

Review Article

Forensic aspects of mass disasters: Strategic considerations for DNA-based human identification

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Abstract

Many mass disasters result in loss of lives. Law enforcement and/or public safety and health officials often have the responsibility for identifying the human remains found at the scene, so they can be returned to their families. The recovered human remains range from being relatively intact to highly degraded. DNA-based identity testing is a powerful tool for victim identification in that the data are not restricted to any particular one to one body landmark comparison and DNA profile comparisons can be used to associate separated remains or body parts. Even though DNA typing is straightforward, a disaster is a chaotic environment that can complicate effective identification of the remains. With some planning, or at least identification of the salient features to consider, stress can be reduced for those involved in the identification process. General guidelines are provided for developing an action plan for identification of human remains from a mass disaster by DNA analysis. These include: (1) sample collection, preservation, shipping and storage; (2) tracking and chain of custody issues; (3) laboratory facilities; (4) quality assurance and quality control practices; (5) parsing out work; (6) extraction and typing; (7) interpretation of results; (8) automation; (9) software for tracking and managing data; (10) the use of an advisory panel; (11) education and communication; and (12) privacy issues. In addition, key technologies that may facilitate the identification process are discussed, such as resin based DNA extraction, real-time PCR for quantitation of DNA, use of mini-STRs, SNP detection procedures, and software. Many of the features necessary for DNA typing of human remains from a mass disaster are the same as those for missing persons' cases. Therefore, developing a missing persons DNA identification program would also provide the basis for a mass disaster human remains DNA identification program.

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1. Introduction

On September 11, 2001, the USA experienced its worst intentional mass disaster in history. Two commercial passenger airplanes were hijacked and deliberately crashed into the twin towers of the NYC World Trade Center (WTC) [1,2]. A third such airplane was deliberately crashed into the Pentagon, and a fourth airplane was forced to crash in a field near Shanksville, PA. These terrorist attacks on the USA resulted in more than 3000

deaths. Mass disasters also can result from other intentional events, e.g. poisonous gas attacks such as that perpetrated in 1995 by the Aum Shinrikyo in a Tokyo subway [3], car bombs, or wars [4–7]; from natural events, such as the devastating tsunamis on December 26, 2004 that resulted from a 9.0 earthquake near Sumatra; and from accidents, such as fires [8–10] and airplane crashes, e.g. Swissair Flight 111 [11]. Many of these types of mass disasters result in loss of lives, and the recovered human remains range from being relatively intact to highly degraded. High impact disasters, such as airplane crashes, typically result in severe fragmentation and degradation of human remains [1,2,11–14].

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Situations involving individual missing persons also can be thought of as mass disasters that occur over a longer time period. Each year thousands of children and adults vanish without a trace or under suspicious circumstances. Spain was one of the first countries to initiate a national program to identify human remains of which there are at least 1000 unidentified remains [15]. In the USA alone there are 5000 unidentified recovered skeletonized remains registered on the National Crime Information Center (NCIC) [16]. Since only a small portion of unidentified remains currently are registered on the NCIC, the actual number may be five to ten times higher.

One aspect that falls under the responsibility of law enforcement and/or public safety and health officials after a mass disaster or a missing person investigation is the individualized identification of human remains found at the scene, so they can be returned to their families. Characteristics or traits used to assist in the identification of the human remains include, but are not limited to: skeletal features, dental comparisons, fingerprints, distinguishing marks (tattoos and scars), medical devices and implants, personal effects, and DNA profiles.

Not all human remains will be suitable for the more traditional identification approaches, especially if there is substantial fragmentation of the remains. Large numbers of mass casualties (such as occurred with the WTC) present a daunting task to rescue workers and government agencies in recovering and identifying the literally thousands of compromised human remains. Fingerprints or dentals record are often not useful in identification of such remains. In contrast to traditional comparisons, DNA-based identity testing is not restricted to any particular one to one body landmark comparison (e.g. friction ridge details in fingerprints). Furthermore, DNA profile comparisons can be used to associate separated remains or body parts. As long as the recovered human tissue contains a sufficient quantity of typeable DNA, important data can be obtained to assist in victim identification.

The DNA profiles from recovered mass disaster remains are compared with the DNA profiles from reference samples such as known personal effects of the victims or family member reference samples. Personal items (such as unlaundered clothing, a toothbrush, PKU cards from State mandated newborn genetic screening programs, or even archival pathology tissue) can serve as direct reference samples and may be available from which DNA can be extracted to attempt to identify the victims. In some situations, such direct DNA comparisons are not possible, and therefore family members must provide reference samples for indirect identification using kinship analysis [17].

While DNA-based human identity testing can sometimes be an uncomplicated process, mass disasters are almost always unexpected and therefore unpredictable. They place tremendous stress on the families of

the victims, health care workers, government agencies and the community. In the immediate aftermath of a mass disaster, the chaotic environment can complicate effective identification of the remains. However, with some planning, such as in organization and surge capacity, a priori, instead of after the event, stress by those affected by the mass disaster can be reduced. Experiences from previous mass disasters, primarily from September 11, 2001, handling of missing persons cases, and identification of soldiers or military personnel from past wars can help better prepare the laboratory personnel responsible for the task of identification using DNA analysis, when the next mass disaster occurs [1–14, 18–22]. Herein we provide general guidelines to consider in developing an action plan for identification of human remains from a mass disaster by DNA analysis. We also discuss some of the key technologies that may facilitate the identification process.

2. External oversight committee/advisory board

When a mass disaster occurs, a number of agencies, institutions, and individuals, both public and private, readily offer humanitarian assistance to the laboratory tasked with the identification of the victims. The support and assistance offered covers every aspect of the operation from providing advice to services to offering new technologies for analysis. Any laboratory faced with the daunting task of mass disaster response can become overwhelmed with proposals and quick fix solutions. First and foremost, the laboratory director should consider assembling an appropriate external oversight committee (or advisory board) composed of recognized experts (both forensic and others). The board can meet regularly to help evaluate meaningful ideas, proposed processes, address specific scientific challenges and questions that will arise, and review and advise on the validity of novel technologies. Members of such an advisory board provide the laboratory with: a focused reality check on possible decisions; additional views; assistance in screening the approaches by those who offer assistance; ready access to recognized expertise; and greater confidence in the direction(s) taken by the laboratory. Typically, such advisory board members will agree to serve pro bono, but travel and accommodation costs need to be considered.

