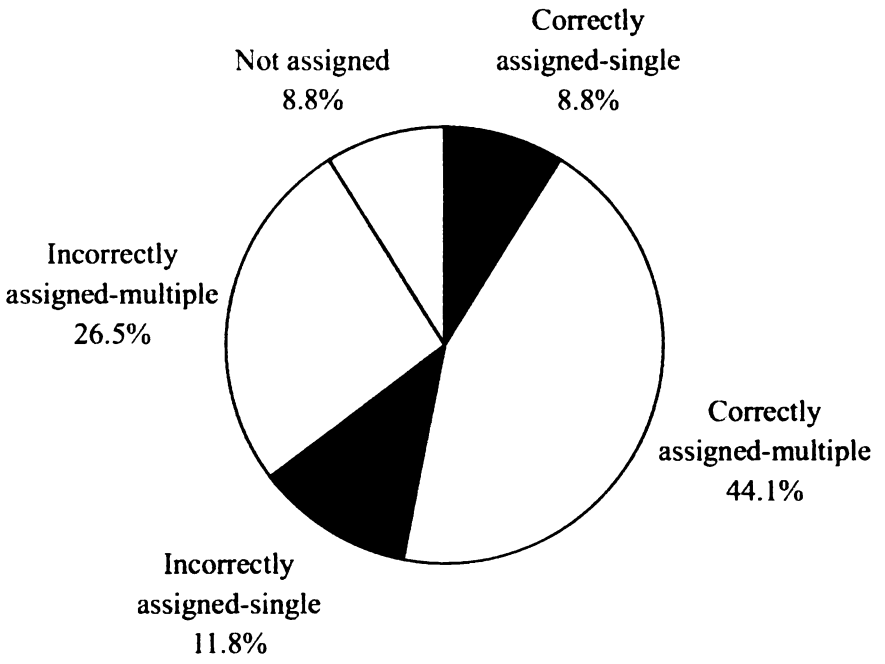

Figure 4. Correctly and incorrectly assigned pipe bombs at 1000 RFU using miniSTRs. Segments of the chart represent the percentage of bombs that were assigned to single or multiple individuals.

The two control pipes that were deflagrated had alleles amplify. The steel control pipe had 11 reproducible alleles at both the 50 and 100 RFU thresholds and seven at 1000

RFU. The PVC control pipe had two reproducible alleles at 50 RFU and one each at 100 and 1000 RFU. These results, like the extraneous alleles observed on the handled bombs, could not be completely attributed to any of the investigators in the study. For example, the steel control pipe produced alleles 8, 9, and 11 at D16S539. One investigator had a 9 allele at this locus; however the other two alleles were not attributable to any of the investigators.

MtDNA sequencing and bomb assignation

HV1 sequences were obtained for all bombs and HV2 sequences were acquired for all but one bomb. Eleven of the 34 bombs were correctly assigned to a single individual (32.4%) and ten others were correctly assigned to a group of two people (29.4%) (Figure 5 and Table 4). Nine bombs could not be assigned because a haplotype was produced that did not match any of the subjects (26.5%). Fewer mis-assignments were made using mtDNA (11.8%) than miniSTRs (38.3% to 44.1%).

Table 4. Summery of bomb assignations using mtDNA. The number of pipe bomb assignations as well as the percentage of the total is indicated for each category.

	Number of bombs	Percent
Correctly assigned to a single person	11	32.4%
Correctly assigned to a subset of people	10	29.4%
Incorrectly assigned to a single person	2	5.9%
Incorrectly assigned to a subset of people	2	5.9%
Not assigned	9	26.5%

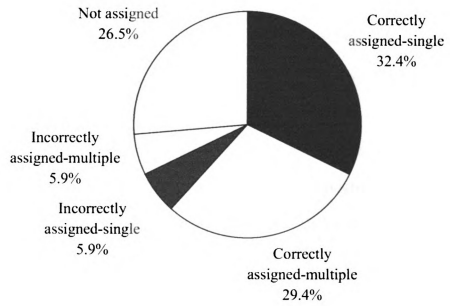

Figure 5. Correctly and incorrectly assigned pipe bombs using mtDNA. Segments of the chart represent the percentage of bombs that were assigned to single or multiple individuals.

One PVC and one steel bomb were swabbed prior to handling by subjects to see if any preexisting DNA that may have been on the bombs was destroyed, and neither bomb generated a mitochondrial PCR product. The two control bombs that were deflagrated each yielded a mtDNA profile. The PVC bomb produced a mixture of haplotypes from unknown individuals: C and T at 16179, 16224 and 16311. The steel control pipe bomb had mixtures of C and T at 199, 204, and 250, which is consistent with the profile of individual 307 and an unknown source. Twenty-four of the 34 experimental bombs showed signs of mtDNA contamination in their profiles. Some of the contamination could potentially be attributed to the investigators, as a mixture of haplotypes that was consistent with originating from both a study participant and an investigator was present. Six of the 24 presumably contaminated bombs were correctly assigned to a single

individual and eight were correctly assigned to a group of two individuals, as a minor profile was subtracted. Seven of the nine bombs that were not assigned showed mtDNA contamination. Three bombs had mixtures of haplotypes consistent with having been contaminated prior to deflagration by the investigator responsible for filling the pipe bombs with powder and placing them in the deflagration room. The investigator who performed the remainder of the analyses in the study may have contaminated three samples during the set-up of PCR, as contamination was not found in all three sequences that were produced. Two of these samples, 2S and 11P, had a mixture in HV1, with both a C and T at 16179, 16291 and 16356, which was consistent with coming from the primary investigator and the subject, but no contamination was found in HV2. A DNA sample from bomb 5P showed a mixture of bases only in HV2, at 309.1C, consistent with the primary investigator. The same investigator may also have contaminated four other samples, however the step when this occurred could not be determined because contamination was found in all three sequences produced, thus contamination could have happened during any step. Fourteen samples contained an extra haplotype or haplotypes that were not attributable to any of the investigators, based on the mixture of bases found. Five of these had only one position with a mixture of bases that was different from the subjects' haplotype. The remainder of the bombs had between two and six positions that had a mixture of bases different than their corresponding reference samples.

