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The use of forensic DNA analysis in humanitarian forensic action: The development of a set of international standards

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ABSTRACT

DNA analysis was first applied to the identification of victims of armed conflicts and other situations of violence (ACOSV) in the mid-1990s, starting in South America and the Balkans. Argentina was the first country to establish a genetic database specifically developed to identify disappeared children. Following on from these programs the early 2000s marked major programs, using a largely DNA-led approach, identifying missing persons in the Balkans and following the attack on the World Trade Center in New York. These two identification programs significantly expanded the magnitude of events to which DNA analysis was used to help provide the identity of missing persons.

Guidelines developed by Interpol (2014) [1] related to best practice for identification of human remains following DVI type scenarios have been widely disseminated around the forensic community; in numerous cases these guidelines have been adopted or incorporated into national guidelines/standards/practice. However, given the complexity of many humanitarian contexts in which forensic science is employed there is a lack of internationally accepted guidelines, related to these contexts, for authorities to reference. In response the Argentine government's Human Rights Division in the Ministry of Foreign Affairs and Worship (MREC) proposed that the United Nations (UN) should promote best practice in the use of forensic genetics in humanitarian forensic action: this was adopted by the UN in Resolutions A/HRC/RES/10/26 and A/HRC/RES/15/5. Following on from the adoption of the resolutions MREC has coordinated, with the support of the International Committee of the Red Cross (ICRC), the drafting of a set of guidelines (MREC, ICRC, 2014) [2], with input from national and international agencies. To date the guidelines have been presented to South America's MERCOSUR and the UN and have been disseminated to interested parties.

1. Introduction

The dignified management of human remains is a moral obligation in all contexts; the ultimate aim is the correct identification of the individuals so that they can be returned to their families, enabling religious rites to be administered and any legal aspects relating to the deceased finalized. In the absence of this closure the psychosocial impact of missing persons can be severe, and in contexts following on from armed conflicts and other situations of violence (ACOSV) can present a barrier to peace-building efforts [3]. In reality, in most cases where persons are missing following on from ACOSV they have been killed and only through the identification of human remains can the families know their fate. There are however some situations, in particular involving the unlawful separation and disappearance of children from parents, where forensic genetics can play a role in identifying living persons and helping to restore family links [4–6].

While in domestic contexts identification of the deceased individuals is often straightforward and, in many cases, requires limited input from forensic practitioners the situation is much more challenging in many situations where deaths have occurred as a result of ACOSV. Myriad complications arise from aspects such as fragmentation of bodies, deposition of bodies in clandestine/mass graves with the potential for relocation of bodies from the original gravesite (resulting in increased fragmentation), large numbers of deceased persons, limited contextual information, time between death and recovery/identification and limited ante-mortem data. The increased complexity typically makes it much more difficult to formulate a realistic hypothesis of identity for a given set of human remains and necessitates a greater input from forensic practitioners to enable robust identifications. Further complications arise where cross-border cooperation is required between parties formally or currently engaged in conflict.

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Forensic practitioners, including anthropologists, odontologists, pathologists, fingerprint experts and geneticist, have a number of tools at their disposal to assist with identification of human remains [1]; in the early 1990s DNA analysis was added to the arsenal of methods that could be deployed. DNA profiling was first carried out in 1984 [7–9], but not successfully applied to human remains identification until 1991 [10,11]. Ongoing developments in the methods used by forensic geneticists has enabled DNA profiling to be applied to increasingly challenging cases involving multiple casualties ranging from, for example, air crashes [12] to contexts with thousands of missing/unidentified persons [13–16].

This paper reviews the role of forensic genetics in the identification process, highlighting technical areas where the practice of forensic genetics differs from that employed in crime scene investigation, and the background to the new set of guidelines produced by the Argentine government, with input from the international community.