3. Value of non-DNA methods

Any information that supports a correct identification is invaluable and should be considered. Forensic scientists who are primarily trained in the field of DNA analysis often believe that DNA typing is the one and only reliable method for identification. DNA analysis

is but one of several tools available that can be applied to the identification of human remains. While we focus on DNA analysis, one should recognize that other traditional identification methods will also be necessary. When possible, some of these identification approaches can eliminate the need for the more labor intensive and costly DNA analysis or reduce the need for re-analysis of some remains. Additionally, initial anthropological screening is extremely beneficial for selecting the best samples for DNA analysis and eliminating non-human remains from further analysis.

4. Chain of custody

It is essential to establish a documented chain of custody on all samples beginning with collection at the disaster scene, regardless if the disaster is due to a criminal act, negligence, or an accident. A large part of the processing of samples from a mass disaster involves the gathering, collating, checking, curation, and storage of data. Maintaining proper chain of custody on all samples, primarily for quality assurance (QA) and control (QC), will reduce sample mix-up and tampering and will often be significant for subsequent legal procedures (e.g. criminal prosecutions or civil matters including estate settlements and parentage determination). With contemporary computer capabilities, digitized data can be stored electronically and tracked readily. If different data tracking and archive systems are used, they should interact as seamlessly as possible. While it would be more effective if one specimen accessioning system is

used, often multiple agencies are involved and they often do not have the same software systems. If the data in a reference sample database could not be compared directly with that in the missing persons database or if samples have been divided and distributed to different laboratories for analysis, a substantial amount of additional work would be required to collate the data. Therefore, customized software may be necessary to avoid confusion with sample tracking (see below).

5. Family liaison and DNA collection from relatives

From the onset of the response, the victims' families should be quickly informed of the processes and resources for identification that are available to them. A family consultation service should be established. Files containing documents that have been gathered over the course of the disaster processing pertinent to the victim and family should be readily available. Any family member should be afforded opportunity to schedule an appointment with an investigator and discuss the family member's file and progress in the analysis. A telephone hotline should be implemented and be operational seven days a week to provide consultation about the identification process or the development of new DNA techniques (Table 1). The fostering of a positive supportive relationship with family members through education and awareness will help facilitate the collection of appropriate reference samples and help maintain the public support for the activities of the laboratory. It will also inform family members on privacy issues regarding the potential use of their DNA samples and whether or not their DNA profiles

Table 1

Frequently asked questions a DNA hotline should be prepared to address

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1. When I called the DNA hotline, I was told that the reference samples I submitted months ago were not listed. What happened to them?
 2. I called the hotline and was told that the reference samples I submitted did not yield DNA. Why didn't anyone contact me?
 3. I provided a DNA sample and was asked to provide a second sample because the first one did not yield a result. Why did it not yield a result?
 4. Remains were identified for my family member. Will DNA testing be used to identify any additional remains?
 5. Will DNA be taken from all the remains found at the disaster?
 6. If I submit personal items for DNA, will they be returned to me?
 7. How should personal items be prepared for submission?
 8. Can someone come to my house to collect reference samples?
 9. Can I get a copy of my DNA profile from my reference sample?
 10. After I submit the reference samples how long will it take to identify my relative?
 11. How long will the identification process continue?
 12. How does the length of time between the disaster and sample collection or storage impact the capability of identifying a relative?
 13. What are the criteria for positive identification of a victim?
 14. Can DNA information for positive identification be obtained from a single strand of hair? If the hair follicle is missing or damaged, can DNA analysis still be performed?
 15. What precautions are taken during collection or processing to prevent DNA contamination of samples?
 16. How accurate and/or sensitive are the methods used for DNA analysis? Can the components of a mixed sample be identified?
 17. Who should provide reference DNA samples?
 18. Does temperature impact the integrity of DNA?
 19. Can personal information be derived from my DNA sample?
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Questions modified from Ref. [73].

can be searched against DNA profiles from unsolved criminal case files.

6. Sample collection

In order to determine if the disaster was accidental or intentional, law enforcement officials must attempt to reconstruct the events through the collection of all available evidence from the scene. However, the primary concern at a mass disaster scene, even if it is the result of a criminal act, is the recovery, rescue and treatment of surviving victims. Under circumstances in which some victims have survived but are injured, assisting the victims may compromise the quality of crime scene evidence. While the evidence may still yield meaningful identification information, the most important responsibility of officials responding to a mass disaster scene is saving victim's lives and protecting the safety of rescue workers.

Evidence recovery and preservation are critical steps in the identification of human remains, especially in cases where the remains are highly fragmented. If at all possible, human remains should be collected immediately and in an organized fashion. Some disaster scenes, though, are difficult or even treacherous for investigators. Thus, sample collection could take weeks or months. Although improper preservation methods will not lead to a wrong result, it can cause destruction of intact DNA such that crucial data may not be available to render an identification of a victim. An example of such improper methods is placing a wet bloodstained garment in a plastic bag (instead of drying it first and then placing in a paper bag). Such action allows bacterial and fungal growth that leads to DNA degradation [23].

Because the scene of some mass disasters initially can be very chaotic, early development and implementation of appropriate sample handling, evidence collection and preservation procedure strategies will lead to more successful laboratory analyses. While the initial organization of evidence collection is often not the responsibility of the DNA typing laboratory, having a laboratory representative interacting with the investigators at the scene as early as possible is very useful, particularly for sample accession and inventory control.