Bomb assignations using both miniSTRs and mtDNA

MiniSTRs and mtDNA were used in combination to assign pipe bombs to the individuals shown in Table 5. At a peak height threshold of 50 RFU, 17 bombs (50%)

Table 5. Bomb assignations using miniSTRs, mtDNA, and both mtDNA and miniSTRs. The bombs (P = PVC; S = steel) and study participants ("Indiv.") are listed along with the individual or sets of individuals to which each bomb was assigned. The individuals that were correctly associated with a bomb are indicated in bold. Table includes data from Table 2.

Bomb	Indiv.	mtDNA assignation	50 RFU	100 RFU	1000 RFU	50 RFU and mtDNA	100 RFU and mtDNA	1000 RFU and mtDNA
1P	313	**313**	**313**, 920	920	**313**, 485	**313**	**313**	**313**
2P	124	-	009, 177, 211, 875, 920	009, 177, 211, 875, 920	211, 875	009, 177, 211, 875, 920	009, 177, 211, 875, 920	211, 875
3P	009	-	-	-	-	-	-	-
4P	307	-	406	-	-	406	-	-
5P	211	177, **211**	**211**, 875	124, **211**, 215, 313, 398, 485, 522, 622, 875, 920	124, **211**, 215, 313, 398, 485, 522, 622, 875, 920	**211**	**211**	**211**
6P	398	**398**, 736	**398**	**398**	**398**, 622	**398**	**398**	**398**
7P	522	-	124, 211, 215, 313, 398, 485, **522**, 622, 875, 920	124, 211, 215, 313, 398, 485, **522**, 622, 875, 920	124, 211, 215, 313, 398, 485, **522**, 622, 875, 920	124, 211, 215, 313, 398, 485, **522**, 622, 875, 920	124, 211, 215, 313, 398, 485, **522**, 622, 875, 920	124, 211, 215, 313, 398, 485, **522**, 622, 875, 920
8P	209	**209**, 406	920	920	**209**, 920	**209**, 406, 920	**209**, 406, 920	**209**
9P	736	406	177, 209, 398, 406, **736**	177, 209, 398, 406, **736**	177, 406, 446, **736**	406	406	406
10P	215	**215**	211, 485	211, 485	211	**215**	**215**	**215**
11P	622	**622**	485, 875	485	009, 211	**622**	**622**	**622**
12P	875	-	009, 211, 398, 485	009, 124, 177, 211, 313, 398, 446, 485, 522, **875**, 920	398, 920	009, 211, 398, 485	009, 124, 177, 211, 313, 398, 446, 485, 522, **875**, 920	398, 920
13P	920	**920**	622, 875	875	398, 622	**920**	**920**	**920**
14P	446	**446**, 522	875	875	209, **446**, 485, 875	**446**, 522, 875	**446**, 522, 875	**446**
15P	485	398, 736	406, **485**	406, **485**	211, 875	398, 406, **485**, 736	398, 406, **485**, 736	211, 398, 736, 875
16P	177	**177**, 211	**177**	**177**	**177**, 485	**177**	**177**	**177**

Table 5. (Continued)

Bomb	Indiv.	mtDNA assignation	50 RFU	100 RFU	1000 RFU	50 RFU and mtDNA	100 RFU and mtDNA	1000 RFU and mtDNA
17P	406	**406**	**406**	**406**	211	**406**	**406**	**406**
1S	313	**313**	**313**	**313**	211, 485	**313**	**313**	**313**
2S	124	-	177, 211, 209	177, 211, 215	177, 211, 313, 522	177, 209, 211	177, 211, 215	177, 211, 313, 522
3S	009	124	485, 736	211, 485, 736	211	124	124	124
4S	307	**307**	**307**	**307**	009, **307**, 485	**307**	**307**	**307**
5S	211	177, **211**	**211**	**211**	**211**	**211**	**211**	**211**
6S	398	-	485	485	177, **398**, 485	485	485	177, **398**, 485
7S	522	446, **522**	875	875	**522**	446, **522**, 875	446, **522**, 875	**522**
8S	209	**209**, 406	622	622	**209**, 622	**209**, 406, 622	**209**, 406, 622	**209**
9S	736	-	215, 307, 398, 875	215, 307, 398, **736**, 875	211, 209, 215, 307, 313, 398	215, 307, 398, 875	215, 307, 398, **736**, 875	211, 215, 307, 313, 398, 209
10S	215	-	177, 209, 211, 522, 622, 875, 920	209, 211, 622, 875, 920	209, 211, 622, 875, 920	177, 209, 211, 522, 622, 875, 920	209, 211, 622, 875, 920	209, 211, 622, 875, 920
11S	622	**622**	485	485	209	**622**	**622**	**622**
12S	875	**875**	211, 406, **875**	211, **875**	211, **875**	**875**	**875**	**875**
13S	920	**920**	-	-	-	**920**	**920**	**920**
14S	446	**446**, 522	398, 406, **446**, 485	398, 406, **446**, 485	406, **446**	**446**	**446**	**446**
15S	485	398, 736	**485**	**485**	**485**	398, **485**, 736	398, **485**, 736	398, **485**, 736
16S	177	**177**, 211	**177**	**177**	**177**, 211, 875	**177**	**177**	**177**, 211
17S	406	**406**	177, 211, 398, **406**, 736, 875	177, 211, 398, **406**, 736, 875	177, 211, 398, **406**, 736, 875	**406**	**406**	**406**

were correctly assigned to a single individual and seven (20.6%) were correctly assigned to a set of 3 – 10 individuals (Table 6). Four pipe bombs (11.8%) were mis-assigned to a single individual and five (14.7%) were incorrectly assigned to a group of 3 – 7 individuals. Pipe bomb 3P could not be assigned because neither a miniSTR nor a mtDNA profile was produced.