2. The identification process

Incorporating DNA profiling into human remains identification, whether for a single person or in large-scale cases, is part of a multi-step process (Fig. 1). Location and collection of the remains is evidently a key component, but one that can be very complex in ACOSV in terms of identifying the location of bodies and accessing sites. Bodies will be in varying conditions depending on the cause of death and any subsequent post-mortem trauma the bodies' experience. When the remains are skeletal the recovery is more complex and requires a greater level of specialist skill to maximize recovery of skeletal elements and also minimize commingling when relevant [17]. Once the remains have been recovered, and when necessary re-associated, collection of post-mortem data can begin. Any information that can contribute to identification should, in ideal circumstances, be collected. The collection of post-mortem data should be mirrored by the collection of ante-mortem data; again the type of ante-mortem data available/collected will be context and case specific [17].

A key step in the identification process is to generate a hypothesis of identity for each victim; this can be through artefacts, such as documents or identification tags, eye-witness testimony, or comparison of ante- and post-mortem data. Once a hypothesis of identify has been established for a victim then the hypothesis should be tested using all available data. There is a need to examine the weight-of-evidence, ideally using a mechanism such as an Identification Committee [1] that can evaluate all the information in a specific context and through this measure maximise the potential for producing reliable identifications.

3. Role of forensic genetics

The reality in many cases, especially when large numbers of individuals are involved, is that DNA will contribute to robust identifications; fingerprint evidence and odontology can be useful in some cases to contribute to highly reliable identifications [18–21], but in many instances of ACOSV fingerprints are not available through decomposition and limited ante-mortem dental records are available.

The analysis used for identification of human remains has many commonalities with the methodology employed for analysis of crime scene evidence and kinship testing. However, some aspects of the analytic process are more specific to human remains identification, and are summarized below.

3.1. Sample selection and storage

Once human remains are recovered sampling for DNA analysis is necessary. When the body is not decomposed, muscle tissue is relatively easy to take, with deep red muscle preferable [1]. If the remains show a high degree of degradation DNA can still, in many cases, be recovered from muscle tissue [22], but this is dependent to a large degree on the ambient temperatures post-mortem [23]; fingernails, ligaments and tendons can be used in some cases where the muscle tissue is too decayed [24]. In some circumstances, for cultural or logistical reasons taking soft tissue samples may not be practicable, and in such circumstances fingernails have been used successfully [25,26]. Once sampled the biological material has to be stored unless DNA extraction commences immediately. For short-term storage refrigeration will help to preserve soft tissues; however, for longer-term storage freezing is necessary; preservation using buffers or alcohol is an alternative solution when access to stable low temperature is not possible [27,28].

Skeletal elements act as a harbor for DNA, greatly reducing the rate of degradation in comparison to soft tissue; this is in part due to the physical barrier against bacteria and fungi that the hard tissues afford. In addition, the chemical composition of bones and teeth, which contain high levels of hydroxyapatite/apatite offers some protection from enzymatic degradation [29]. Not all skeletal elements are equally effective at preserving DNA: data are available from a large number of cases that provide a hierarchy of preference when choosing which element(s) to use for DNA

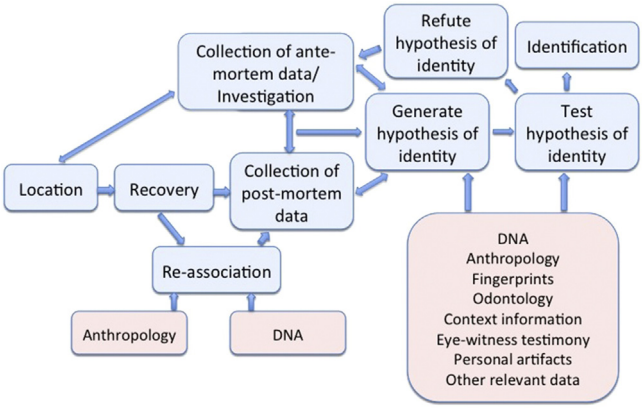

Fig. 1. Schematic representation of the different stages in the identification process. A hypothesis of identification can be generated from a wide variety of sources and then tested using all the available data.

analysis [17,30–34]. While DNA in skeletal elements is more stable if bone is 'wet' then it requires special storage conditions after sampling to prevent further degradation of DNA, typically low temperature storage; DNA in 'dry' bone is less prone to rapid degradation, but will potentially continue to degrade over time.