For DNA-based identification of human remains, reference samples from the victims or their family members are needed. As mentioned previously, samples collected from family members can be used for indirect genetic identification by kinship analysis [24–29]. Establishing the true biological relationship of all the donors and the victim is necessary. Some people when asked (and also those inquiring) may not understand the designations for a specific family member relationship (such as half-sib) or that an adopted child cannot be genetically related to the parents of the family. Family history forms that are simple and straight forward to complete should be

developed that clearly define and enable documentation of these relationships. Software tools that assess pedigree and genetic data can ensure that the biological data comport before evaluating the victim relationship (see below).

There will be times when the purported genetic relationship is inconsistent with the genetic data. For instance, consider a hypothetical family in which the victim has two living parents and three siblings. The family members provide DNA samples for analysis, and it is discovered that the father cannot be the biological father of one of his children. There may be several explanations for the discrepancy, such as the child is from the mother's previous marriage. Great care should be exercised to avoid violating privacy issues in such cases. The laboratory representative should be prepared for such situations and must be discrete, no matter what the laboratory official's personal convictions may be. Forensic scientists should put in place guidelines that protect the privacy rights of the victim and his/her family.

As long as sufficient genomic DNA can be recovered, items that the victim previously handled or contacted may potentially serve as a reliable known reference sample for a direct DNA-based identification. Direct comparisons are straightforward and often require typing of fewer genetic loci to effect a reliable and valid identification than are needed in situations requiring indirect identification using kinship analyses [30]. Potential direct reference biological material may be found on items such as hairbrushes, stamps, envelopes, toothbrushes, razors, and clothing. Children may also leave biological material on pacifiers and toys (e.g. stuffed animals and dolls). Bloodspot cards collected in newborn genetic screening protocols may also be available, especially for infants and young children. Such bloodspot cards also are available for most USA military personnel. Some individuals may have banked donated tissue samples (e.g. donated in a bone marrow drive), or a tissue biopsy sample may reside in the pathology archives of a hospital. One caveat to consider when using personal items is that there is a lack of a prior chain of custody on the personal effect; thus, the confidence regarding the source of the sample may be somewhat reduced unless additional information is available. Personal items are usually very limited and often yield relatively small amounts of DNA; therefore care should be exercised to preserve them as best as possible.

Investigators should attempt to collect as many samples as possible for analysis; some samples may be better suited than others. Those collecting the samples, for example law enforcement agencies or family assistance groups, may not be sufficiently experienced to make an assessment of what constitutes a good reference sample. DNA extracted from some personal item samples may be too degraded, which would not become evident until part way through

the analytical process. Also, some samples may need to be divided for multiple analyses and for quality control purposes.

As a result of the disaster incident, the victim's family members have been traumatized; therefore any undue stress should be avoided. To limit the stress placed on a family member, reference samples should all be collected at one time, if possible. Therefore, proper collection, storage and handling of these samples are as important as the practices employed for evidence samples. Requesting additional samples on multiple occasions from the family can raise doubts about the process to identify their relative and bring into question the competence of the various agencies, the government, and the laboratory scientists.

To avoid some of the problems that can arise when collecting and packaging samples, a standardized sample collection kit(s) for putative relatives and unidentified remains should be developed, and can be modeled on those developed for missing persons cases. A family member reference kit should include all the supplies for the collection, packaging and shipping of a biological sample. The best design is one with a non-invasive collection device for the safe and effective acquisition of the appropriate family reference samples, such as a buccal swab or a slightly more intrusive blood sample can be obtained by a finger stick. The sample collection tools should be compatible with both long-term archival storage as well as the use of robotics for high throughput processing and analysis. The kit also should contain (1) collection instructions (Appendices A and B)¹; (2) a form for recording demographics of the person providing the family reference sample which includes a pedigree describing the relationship with the missing person (Appendices C and D); (3) a standardized multilingual release form (for example, at a minimum for the USA English and Spanish) that authorizes the collection, testing and entry of the DNA profiles into a database and describes the permissible use of the sample (Appendix E); and (4) an evidence submission form (Appendix F). In addition, a multilingual informational brochure and a collection training video or CD should be prepared. The brochure should describe the services offered by the laboratory including the use of DNA in identity testing, potential sources of DNA (e.g. bone, teeth, hair, blood, etc.) (Appendix G), the most informative individuals that may provide a family reference sample, information on the types of individuals/samples included in the database, how the data are maintained in the database system and how the entire process works. A videotape or CD presentation can provide officials with a visual guide through the process of completing the form, as well as detailed correct techniques required to collect and package an appropriate

reference sample for DNA analysis. In addition, the DNA laboratory might consider providing training sessions for all individuals involved in the DNA process, as well as family members. Visual tools are usually much better for educating those involved than written instructions alone. While it can require a good deal of work, training and education tools can dramatically reduce errors in sample collection, packaging, and shipping.

A second kit for the collection, transportation, and storage of human remains samples should also be developed. The human remains will vary in size and condition (Appendix H). Thus, multiple size boxes should be made available. Acid free wrapping paper for wrapping individual bone fragments could be part of the kit. For personal effects, standard crime scene collection kits would suffice. All kits should include bar coding or some other tag to assist in the identification and tracking of samples by a Laboratory Information Management System (LIMS). Lastly, it would be desirable to standardize kit formats within a country or jurisdiction to ensure the minimum requirements are contained within each kit. Kits designed for missing persons cases could be the model system.

When sample recovery at a disaster site takes an inordinately long time, tissue decomposition is less controllable. To obtain a sufficient quantity of DNA from more compromised samples, larger portions of the samples, if they are available, should be collected and sent to the laboratory for analysis. The analysis of a larger sample will impact the extraction process; modifications to accommodate the larger sample size will be necessary.

7. Sample storage

Once collected and packaged, the biological samples are sent to the laboratory for storage and analysis. The number of samples received by the laboratory often will be overwhelming, particularly in high impact events. The receipt and handling of the samples can be a drain on the laboratory's resources. Crime laboratories already have a backlog of cases and cannot readily accommodate the surge capacity required. To ensure samples are not lost, mixed-up, or improperly stored, and that associations made are reliable, the laboratory must have a quality assurance and quality control process in place. Particularly, an effective inventory system and LIMS should be implemented. Current chain of custody practices can be used, and no new procedures need to be implemented for maintaining the chain; however, they can be enhanced with well-designed software tools.