Table 6. Summery of bomb assignations using both mtDNA and miniSTRs. The number of pipe bomb assignations as well as the percentage of the total is indicated for each category at all three RFU values.

	50 RFU and mtDNA	100 RFU and mtDNA	1000 RFU and mtDNA
Correctly assigned to a single person	17 (50.0%)	17 (50.0%)	20 (58.8%)
Correctly assigned to a subset of people	7 (20.6%)	9 (26.5%)	4 (11.8%)
Incorrectly assigned to a single person	4 (11.8%)	3 (8.8%)	2 (5.9%)
Incorrectly assigned to a subset of people	5 (14.7%)	3 (8.8%)	6 (17.6%)
Not assigned	1 (2.9%)	2 (5.9%)	2 (5.9%)

When the peak height threshold was raised to 100 RFU for the miniSTR profiles used in conjunction with mtDNA, 17 bombs (50%) were correctly assigned to a single individual and nine (26.5%) were correctly assigned to a group of 3 – 11 people (Table 6). Three bombs (8.8%) were incorrectly assigned to an individual and three others (8.8%) were incorrectly assigned to a group of 3 – 5 individuals. Two bombs could not be assigned. Thirty-one bombs remained in the same classification using 50 or 100 RFU threshold values, where two went from being incorrectly assigned to a group of individuals to being correctly assigned to a group of individuals, while a third bomb was incorrectly assigned to a single individual at 50 RFU and was not assigned at 100 RFU.

The highest number of bombs that were correctly assigned to single persons, 20, occurred using 1000 RFU and mtDNA (Table 6). Four bombs (11.8%) were correctly assigned to a group of 2 – 10 individuals. Two pipe bombs (5.9%) were incorrectly assigned to a single individual and six (17.6%) were incorrectly assigned to a group of 2 – 6 individuals. Two bombs could not be assigned to any individuals using both mtDNA and miniSTRs. Between 100 and 1000 RFU, nine bombs changed classifications; four went from being correctly assigned to a set of people to being correctly assigned to a single individual. Three went from correctly assigned to incorrectly assigned, and the remaining two from being assigned either correctly or incorrectly to a single individual to being correctly assigned to a set of individuals.

MiniSTR amplicon size vs. number of alleles amplified

The size of each amplicon was compared to the average total number of alleles present at each locus in every bomb's STR profile. The profile encompassed alleles that were present in at least two of the triplicate reactions performed. With a range of 51 to 98 bp, TH01 was the smallest amplicon utilized in this study, and at a value of 50 RFU, an average of 3.03 alleles per bomb amplified (Figure 6). As the size of a locus's amplicon increased, the number of alleles amplified tended to decrease (Table 7).

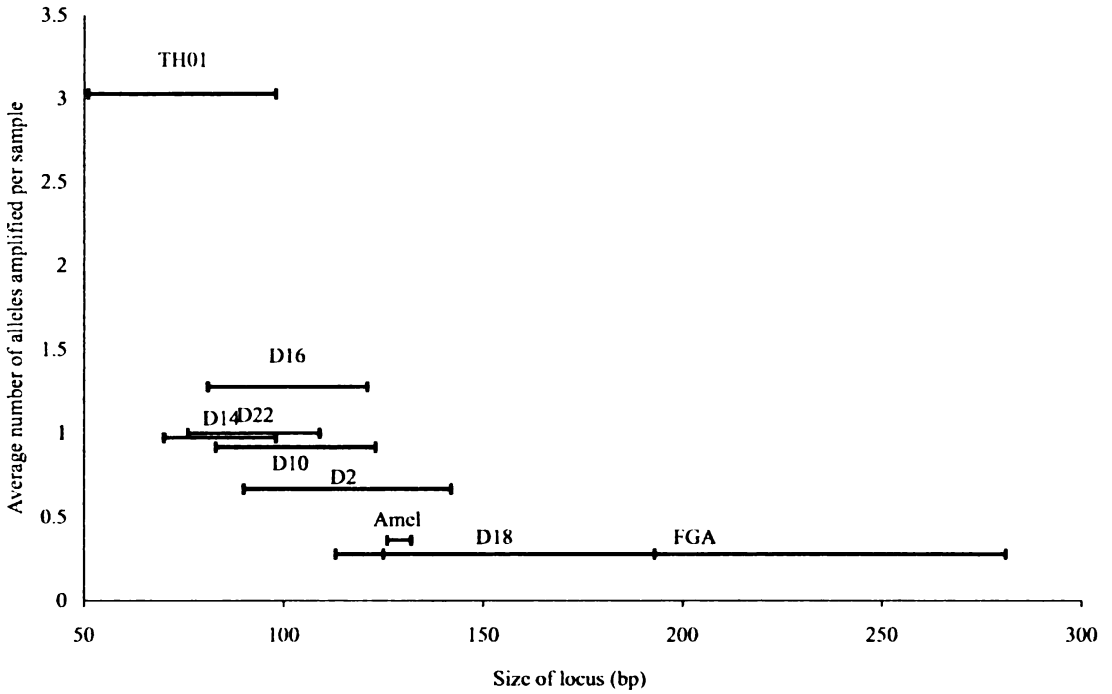

Figure 6. Average number of alleles amplified per locus at 50 RFU in bomb profiles. Each bar represents the size range in basepairs of the amplicon produced at the locus indicated.

Table 7. Summery of the number of alleles amplified per locus. The average number of alleles, correct alleles, and incorrect alleles amplified at each locus in the bomb profiles for the three peak height threshold values (50, 100, and 1000 RFU) is shown. The loci are listed across the top of the table in ascending order of average size.