3.2. Extraction and quantification of DNA

Extraction of DNA from muscle tissue and other soft tissue, such as ligaments is relatively straightforward and a plethora of methods can be applied. Nails similarly can be extracted using a range of methods that incorporate dithiothreitol (DTT) to facilitate the breakdown of nail material [25,26].

Hard tissues present a greater technical challenge, as it requires mechanical disruption prior to DNA extraction. However, the hard nature of skeletal elements does offer an advantage over soft tissues in that the surfaces can be cleaned using both chemical (typically bleach) [35] and physical methods, i.e. the mechanical removal of the surface of the bone. Both the chemical and physical cleaning removes exogenous DNA that may have contaminated the bone, thereby providing an increased level of confidence that the DNA profile produced is from the bone, and not contamination [36,37]. A commonly employed final step in cleaning is exposure of the skeletal elements to high levels of UV light, which cross-links residual exogenous DNA. Following cleaning, several approaches are available for physically disruption of bone/tooth material; the most commonly employed method is to grind the bone/tooth under liquid nitrogen using an impact grinder [38]. Drilling of the sample is an alternative method, requiring less equipment, and is preferred by some laboratories, although care has to be taken not to generate excessive heat when drilling, especially when processing teeth.

The methods used for DNA extraction from bone are varied. If the post-mortem interval has been short most methods will yield a high amount of DNA. However, if the bone is aged or has been subjected to extreme environmental conditions then low yields of DNA are expected and more care has to be taken with the selection of the extraction method. Demineralization of powdered samples is a standard step when handling poor quality samples; powdered hard tissue is incubated with EDTA, typically overnight, which helps to breakdown the calcium phosphate, a major component of the mineral portion of the bone/tooth, leaving decalcified material for subsequent extraction. Alternately the powdered material can be subject to total demineralization with the addition of sodium lauryl sulphate and proteinase K to the EDTA, which leads to complete breakdown of the hard material and increases the yield of DNA [16,36,39,40]. After demineralization various methods can be employed: for many years the organic phenol:chloroform extraction was preferred by many laboratories [36,41], but this has been largely replaced by DNA capture methods [42,43]. In addition to recovering the maximum amount of DNA from samples, especially those that are likely to have low amounts of DNA, the extraction also has to remove chemical that may have entered the bone post-mortem. In contexts where the skeletal elements have been recovered from soils, especially if the soil is rich in organic materials, chemicals can enter the hard tissues and can be co-extracted with the DNA. If the extraction method does not remove these chemicals the DNA profiling may be inhibited [44].

Quantification of extracted DNA is carried out using real-time PCR. Several commercial kits are available [45]. The real-time PCR quantitates human DNA, differentiating the non-human component in the DNA extract. When processing decomposed soft tissues and hard tissues a significant proportion of the extracted DNA can be from bacteria and fungi, making human-specific quantitation highly desirable.

3.3. Collection of reference samples

Obtaining a DNA profile from human remains in itself is of limited use — as with all forms of identification ante-mortem data is required for comparison. Comparison with the missing persons' profile, i.e. a direct reference, will provide the strongest evidence for identification when a match is found. These can be available in some circumstances, for example, US military personnel provide a blood sample that is stored as a reference, should that individual go missing in action and need to be identified. Other examples include entries in to a national DNA databases, where legislation allows these profiles to be accessed, or results from prior DNA testing, such as paternity tests, which could be provided by the missing person's family. Biopsy and other archived medical samples can in some cases be recovered and profiled, when they can be located and permission for their use obtained. Personal possessions, such as hairbrushes, toothbrushes and razors, are alternative sources of ante-mortem DNA; however, reliably establishing the identity of the personal possessions can be challenging [46].

Following conflicts accessing ante-mortem DNA profiles/biological samples or obtaining personal belongings, in most circumstances, is unrealistic for various reasons, including displacement of people, destruction of property and a general deterioration of infrastructure. Therefore, ante-mortem DNA data, in most cases, can only be obtained through relatives donating biological samples to the authorities carrying out the identification. Parents and children (even more so when the spouse is also available) are typically the most valuable relatives followed by siblings [47,48]; as the relatives become more distant the value of ante-mortem DNA data diminishes.