If at all possible, the number of samples that may be collected should be estimated. This will enable the laboratory to better address storage space needs, which ideally should be in place before any samples arrive. It is unlikely that sufficient storage facilities can be obtained immediately after the disaster or before the receipt of the samples. One way to mitigate the storage space needs

¹ Appendices A-H are published on the web version: www.sciencedirect.com, as supplementary material.

(as well as other demands placed on the laboratory) is to develop a proactive plan of cooperation with other agencies to share some responsibilities when a disaster occurs. For example, some samples could be stored at another crime laboratory until the laboratory responsible for the analysis of the disaster samples has enhanced storage capabilities. Also, typing responsibilities can be divided among a number of laboratories. If samples (at any stage of the analysis) are sent to other laboratories, the same QA/QC and chain of custody practices need to be maintained and, if feasible, similar sample labeling strategies and software should be implemented to allow efficient sample tracking.

Proper storage of samples should be based on past experience and best practices. To minimize decomposition, if at all possible, all samples should be stored in low temperature freezers (e.g. -20°C). Although freezing is ideal, dried stains can be stored at room temperature in a low humidity environment for a long period of time. Skeletal remains, if necessary, can be stored at room temperature.

Proper storage of reference samples is as important a consideration in the identification process as it is for the remains; these samples are precious and can be limited. Lastly, additional refrigerators/freezers will be needed to be properly maintain samples generated during extraction and analysis.

8. DNA analysis

Laboratories have to accommodate the mass disaster analysis while still maintaining an active casework load. To begin the analysis, sufficient additional sample processing area is needed, which includes adequate bench space and safety hoods. Supplies and additional analytical tools will need to be acquired. The laboratory should seriously consider implementing a robotics system. In addition to increasing throughput, robotics can reduce human error, minimize contamination, and facilitate tracking of samples throughout the analytical process. Robotics systems are widely available and were essential for handling the tremendous number of samples recovered from the WTC site.

Due to the environmental conditions to which mass disaster samples are often exposed, many could be limited in quantity or quality. The quality of the samples obtained from mass disasters will vary substantially, from apparently pristine to highly degraded to substantially contaminated (i.e. co-mingled with tissues from other victims or containing materials that may inhibit portions of the analytical process). Sample contamination and mixtures may be unavoidable. Therefore, DNA typing methods need to be robust. The standard operating procedures used in forensic applications have been designed to accommodate forensic samples and have

been through extensive validation. Protocols for testing short tandem repeat (STR) loci and mitochondrial DNA (mtDNA) are well developed and figure prominently in identification cases [31–37]. However, additional demands may be placed on analytical processes particularly because of the extreme condition of some of the samples. Consideration might focus on the extraction procedures, alternate analytical methods for challenging samples, as mentioned above automation for handling high throughput analyses, and expert system software to facilitate interpretation of results.

The first step in the analytical process is to extract DNA from the reference and disaster samples. It is standard practice in forensic cases to first extract the unknown evidentiary samples prior to the extraction of any known or reference samples. The practice of extracting DNA from human remains first may not always be possible with disaster samples. Regardless, the extraction of DNA from the disaster samples should be separated from the extraction of DNA from the reference samples by time and/or space. The success of DNA typing relies on isolation of DNA of sufficient quantity, quality, and purity. As already mentioned, the sample condition often is out of the control of the scientist. Some samples will yield sufficient high molecular weight DNA without chemical contaminants that might inhibit the analytical process. For others, the environmental insults may be so great that little or no DNA may be available for subsequent typing. Extraction methods that minimize loss of the DNA are the most desired. DNA extraction protocols that overcome, remove, or dilute enzymatic inhibitors, also should be considered. Standard DNA extraction procedures exist for the types of materials that may be encountered and include organic solvents, chaotropics, and ion exchange resins [32,38].

These current extraction techniques are time consuming, require multiple centrifugation steps, may use toxic organic solutions, and/or may not be efficient at removing all PCR inhibitors. A recently developed method that was used for extraction of DNA from WTC samples, is based on a resin that binds DNA so that inhibitors and impurities can be washed away. The DNA IQ™ System (Promega Corp, Madison, WI) uses a paramagnetic resin to capture DNA and has a strong denaturing agent that disrupts many types of cells/tissue in preparation for DNA purification [39,40]. The purified DNA is subsequently eluted from the resin and is ready for analysis without further preparation. Because the system efficiency increases as the sample size decreases, it is particularly suitable for mass disaster samples. The extraction process minimizes loss of DNA, and it is amenable to automation.

Bone and teeth are relatively resilient materials from which to obtain non-degraded DNA, especially compared with soft tissue samples. Procedures for extraction of DNA from bone and teeth are well established [32,38]. However, the process is laborious and time consuming.

This demand is exacerbated in a mass fatality event, because a large number of bone samples often need to be analyzed. If it is decided (by policy) that there will be an attempt made to type every recovered sample, there can be an inordinately large number of bone samples that will need to be extracted for some mass disasters. Thus, there may be a desire to implement a rapid, high throughput procedure so that hundreds to thousands of bone fragments can be analyzed in a relatively short time frame. However, caution should be exercised with such practices. The sanding and grinding of bones generate bone dust which can lead to sample cross contamination. Manual processing of single bone samples reduces the chances for sample cross contamination compared with batch processing. Strict measures are needed to minimize cross contamination, if a batch process is considered. Even though the identification of all possible remains may take a long time, speed should never be selected at the expense of quality.