	TH01	D14	D22	D16	D10	D2	Amel	D18	FGA
Total number of alleles amplified									
50 RFU	3.028	0.972	1.000	1.278	0.917	0.667	0.361	0.278	0.278
100 RFU	2.944	0.944	0.917	1.250	0.889	0.556	0.333	0.278	0.222
1000 RFU	2.083	0.694	0.583	0.528	0.639	0.222	0.111	0.000	0.056
Number of correct alleles amplified									
50 RFU	1.111	0.083	0.361	0.611	0.472	0.417	0.306	0.139	0.194
100 RFU	1.139	0.083	0.361	0.611	0.472	0.389	0.306	0.139	0.167
1000 RFU	0.972	0.083	0.250	0.250	0.417	0.194	0.083	0.000	0.056
Number of incorrect alleles amplified									
50 RFU	1.917	0.889	0.639	0.667	0.444	0.250	0.056	0.139	0.083
100 RFU	1.806	0.861	0.556	0.639	0.417	0.167	0.028	0.139	0.056
1000 RFU	1.111	0.611	0.333	0.278	0.222	0.028	0.028	0.000	0.000

The number of alleles that matched the individual who handled the bomb was determined at each locus for every bomb profile and an average value was calculated across all bombs, which was then compared to the size of the locus. At all threshold value (Figure 7 and Table 7), the locus with the highest average number of correct alleles was the smallest, TH01. There was a trend in which smaller loci tended to have the greatest number of correct alleles amplify. Similarly, TH01 had the greatest average number of incorrect alleles amplify (Table 7).

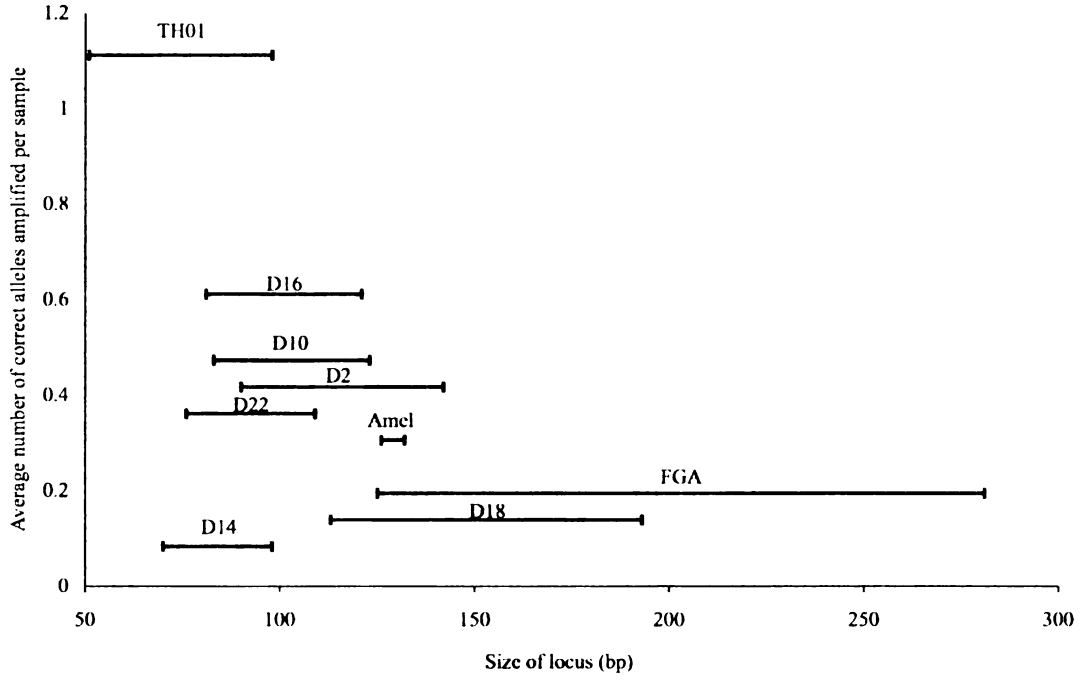

Figure 7. Average number of correct alleles per locus at 50 RFU when bomb profiles were compared to reference profiles. Each bar represents the size range in base pairs of the amplicon produced at the locus indicated.

Examination of PVC and steel pipe bombs

A comparison of successfully assigning PVC and steel bombs showed that they were similar. Ten PVC bombs (29.4%) and eleven steel pipe bombs (32.4%) were correctly assigned to an individual using only mtDNA (Table 8). Both PVC and steel

pipes had two incorrectly assigned bombs when using mtDNA profiles. Eight PVC bombs (47.1%) and eight steel bombs were correctly assigned at 50 RFU (Table 8). Two PVC bombs (11.8%) and one steel bomb could not be assigned at the 100 RFU threshold value, however eight PVC and nine (26.5%) steel bombs were correctly assigned. When the threshold value was raised to 1000 RFU, 11 PVC (32.4%) and 12 steel (35.3%) bombs were correctly assigned and four PVC and four steel bombs (23.5%) were incorrectly assigned to an individual or set of individuals (Table 8).

Table 8. Comparison of PVC and steel pipe bomb assignations. The number of bomb assignations in each category is shown along with the composition of the bomb (PVC or steel) and the various analyses used to make the assignations.

	mtDNA		miniSTR at 50 RFU		miniSTR at 100 RFU		miniSTR at 1000 RFU	
	PVC	Steel	PVC	Steel	PVC	Steel	PVC	Steel
Bombs correctly assigned	10	11	8	8	8	9	11	12
Bombs incorrectly assigned	2	2	8	8	7	7	4	4
Not assigned	5	4	1	1	2	1	2	1

Finally, steel and PVC bomb assignations were compared utilizing combined mtDNA and miniSTR data (Table 9). Twelve PVC and twelve steel bombs were correctly assigned at a miniSTR threshold of 50 RFU. Four PVC bombs and five steel bombs were mis-assigned and one PVC bomb could not be assigned. Thirteen PVC bombs and 12 steel bombs were correctly assigned at 100 RFU, while two PVC and five steel pipe bombs were mis-assigned. Two PVC bombs could not be assigned at 100 RFU. Eleven PVC and 13 steel bombs were correctly assigned at 1000 RFU, whereas four PVC bombs and four steel bombs were not assigned to the correct individuals. Two PVC bombs could not be assigned whereas all steel bombs were assigned.