After DNA extraction, the quantity of recovered DNA should be determined, if possible. Knowing the quantity of DNA facilitates obtaining the best analytical results. While beneficial, particularly for the polymerase chain reaction (PCR), determining the quantity of DNA should not be carried out blindly on all samples. Instead, it should be based on estimates of available biological material. Some recovered samples may be so limited in quantity that consuming any material for DNA quantitation may compromise successful typing. The most sensitive and specific (i.e. human specific) assay that consumes the least amount of sample should be used for DNA quantitation.

The traditional forensic method for human-specific quantitation of genomic DNA is known as slot blot hybridization. In this method, denatured extracted DNA is immobilized on a nylon membrane and subsequently hybridized with the primate-specific aliphoid DNA repeat probe D17Z1 [41–43]. The slot blot procedure can be completed within one working day, enables the simultaneous analysis of a large number of samples, and can detect subnanogram quantities of human DNA.

More recently, a real-time PCR method was developed that can estimate the amount of DNA in a sample. The Quantifiler™ Human DNA Quantification Kit (Applied Biosystems, Foster City, CA) has a dynamic range of 23 pg to 50 ng/5µl and is human (or higher primate) specific [44]. The procedure is semi-automated and sensitivity of the assay exceeds that of the slot blot hybridization method (which at best can detect 100 pg of DNA). Unlike the slot blot procedure, the presence of PCR inhibitors can be detected using the real-time PCR method by monitoring its internal control. This quantitative method is far superior to slot blot hybridization and is currently the method of choice for quantifying mass disaster samples.

After extraction, but prior to developing a DNA profile for comparison, the DNA is first subjected to amplification by the PCR [45]. The PCR is particularly useful for the analysis of materials that may contain degraded DNA. If possible, the components of the PCR should be optimized to overcome the vagaries of environmentally contaminated samples. Therefore, cost-cutting practices such as using reduced reaction volumes, as is done in some databasing laboratories, may not be advisable. A larger reaction volume dilutes inhibitors that impact PCR. Additives, such as bovine serum albumin (BSA), also could be included routinely in the PCR to overcome the effects of some inhibitors that may be present [32,46].

The primary method of analysis will be the technology predominantly used in the forensic laboratory at the time of the mass disaster (i.e. STR typing). Routine standard operating protocols for typing should be attempted first, because they are often robust and the laboratory scientist is experienced with the analytical and genetic marker system(s). Many disaster samples will be typeable by STR analysis using commercially available kits, such as AmpFLSTR® Profiler Plus® ID and COfiler® PCR Amplification Kits, AmpFLSTR® Identifiler® PCR Amplification Kit, the AmpFLSTR® SGM® Plus PCR Amplification Kit (Applied Biosystems, Foster City, CA), and the PowerPlex® 16 System (Promega Corp., Madison, WI). The use of these kits enables typing of 10–17 STR loci (CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, Penta D, Penta E, TH01, TPOX, and vWA) [33–35].

However, there will be situations in which the environmental insults to recovered biological samples are so great that standard STR typing protocols will not yield sufficient results to obtain a reliable identification. Alternate DNA analysis techniques may be needed. One such method involves mtDNA sequencing. The evaluation of DNA profiles from unidentified human remains often relies on typing mtDNA. The copy number of mtDNA molecules in a cell is in the thousands compared with only two copies of each chromosome in the nucleus [47], and the circular nature of the molecule may protect it from degradation. In cases where the amount of extracted DNA is very small or degraded, it is more likely that a DNA typing result can be obtained by typing mtDNA than by typing STR loci. In fact, mtDNA typing is successful for greater than 95% of skeletal samples in missing person cases (unpublished data). Additionally, mtDNA analysis is more flexible than autosomal markers regarding reference samples and kinship analysis, since more distant maternal lineage relatives can serve as genetic references. However, the mode of inheritance and its genetic diversity make it such that mtDNA often does not by itself yield the magnitude of certainty required for a positive identification.

Common mtDNA haplotypes are found in various populations and therefore adventitious mtDNA-based associations occur, which increases as the number of victims to identify increases. However, if the mass disaster is a closed system (i.e. all victims are known, and all victims have a different maternal lineage), it may be plausible to identify all only using mtDNA typing. Another limitation of mtDNA typing is only maternally related individuals can serve as a reference source. Because fathers do not contribute mtDNA to their offspring, mtDNA types of children of male victims (or fathers of victims) provide no relevant mtDNA information.

Efforts in the identification of WTC victims demonstrated that the combination of current mtDNA and STR technologies was not sufficient to provide a level of certainty required for a positive identification of the most degraded samples. There will be situations in which STR typing fails to yield a result and even the more sensitive mtDNA typing does not provide sufficient genetic information (or also fails to yield a result) to allow a DNA-based victim identification. When this occurs, new technologies might be sought. Molecular biology is a dynamic field, and novel analytical tools are continuously being developed. While it would be ideal for the laboratory to use the latest technologies, it would be impractical to readily implement new non-validated technologies during the analysis of mass disaster samples. Some seemingly useful new technologies may not be sufficiently reliable, and the laboratory may not have the resources during a mass disaster to assess the efficacy of the new technologies.

Typically, a forensic laboratory employs standard operating procedures that have been validated as defined by its quality assurance practices. Validation is a process by which a procedure is evaluated to determine its efficacy and reliability for analysis. The QA requirements place limitations on the use of non-validated procedures for criminal investigations. Thus, procedures that have not yet been validated by standard means for forensic testing purposes are often not considered, even though the identification of human remains may be a humanitarian effort. A full validation of a seemingly promising technology could take a longer time than is allotted to identify the victims of a mass disaster. Therefore, using the aforementioned advisory board of experts, the laboratory might consider implementing the procedure (for identification of victims only) by carrying out a 'preliminary validation' [48,49]. In this type of validation, limited test data are acquired to enable an evaluation of the method and a board of experts assesses the utility of the method. If deemed usable, the board makes recommendations defining the limits of interpretation and conclusions that can be drawn.

A limiting factor in attempting to use additional typing technologies or strategies is having sufficient extracted

material to support the new analysis, as well as any QC inquiries that may arise. Obviously, the more compromised the sample is the more difficult it will be to obtain enough material for any or all subsequent novel assays. Yet, for some samples the amount of DNA will not be a limiting factor; it is only that the DNA is too degraded for standard STR analysis.

9. New methods and alternative approaches

Several alternate approaches can increase success of obtaining genetic typing data from highly degraded samples. Two such methods were used in the WTC victim identification process: reducing the size of STR amplicons (known as mini-STRs) [2] and typing of nuclear single nucleotide polymorphisms (nuSNPs) [50]. The major premise for considering each of these methods rested on the interrogation of smaller DNA target regions than is possible with the standard STR kits used by the forensic community.

Currently, STR loci are the most informative genetic markers for identity testing. To improve success in STR typing of degraded DNA, the PCR primers for the STR loci can be repositioned so they reside closer to the repeat region [2,51–54]. Thus, the amplified PCR product (amplicon) will be reduced in length, and if smaller than some of the fragmented DNA template molecules, genetic characterization of the sample may then be possible. The general assay procedure for these modified STR primers is similar to that used for the forensically validated STR systems, so no additional equipment and training are required for implementation. Even so, these procedures still require validation for robustness and for potential primer binding mutation sites. There are a number of documented cases in which one of the PCR primers in a set hybridizes to a genomic site at which there is a common SNP variant in the population (i.e. a primer binding site mutation) [55–58]. DNA from individuals with such an allelic variant type may present no recognizable PCR product (or a null allele) because of destabilization of the primer during hybridization. Null alleles can complicate a kinship analysis and/or reduce the likelihood of the kinship association between the victim and true relatives. Since such allelic variants cannot be predicted a priori, sample population studies are needed which compare allele designations using the new sets of primers along with established primer sets. The availability of reduced amplicon size STR multiplexes was invaluable for analyzing some of the more degraded samples recovered from the WTC (2).

Although several of the adopted forensic core STR loci have been converted to a mini-STR format, additional loci may be needed in order to provide the increased power of resolution that is required in mass disaster

scenarios. A different class of genetic markers, known as nuSNPs, can be typed in much smaller amplicons than those of mini-STRs. nuSNPs are base substitutions, insertions, or deletions that occur at single positions in the nuclear human genome (at about 1 SNP/1000 bp of DNA). Since approximately 85% of human genomic variation is based on nuSNPs [59–62], there is an abundant supply of nuSNPs for identity testing. Most nuSNPs are bi-allelic, making them less informative for identity testing compared with forensically validated STR loci. A larger number of nuSNP loci is required to provide sufficient statistical power for a positive victim identification. Regardless, if the amplicon sizes can be reduced to 60–80 base pairs in length, DNA that was degraded beyond the limits of STR typing may become typeable.

The technology known as SNPstream[®] Ultra High Throughput System (Orchid Cellmark, the forensic unit of Orchid Biosciences, Dallas, TX) was used to analyze nuSNPs from some of the most challenging samples from the WTC. It combines the features of a chip array, the primer extension assay, and universal tags in a multiplex assay (70 total nuSNPs were used) [50]. The microarray consists of a flat bottom microplate in which each well contains a total of 16 individual anti-tag sequences for 12 nuSNPs and four controls. Basically, a SNP extension primer, about 45 bases long, is comprised of a 25-base long segment that is complementary to the area immediately adjacent to the nuSNP site and a 5' 20-base long sequence (i.e. the tag sequence) that is complementary to an anti-tag sequence attached to the bottom of a well. After PCR, a SNP primer extension product is generated, transferred to the microplate, and allowed to hybridize in an array of anti-tags. Immobilized fluorescently labeled allelic products are then detected using a laser-based detection system. The instrumentation required is specialized and is outside of the financial reach of most crime laboratories. Fortunately, these nuSNP loci are amenable to testing using other primer extension methods, such as the SNaPshot[®] kit (Applied Biosystems), which can be conducted on the capillary electrophoresis systems available in most crime laboratories [50].

Using a large battery of nuSNPs requires modifications in the statistical interpretations of DNA profiling results. Ideally, all nuSNPs (and STR loci) on the same chromosome should be located far enough apart (at least 50 cM) to behave in accordance with Mendel's Second Law of Independent Assortment. For a panel of 70 nuSNPs (as can be assayed by the SNPstream[®] Ultra High Throughput System), this requirement cannot be met due to the size of the genome and the number of chromosomes (22 pairs of autosomes). Thus, genetic linkage studies should be performed to examine the independent segregation of the particular loci employed, so that proper statistical weight is used for any identity assignments.

The primer extension assay utilized with the nuSNP technology described has some limitations when applied to mtDNA-based SNP analysis. The regions immediately flanking many mtDNA SNP sites also contain SNPs. The proximity of these surrounding SNPs complicates the design of SNP extension primers. As the amount of genetic information in mtDNA is limited, one cannot simply reject the SNP of interest and seek another site to assay. Sometimes in order to deal effectively with the presence of such variants near the SNP of interest, redundant primers are used [50,63]. This approach complicates assay design and to be effective most of the population variation must be known a priori so all possible redundant primers can be made. However, making every possible redundant primer is not practical. Consider that there are four variable bi-allelic sites residing at the region where the SNP extension primer resides. That would require 16 different redundant primers to be constructed and ideally all should have similar annealing temperatures. Such a design would be very challenging especially if a multiplex assay were to be employed.

SNP assays inherently only assess a small portion of the total variation within the variable regions of the mtDNA. The majority of the mtDNA variation in the population is not at the signature SNPs (that define haplogroups) but are private mutations (which is the major contribution of intra-individual variation). Since SNP assays for mtDNA cannot target private mutations effectively, the power of mtDNA SNP-based assays can never approach the level of discrimination power that is enjoyed by sequencing. However, a SNP assay method is now available that offers a level of resolution approaching that of sequencing, and it is amenable to multiplex analyses, requires less assay design than typical SNP methodologies, is automatable, and does not require extensive laborious manual data interpretation. Hofstadler et al. [64] and Budowle [63] described an automated electrospray ionization mass spectrometry method that can detect a number of clustered SNPs within an amplicon without a priori knowledge of specific SNP positions, and it is a quantitative assay. With mass measurement accuracy of 1–25 parts per million, mass of a fragment can be used to determine the base composition of amplicons less than 140 nucleotides in length. Differences in base composition are then used to differentiate samples. By base composition alone, this assay approaches the discrimination power of DNA sequencing. This mass spectrometry method would be a good choice to consider for mtDNA typing because of its ability to increase throughput and reduce labor with little loss in identity power.