Table 9. Comparison of PVC and steel pipes combining miniSTR and mtDNA data. The number of bomb assignations in each category is shown along with the composition of the bomb (PVC or steel) and the various threshold values used to make the assignations.

	50 RFU and mtDNA		100 RFU and mtDNA		1000 RFU and mtDNA	
	PVC	Steel	PVC	Steel	PVC	Steel
Bombs correctly assigned	12	12	13	12	11	13
Bombs incorrectly assigned	4	5	2	5	4	4
Not assigned	1	0	2	0	2	0

Discussion

The laboratory analysis of a deflagrated IED can help identify the individual who assembled it, however this can be difficult as much of the physical evidence has only class characteristics, while individualizing features such as fingerprints are frequently destroyed. Since cells can be deposited onto the bomb by the assembler, molecular evidence may provide definitive information regarding their identity. However, DNA is often found in low quantities and will likely be severely degraded following deflagration, making its examination challenging. The recovery and subsequent analysis of DNA from pipe bombs was examined in two previous studies. Esslinger et al. (2004) used traditional STR kits and showed that it was possible to amplify nDNA gathered from deflagrated pipe bombs, although the success rate was quite low. Gehring (2004) demonstrated that mtDNA analysis can result in a fairly high success rate when attempting to identify individuals who handled a pipe bomb, however mtDNA is not individualizing evidence. Owing to this, the current study revisited nDNA analysis using newly introduced miniSTRs, which assay smaller DNA templates to better amplify degraded DNA, along with traditional mtDNA testing.

All bombs produced partial nDNA profiles in the current research, although none produced full profiles. Overall, this was an improvement over the findings of Esslinger et al. (2004), where eight of 20 bombs (40%) produced either a full (1) or partial (7) profile, following DNA concentration. Several factors could result in the differences between the two studies. One was the DNA primer sets used for amplification. MiniSTRs resulted in more amplified loci and thus more profiles produced. Another was the procedure used for DNA extraction, STR amplification, and capillary electrophoresis. The use of LCN

DNA analysis in the field of forensic science is a relatively recent development and thus has not yet been incorporated into most crime laboratory systems. Esslinger et al. (2004) employed procedures that are standard in most crime laboratories, which are not adjusted for use with LCN DNA. Research has just begun to offer suggestions for modifying methods for better amplification (Taberlet et al., 1996; Wiegand and Kleiber, 1997; van Hoofstat et al., 1998; and Budowle et al., 2001). The current study included several modifications that reflect contemporary LCN practices, including a reduction in PCR volume, increased PCR cycles, post-amplification filtration, addition of more PCR product to the formamide, and performing PCR reactions in triplicate. The sum of these modifications likely improved the ability to produce accurate profiles from deflagrated materials. For example, post-amplification filtration was found to reduce the amount of background noise in electropherograms, making identification of alleles easier. Finally, there may have been a difference in deflagrated bomb fragment recovery, allowing for more DNA to be collected in the current study. Esslinger et al. (2004) placed each bomb in a hole in the ground and covered it with a large rock to contain the fragmented pieces produced during deflagration. In some instances fragments escaped from the hole and were not collected. In the current study, the bombs were deflagrated in an enclosed room, which contained the fragments. This allowed for all fragments that were large enough to swab to be collected and potentially more DNA to be recovered.

The current study also incorporated analysis of mtDNA, which can be recovered from post-blast materials and successfully amplified and typed, as originally described by Gehring (2004). Within that research, 50% of the bombs were correctly assigned to a single individual and 19% more were assigned to a subset of three individuals, for a total

of 69% correctly assigned. MtDNA-based research presented here demonstrated that 32% of bombs were correctly assigned to a single individual and 29% to a subset of two people, or 62% correctly assigned. Of the 18 subjects that participated in the Gehring (2004) study, six (33.3%) had non-unique haplotypes and therefore the bombs handled by them could not be individualized. Eight of 17 (47.1%) subjects had non-unique haplotypes in the current research; thus the individualizing capability was necessarily less than Gehring (2004). The two studies had similar total percentages of bombs correctly assigned, the most important factor to consider when comparing them. This was not surprising as the methods used here were heavily based on those from Gehring (2004), with only a few changes made to the procedures to decontaminate, assemble, and deflagrate the bombs. It is possible that this methodology could be improved by exploring alternative methods to increase the amount of DNA recovered from the post-blast material, which may allow more profiles to be produced. For example, soaking the fragments in buffer instead of swabbing could increase DNA yields, as even very small pieces could be assayed. Further, smaller mtDNA amplicons could be used to circumvent the problems of DNA degradation and sequencing of long stretches of DNA (Gabriel et al., 2001). These would provide for fuller mtDNA profiles, which can be used to better differentiate among individuals.