10. Interpretation of DNA profiling results

While a sufficient number of loci are available for individualized identification purposes, there are limitations

to consider. First, if the sample has been degraded or is too small to analyze, only a partial DNA profile might be obtained. Thus, the statistical power to identify the source of that sample will be reduced. Second, the reference samples obtained are critical in effecting identification. Even if a mass disaster sample yields a complete DNA profile, an identification may not be possible if there are insufficient reference samples for kinship analysis to yield a selected level of statistical confidence. For example, it may be relatively unproblematic to identify a missing child when his/her two biological parents and two siblings are typed. In contrast, if the only relative available for comparison is a half-sib, the genetic information will have reduced statistical power and a convincing DNA-based identification would be unlikely. Thus, every effort should be made to obtain as many immediate family members as possible.

Personal effects enable direct comparisons of profiles. Obviously, information about these personal items and multiple personal effects will increase one's confidence for use as reference samples. Moreover, because of the lower confidence in attribution for direct samples, when possible, direct comparison samples should be confirmed by kinship analysis, if possible. Even if the kinship analysis does not meet threshold requirements for identification, it can often increase the overall confidence of a specific identification.

When a DNA profile obtained from a mass disaster specimen matches that of a reference sample(s) or is included within a reference family pedigree, a statistical computation (of the likelihood of such an occurrence) is calculated. Most genetic markers used for identification reside on autosomal chromosomes. If they reside sufficiently far apart on the chromosomes (or reside on different chromosomes), the markers are presumed to segregate independently. Thus, for statistical purposes, the product rule (with modifications) is employed to estimate the frequency of the multi-locus STR profile [26].

Estimating the frequency of a mtDNA profile (or of a Y-chromosome profile) is performed differently than for autosomal DNA markers [65–67]. Since there is little evidence for recombination between sites contained in the mtDNA genome (or the Y chromosome), these profiles are treated as a single locus. For statistical purposes, the number of times a particular sequence is observed in a database(s) conveys the rarity of a mtDNA type among unrelated individuals. A confidence interval is computed to estimate an upper bound of the estimated profile frequency among unrelated persons to correct for sampling error [65–67].

A certain statistical threshold should be established to assign DNA-based identity with high confidence [24,68,69]. Generally, this quantitative assessment is based on the circumstances of the case (e.g. the number of unidentified victims) and the level of confidence (or error margin)

tolerated. The WTC Kinship and Data Analysis Panel (the advisory board for the New York Medical Examiner's Office's DNA Laboratory) set a minimum threshold for assigning identity for direct comparisons of WTC remains samples at a point estimate of the combined match probability of 1 in 10 billion. The justification for selecting this value was that it provides a 99% chance of making no errors in 10,000 identifications. Another way to consider determining such a threshold is to obtain the likelihood ratio or frequency needed in a population of N victims (the estimate of number of fatalities) such that the chance of misidentification is less than some specified probability. If one desired a mismatch probability of less than 1 in 10^6 (a policy based value) in a population of 5000 unidentified victims, a threshold of 1 in 10^{10} (a likelihood ratio of 10^{10}) would be necessary. This threshold [68] is derived as follows:

If $P = 1/10^n$, then the probability of no mismatch is $1 - P$. Therefore, the probability of no mismatch in N victims is $1 - (1 - P)^N$. The inequality for the prescribed mismatch probability is $1 - (1 - 1/10^n)^N < 1/1,000,000$. For $N = 5000$, $n = 9.7$ (~ 10). Therefore, $1/10,000,000,000$ would be set as the minimum threshold (i.e. the likelihood ratio in favor of identity) for a population of 5000 unidentified victims. In closed systems in which the identities of the victims are known (e.g. small plane crash with a reliable passenger/crew manifest), such statistical thresholds may not be required at all. All that would be necessary in such cases would be that the genetic typing unequivocally differentiates all victims (and pedigrees).

For kinship analyses, DNA profiles from relatives are compared with the recovered biological sample profiles (for example, a mother and a father for an alleged missing child). Likelihood ratios are generated to evaluate whether there is sufficient evidence to support a biological relationship. The statistical formulas for simple paternity and kinship analyses are well established [24–29]. The same principle applies above, in that prior odds based on the sample size of victims should be considered so that a minimum posterior odds of 1000 (or a threshold of 99.9% certainty for a kinship identification) is obtained. Probabilities and thresholds should be chosen to maximize victim identifications, while minimizing incorrect associations.

Even with sufficient genetic marker typing and sufficient family members, kinship analysis cannot routinely resolve victims that are siblings of the same sex. Direct reference samples are needed to individually identify full siblings.

Because of the destructive nature of mass disasters, victim remains can be co-mingled. In such situations, a profile of a DNA mixture may be observed. The best practice is to avoid interpreting such profiles and to return to the original sample (if any is remaining) and perform a re-extraction and re-analysis of the DNA. However, appropriate statistical DNA mixture analyses can be carried out

much in the same manner as in criminal case analyses [27, 28,70]. The statistical reporting thresholds above also could apply for mixture analyses.

11. Software

As discussed above, for some mass disasters, such as the WTC and identifying war remains, victim identifications can be very complex. Large numbers of samples can be collected, reviewed and analyzed, and a wide variety of analyses can be carried out. Typically, a number of laboratories will be involved, and the process is time consuming. While data generation is emphasized data handling and management are not often given adequate attention. Managing and handling the large amount of data generated in a mass disaster while maintaining the quality throughout, present substantial challenges.