There was a slight difference in the number of bombs within the current study that were correctly assigned using mtDNA versus miniSTRs, however it was not statistically significant. On the other hand, there was a large difference in the number of bombs that were incorrectly assigned. When utilizing mtDNA, only four bombs were incorrectly assigned, whereas more bombs were incorrectly assigned using miniSTRs at each of the

three threshold values (50 RFU: 16; 100 RFU: 14; 1000 RFU: 13). This could have resulted from contamination that occurred during nDNA analysis, as every miniSTR profile contained extraneous alleles. Two negative controls during the miniSTR procedure showed signs of contamination; however all negative controls during mtDNA analysis were negative. Several reagent blank controls had contamination when amplified with both miniSTR and mtDNA primers. In general, the nDNA analysis each sample was conducted prior to the mtDNA analysis, thus contamination found in miniSTR profiles, but not in corresponding mtDNA profiles, was likely introduced during nDNA analysis. The increased contamination in the nDNA analyses made assignments of bombs more difficult than using mtDNA haplotypes, resulting in more incorrect assignments. Further, contamination within mtDNA profiles exhibited as mixtures of haplotypes, which were easier to recognize owing to two bases at one location in the sequence. Extraneous alleles in the STR profiles may have been the same as those in subject profiles by chance, as there are only a limited number of alleles possible. Such alleles increase the likelihood that a bomb would be assigned incorrectly because they appear the same as a true allele in a subject profile.

Another potential reason for the different amounts of incorrectly assigned bombs between mtDNA and miniSTRs was the novelty of the procedures used for miniSTR analysis. Unlike the mtDNA analysis, which was based on procedures by Gehring (2004) that had been optimized for DNA recovery, BSA addition, amplicon size, and primer combinations, miniSTR analysis of DNA from pipe bombs was new. A few steps were taken to enhance to ability to correctly identify the handler using miniSTRs. For instance, reagent concentrations were used that were slightly different from those

suggested by the developers of the miniSTR primers (http://www.cstl.nist.gov/div831/strbase/miniSTR.htm). These included the quantity of BSA that was added to combat inhibition, which was increased from the recommended amount of 0.5μL 3.2 mg/mL BSA to 1.5 μL 10 mg/μL BSA, however this quantity could be optimized to better improve PCR efficiency. Further, more Taq polymerase (1.25 U) than recommended (1 U) was used to enhance PCR efficiency, although optimization was not performed. In the future, procedures for amplification and subsequent miniSTR analysis can likely be refined to allow for more bombs to be correctly assigned. These could include the addition of more Taq polymerase to the reactions, which may improve amplification of the LCN DNA as the polymerase degrades at high cycle numbers thus losing efficiency (Gill et al., 2000). Amplifying for a standard 28 cycles then adding polymerase to the reaction and reamplifying for several more cycles has been proposed as a better approach for amplifying LCN DNA (Kloosterman and Kersbergen, 2003). Also, the number of PCR cycles can be lowered from the 42 used in this study so that allelic drop-in would be lessened, thus reducing the number of erroneous alleles and producing profiles more representative of the handler. Furthermore, the miniSTR primers in this research were constructed at the Armed Forces DNA Identification Laboratory. Recently developed commercial miniSTR kits are more likely to have better quality control, be optimized for degraded DNA, and be subjected to validation studies. These, in turn, could improve pipe bomb DNA amplification and thus produce more complete profiles. Finally, DNA quantification was not incorporated into the methods, therefore exact amounts of recovered DNA were not known. Future studies should include

quantification of the DNA so that the optimal amount of template is added to the reactions.

Another potential reason that mtDNA and miniSTRs had different numbers of incorrectly assigned bombs is the characteristics that each possesses. As discussed by Foran (2006), mtDNA's protected location within the cell might decrease the likelihood that it degrades relative to nDNA. Further, more copies of mtDNA exist within fingerprints (Andréasson et al., 2006). Both the protected location and the increased copy number of mtDNA deposited onto the bombs may have resulted in more of it present post-deflagration, allowing for greater success when assigning the bombs. In this regard, if only one type of analysis can be performed because of limited DNA quantity, it is recommended that mtDNA be assayed, as it is more likely to produce the profile of a handler.

Combining miniSTR and mtDNA profiles increased the success of assigning bombs over using either alone, and allowed for greater discrimination of individuals that handled the bombs. This indicates that casework success could be improved if both analysis methods are applied to determine the identity of the bomb handler. MtDNA testing would allow investigators to narrow down a group of suspects based on the haplotype found, then STRs could be used to hone in on the individual who contributed their DNA to the evidence. Robustness of mtDNA together with the individualizing power of nDNA would enhance the identification of individuals who handled or assembled a pipe bomb. When sample quantities permit, both mtDNA and miniSTR analyses should be performed to garner as much genetic information as possible about the handler.

An integral part of obtaining a correct STR profile is the peak height threshold employed. An optimal threshold should be sensitive enough to detect valid alleles but high enough to prevent the incorporation of spurious low-level signals from background noise or contamination. Three threshold values, 50, 100, and 1000 RFU, were examined in this study to see how they influenced identifications. Fifty and 100 RFU resulted in similar numbers of correctly assigned bombs to both individuals (8 individuals each) and groups of individuals (8 and 9 respectively), whereas three bombs were correctly assigned to an individual and 15 to a group of individuals at 1000 RFU. Further, all three threshold values produced similar levels of both incorrectly assigned bombs (50 RFU: 16; 100 RFU: 14; 1000 RFU: 13) and bombs that were not assigned (50 RFU: 2; 100 RFU: 3; 1000 RFU: 3). As the threshold value was raised, fewer peaks were incorporated into the profiles and thus less information was available to allow for a correct assignation. The number and accuracy of alleles present at each threshold value was also considered. There were more alleles called at all loci using 50 RFU than the other two threshold values; also, 50 RFU yielded the highest number of both correct and incorrect alleles. In summery, more individuals were correctly assigned using RFU values of 50 and 100 because of the larger amount of information available in the profiles.

The relative utility of each threshold value can also be assessed by the number of true and false alleles present in their respective profiles. This was examined by calculating the ratio of correct to incorrect alleles. In this case, the lowest ratio was found at 50 RFU with a value of 0.727 and the highest was at 1000 RFU with 0.883. The higher number of incorrect alleles at 50 RFU can be attributed to allelic drop-in and/or contamination. Given this, if the overarching goal is to reduce the amount of extraneous

alleles present in the profile, a higher threshold value should be used. However, 1000 RFU also had the lowest total number of correct alleles, thus reducing the amount of information available to link a bomb to a handler. Based on these findings, the optimal way to examine a DNA profile from a crime scene if a suspect's profile is available for comparison would be to incorporate a low threshold value that allows for the highest number of alleles, which provides the most information for developing a profile. If, however, no suspect exists, a higher threshold value should be used, which would produce a profile with fewer incorrect alleles and thus more accurate database searching. Further research could help laboratories to determine a threshold value for each instance so that a standard operating procedure can be developed and implemented.

Alleles and mtDNA haplotypes not corresponding to the handler of the bomb may have resulted from contamination. Allelic drop-in is usually not reproducible and therefore was not likely to become part of a profile because only alleles that amplified at least two times were incorporated into a profile. Every bomb had called alleles that did not originate from the handler, and sometimes investigators could not be excluded as the source. However, the investigators were not the only source of contamination as there were many alleles that did not correspond to any of them. Control bombs tested after decontamination by attempting amplification of mtDNA were negative, however they tested positive following deflagration. After decontamination and prior to deflagration, the bombs were handled by the investigator who filled them with smokeless powder and placed them in the deflagration room. Although gloves were worn, other items were handled at the same time, such as the smokeless powder container, PVC glue can, and lighter, which may have carried foreign cells. It is possible that secondary transfer of

DNA occurred between these items and the bombs, which should be examined in the future.

The method used to isolate DNA from the recovered bomb fragments could also be modified in an attempt to lower the number of mixtures. For instance, multiple swabs can be used to recover DNA from various fragments or portions of a bomb. If different DNAs or mixtures are present on portions of a bomb and one set of swabs is used for DNA isolation, the resulting profile will be mixed. However, if only a single DNA source is present on some part(s) of a bomb and it is processed using a new swab, then a single DNA profile would be obtained from that region. The profiles developed from different parts of the bomb can be used to create a consensus profile, which can help in identifying and excluding extraneous alleles (Hoffmann et al., 2008).

Contamination could also have occurred during DNA isolation. Research conducted after this investigation was completed indicated that the sterile swabs used during the isolation process might have been contaminated with human DNA prior to use (Gomez and Hoffmann, personal communications), which could have resulted in the amplification of alleles that did not match those from either study participants or investigators. Further, extraneous alleles become more pronounced when increased PCR cycles are used (Kloosterman and Kersbergen, 2003). The number of cycles in the current research, 42, was more than the recommended 28 – 34 cycles for miniSTRs (http://www.cstl.nist.gov/div831/strbase/miniSTR/updated_NC01_protocol.pdf). This cycle number was chosen to produce more amplicon copies, however it was not optimized and may have resulted in extraneous alleles. There seems to be a trade-off between being able to amplify LCN DNA using increased cycles and introducing

extraneous alleles to profiles. Therefore, it is recommended that other LCN methods (Budowle et al., 2001), some but not all of which were included in this study, be implemented in place of increased cycles to lessen the amount of extraneous alleles in STR profiles.

Gehring (2004) noted that mtDNA mixtures, which may have resulted from contamination, were present in DNA samples. Eighteen of 36 bombs (47.4%) produced mixtures, which was lower than the mtDNA mixture level (70.6%) in this research. Gehring (2004) found that the primary investigator, responsible for decontaminating the bombs, collecting fragments, and completing the analyses, had a haplotype consistent with contributing to the mixture 50% of the time. This investigator had a relatively rare haplotype (0.05%) in the FBI mtDNA population database (Monson et al., 2002) indicating contamination occurred directly (Gehring, 2004). The author noted that although gloves were worn during all stages, facemasks were only worn during the second half of the analyses performed, although this did not appear to affect the rate of contamination, as similar numbers of mixtures were found before and after the introduction of facemasks. The research presented here had a smaller percentage of samples (29.2%) consistent with being contaminated by the primary investigator, whose haplotype was unique within the mtDNA database, indicating that the investigator contributed to the mixtures. Further, two reagent blanks were found to contain the primary investigator's haplotype, thus some contamination occurred following the collection of the post-blast materials. Facemasks, protective clothing, and gloves were worn during all stages of contact with the bombs and DNA samples. These preventative measures, together with sample processing in a laminar flow hood, may have contributed

to the decreased amount of contamination by the primary investigator, however it was still present. This could potentially be reduced further by using automated robotics instrumentation, which can perform DNA extraction, purification, and PCR set-up, thus limiting the contact between the investigator and the samples (Greenspoon and Ban, 2002).

After considering contamination from investigators, there were still a large number of bombs where the contamination could not be traced. As with nDNA, the mixtures found in the mtDNA profiles could have been from exogenous DNA on laboratory supplies. Two bombs were tested to ensure that the decontamination procedure removed DNA from the bombs, and both tested negative. However, the remainder of the bombs was decontaminated in several batches at different times, so it is possible the procedure was not executed properly or did not always produce the intended results. Future studies might incorporate testing of all bomb materials following the decontamination procedure to ensure that they are free of DNA. It is important to note, of course, that bomb component decontamination is unlikely to occur prior to handling by bombers in real life situations. It is plausible that profiles could include alleles and haplotypes from people who handled the components during the manufacturing, transportation, or retail transaction stages, but were unconnected to the construction of the bomb. Therefore, if DNA is recovered from post-blast materials, it is possible that both mixtures and contamination will be encountered, and extreme caution should be taken when managing these materials so as not to introduce further contamination.