Software tools for storing, tracking, comparing, annotating and curating data had never been considered a priority in North America, because there was no previous case of the scale and scope of the WTC. Most previous mass disasters had been relatively small scale and simple spreadsheet approaches were sufficient to organize and track data. Some programs already exist for statistical analyses [11,71]. However, even small-scale disasters require careful attention to data management and even then errors can occur. Data handling systems should be used which integrate custom software and provide a middleware system, so that a number of interoperable functionalities can be performed. The software should be able to: organize, store, and retrieve diverse and different data; integrate different software systems; allow technical and administrative review of data; allow for annotation and recording of problems and resolutions; report various metrics; track samples among partner laboratories; prioritize sample selection and review; generate family pedigrees and calculate likelihood ratios for hypothesized kinships; combine remains with the same profile to facilitate searching; enable profile comparisons and statistical calculations; allow for operators to interact with the interpretation and evaluation of ambiguities; and be user friendly. Fortunately such software was developed with these requirements in mind and in response to the demands of the WTC (Mass-Fatality Identification System (or M-FISys), GeneCodes Corp., Ann Arbor, MI) [72]. Without such software, the success of the WTC identification process would not have been possible.

Another software system that can assist in identification of human remains on a national level is the FBI's combined DNA index system (or CODIS) [31]. Two of the four files in CODIS pertinent to identification of human remains are the missing persons and unidentified human remains index and the reference samples from

personal items and family index. DNA profiles can be searched and compared between these files to either identify the remains or develop investigative leads. Samples taken from family members are used only for searching missing persons and not any criminal indices. The use of mtDNA profiles as an effective screening system is facilitated by the introduction of the CODISmp system. Although designed for missing persons, the same system could be used for searching of DNA profiles from mass disaster cases. The following missing person case example demonstrates the identification process, how DNA can be used to effect an identification, and how traditional identification tools are important to the identification process.

Donna Williamson was last seen alive in August of 1982. Her car was found in a Fort Worth, Texas motel parking lot and no investigative leads were developed. In August 1993, the Johnson County Sheriff's Department found a set of skeletal remains in heavy brush near a highway. For 10 years these unidentified remains were maintained at the Tarrant County Medical Examiner's Office (TCME). With no leads and no distinguishing characteristics, the human remains were kept in storage for the next 10 years. In the fall of 2003, after learning of the existence of the Texas Missing Persons DNA Database (TMPDD) at the University of North Texas Health Science Center (UNTHSC), Detective Enriqueta Garcia of the North Richland Hills, Texas Police Department located the mother of Donna Williamson (Linda Williamson) in Florida. Detective Garcia arranged to have a DNA sample collected from Donna's mother. Her sample was submitted to the TMPDD. Her DNA was typed both for mtDNA and the 13 core STR loci and uploaded into CODISmp in February of 2004. The belief was that, if Donna Williamson was dead, one day her remains would be typed and entered into the TMPDD. The Johnson County Detectives then learned independently about the existence of the TMPDD. They contacted the TCME and arranged to have a bone sample from the 1993 unidentified remains sent to the UNTHSC. The sample was typed for mtDNA, and in May of 2004, during an upload and routine search of the database, a cold hit was made. This hit was between the mtDNA haplotype from the bone sample from Johnson County and that of the reference sample submitted by Linda Williamson. The particular haplotype had not been observed previously in the FBI's database of 5071 mtDNA sequences. The bone sample only yielded a partial STR profile (five of 13 loci). But, Linda Williamson could not be excluded as the biological mother of the individual whose remains were found in Johnson County in 1993. However, the probability of maternity based upon the five STR loci was only 95.5%. The TCME was notified of the 'match' and based on the lead located dental records from Donna Williamson. The dental records matched the dentition of the remains. Based upon both the dental

records and the DNA analysis, in June of 2004, the TCME positively identified the remains from Johnson County as those of Donna Williamson. The case was then ruled a homicide by the TCME. The Donna Williamson case was the first unidentified remains cold hit recognized through the use of the FBI's new CODISmp software. This case shows how a successful partnership between law enforcement, a medical examiners office and a forensic DNA Laboratory using a database and an effective searching software program can help effect an identification in a cold missing person case. The same general practices would apply to a mass disaster, just on a larger scale.

12. Conclusion

DNA analysis is integral to the accurate identification of human remains from mass disasters. Forensic DNA typing allows the identification of any biological sample and the association of body parts, as long as sufficient DNA can be recovered from the sample(s). This is true even when the conditions are such that the victims' remains are fragmented and the DNA degraded. While many efficacious laboratory protocols are available for DNA analysis, the analytical portion is only a small part of the identification process. There are a number of salient features in the process to consider for best practice and to minimize errors. These include: (1) sample collection, preservation, shipping and storage; (2) tracking and chain of custody issues; (3) laboratory facilities; (4) quality assurance and quality control practices; (5) parsing out work; (6) extraction and typing; (7) interpretation of results; (8) automation; (9) software for tracking and managing data; (10) the use of an advisory panel; (11) education and communication; and (12) privacy issues. Developing strategies a priori that address these features of DNA identification will facilitate the process. Yet, human nature is such that it is unlikely an action plan would be implemented prior to a mass disaster. Since many of the features described herein for DNA typing of human remains from a mass disaster are the same as those for missing persons' cases, it may be possible to invest in and coordinate with missing persons identification efforts. Thus, the infrastructure for a mass disaster identification process could be in place and only surge capacity would need to be addressed. Also, to enhance capabilities and performance, a retrospective review should be carried out on past human remains identification cases where DNA typing has been applied. With such efforts, the highest success possible can be attained for identifying victims from mass disasters so they may be returned promptly to their families.

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This is publication number 05–05 of the Laboratory Division of the Federal Bureau of Investigation. Names of commercial manufacturers are provided for identification only, and inclusion does not imply endorsement by the Federal Bureau of Investigation.

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Supplementary data

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