Varying amounts of contaminating alleles were found in different sized miniSTR loci. A trend was seen in which smaller target amplicons yielded higher numbers of both

correct and incorrect alleles than did larger amplicons. Further, there was a higher ratio of correct alleles to incorrect alleles as locus size increased. Gill et al. (2000) noted that spurious alleles were found more often in smaller loci when amplifying LCN DNA using traditional STRs. It appears that, although smaller loci allow for more alleles to be amplified from degraded DNA, they also promote the incorporation of more incorrect alleles into profiles. In a real world situation, the size of the loci utilized may best be chosen based upon other circumstances in the case. If a suspect is unknown, larger loci, or even traditional STRs, can be used, which would tend to yield fewer, but more likely correct, alleles, allowing a profile to be uploaded to a database. On the other hand, a database search would be futile if the evidence profile contained a large number of incorrect or extraneous alleles, as many unrelated hits would cause investigators to waste time and resources. If, however, a suspect(s) is known and a reference sample is available, it may be advantageous to assay smaller loci so that more information may be gleaned from the evidence. Laboratories should develop protocols for each of these situations so that resources can best be maximized.

Somewhat surprisingly, the materials from which the bombs were constructed had no influence on correctly assigning bombs to individuals. Increased DNA degradation was expected to occur on steel bombs, since metal conducts heat more readily than PVC. Also, pieces of the galvanized layer of the metal pipes peeled off during deflagration; therefore, DNA was only recovered from the fragments where the layer remained intact, which may have lowered DNA recovery. The amount of DNA lost due to the missing galvanized layer of metal bombs might be similar to that lost on tiny shards of PVC pipe that were not recovered. DNA was not quantified in this study; therefore it is not

possible to determine how much was recovered from each type of material. Esslinger et al. (2004) obtained the same results, with no difference between the two types of pipe. Since there does not appear to be any distinct difference between them, it is possible that similar amounts of heat are actually transferred to the exterior surface of both PVC and metal pipe bombs resulting in the same amount of DNA degradation. Given this, the two types of bombs should be processed the same in a laboratory. It is important to note, however, that the bombs in this study were subjected to similar experimental conditions, and variations in bomb construction could alter the outcome of comparisons between the materials. Future studies should include various types of IEDs to determine if DNA yield and degradation are similar from different substrates.

Finally, producing a DNA profile from deflagrated pipe bombs is only worthwhile if it leads to identifying the individual responsible for the incident. There are several ways that a DNA profile can be used. The first and easiest is if there is a suspect whose DNA profile is available for direct comparison. If however there is no suspect, the nDNA profile may be uploaded into the Combined DNA Index System (CODIS), a database in the United States that includes profiles from both crime scenes and convicted offenders. The profile from the evidence is searched against all profiles in the database, and if a match is made, either a suspect can be identified from the convicted offender list or the bombing incident can be linked to other crimes. Although other countries have their own national databases, a worldwide database is not currently available. This limits the ability to search profiles from around the world, but it would not preclude international matches from occurring. Several of the STR loci in CODIS are the same as those used in the European Union, and sequencing of the control region is the standard

method for analyzing mtDNA. Investigators must be proactive if they want to match DNA profiles across national borders to maximize the potential for identifying those who utilize IEDs.

Conclusions

The results of this study indicate that combining miniSTR and mtDNA data is a better approach for identifying an individual who handled a pipe bomb than is using either of the two alone. Over 70% of the bombs were correctly assigned to a single individual or a set of two individuals with the combined analyses, whereas around 50% were correctly assigned with miniSTRs and 60% with mtDNA. The potentially degraded nDNA from the pipe bombs was amplified at smaller miniSTR loci; however, more incorrect alleles were amplified at smaller loci than larger loci. Finally, both PVC and metal pipe bombs show similar results with regard to assigning them to individuals.

The techniques in this study were highly sensitive, producing profiles from low amounts of template DNA. An unwelcome consequence of the sensitivity was the large number of extraneous alleles amplified from the sample DNA. Encounters with pipe bomb evidence by both responders in the field as well as laboratory personnel will need to include precautions to prevent or minimize exposure of the evidence to extraneous sources of DNA. Gloves, facemasks, and other personal protective equipment should be worn during evidence collection, and changed if contact with items other than the evidence occurs. It would be best to perform all subsequent analyses in areas that reduce the possibility of contamination. These safeguards can improve the likelihood that only the handler's profile, and not those of the investigators, is obtained from post blast pipe bomb evidence.

Appendix A

Bomb	miniSGM	miniNC01
1P	M M M	M M M
2P	M M M	M M M
3P	M M M	M M M
4P	M M M	M M M
5P	M M M	M M M
6P	M M M	M M M
7P	M M M	M M M
8P	M M M	M M M
9P	M M M	M M M
10P	M M M	M M M
11P	M M M	M M B
12P	M M M	M M UC
13P	M M UC	M M M
14P	M M M	M M B
15P	M M M	M M M
16P	M M M	M M M
17P	M M M	M M M
1S	M M M	M M M
2S	M M M	M M M
3S	M M M	M M M
4S	M M M	M M M
5S	M M M	M M M
6S	M M M	M M M
7S	M M M	UC UC UC
8S	M M M	UC UC UC
9S	M M M	UC UC UC
10S	M M M	UC UC UC
11S	M M M	UC UC UC
12S	M M M	UC UC UC
13S	M M M	UC UC UC
14S	M M M	UC UC UC
15S	M M M	UC UC UC
16S	M M M	UC UC UC
17S	M M M	UC UC UC
Control P	M M M	M M M
Control S	M M M	M M M

Method used to purify each miniSTR PCR following amplification. MiniSGM and miniNC01 refers to the two sets of miniplexed primers used to amplify the bomb DNA, which were performed in triplicate. "M" indicates the PCR was cleaned using a Montage® PCR Unit, "UC" indicates the PCR was cleaned using a UltraClean™ PCR Clean-Up Kit, and "B" indicates that the PCR was cleaned using both the Montage unit and the UltraClean unit. P indicates the bomb was constructed from PVC pipe and S indicates the bomb was made of steel.

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