The means of collection are variable; however, most large-scale collections have utilized card-based systems where blood or saliva/buccal scrapes are deposited onto a card that preserves DNA [49,50]. Once properly dried, the biological material on the card is stable at room temperature for years; DNA on the cards can be profiled directly or extracted, quantified and then profiled [51].

3.4. PCR-based DNA profiling

Since the early 2000s two commercial short tandem repeat (STR) kits that incorporated the Combined DNA Index System (CODIS) loci [52] have been widely used: PowerPlex 16 (Promega Corporation, USA) [53] and Identfiler (Applied Biosystems, USA) [54]. Several kits have since become available, for example, the GlobalFiler (Applied Biosystems), PowerPlex Fusion System (Promega Corporation), and Investigator (Qiagen); these all enable 20 plus autosomal STR loci to be analyzed in a single reaction. The choice of loci used in crime scene casework is often determined by national requirements/legislation; however, there is typically more flexibility with the loci used for kinship analysis and more loci can be analyzed as required.

Post-PCR processes are essentially the same as used for crime scene analysis; using capillary electrophoreses to detect the amplified DNA. The power of DNA profiling is that each profile can be broken down into a series of numbers, which describe the structure of STRs in each individual. The numeric DNA profile will be the same regardless of which laboratory has undertaken the work, allowing centralization of profiles in databases. Other forms of DNA analysis can be used, usually to supplement the autosomal STR typing, including Y chromosome and mitochondrial DNA (mtDNA) analysis — neither of these can individualize because multiple individuals in any given population will have the same Y chromosome/mtDNA [55]; however, both can either refute matches or increase the significance of a match [56]. New DNA profiling systems have become available that are based on next-generation sequencing, and allow large amounts of loci to be

characterized for each sample, and are potentially very highly discriminating; however, their use is currently limited and will not be considered in detail here.

4. The identification process: incorporating DNA data

The complexity of identification ranges from one to thousands of individuals, potentially with a high degree of fragmentation. While the technical process employed typically changes little, the framework in which identifications take place can vary, and so can the manner in which DNA is used in the identification process.

The most widely used approach, sometimes referred to as the classical approach, is to use DNA to support or refute a hypothesis of identity generated through different lines of evidence, such as visual recognition, eye-witness reports, and any other relevant data (Fig. 1). At the other end of the spectrum is the DNA-led approach where a database of ante-mortem data (typically from relatives) is compared to a database of post-mortem data generated from the human remains. Cross-checking the two sets of data, when relatives have been used to generate ante-mortem data, generates potential matches that have to be examined in more detail using specific pedigree information, and at this point can provide a numerical qualification to express the strength of a given match. When conditions allow, further checks should be carried out, for example, ensuring that the biological profiles of the human remains are sufficiently similar to those of the identified person; this necessitates collection of ante-mortem data for the missing persons, such as stature, age, pathologies etc. — typically from relatives of the missing persons through interview. This process, although laborious, reduces the potential for misidentifications [17]. The process of data consolidation varies from context to context and is dependent on the type and quality of data available as well as the legal structures in place, and guidelines followed in any given context. Interpol guidelines recommend that when data reconciliation results in strong evidence for the identity of a particular individual, the case should be presented to an identification board (however named), whose role is to evaluate the evidence and if appropriate make the legal identification [1].

4.1. Assessing the strength of DNA evidence

In crime scene work DNA is routinely presented in many criminal jurisdictions to link a person to a crime scene. The strength of a given match is presented as a match probability, or more preferably a likelihood ratio. When full profiles match at 15 or more loci then the match likelihoods are in the order of billions of times more likely (e.g. full GlobalFiler or PowerPlex Fusion profiles provides likelihood ratios in the sextillions), providing extremely strong support [57] for the identity of the biological material. Just as with crime scene investigation, when applied to the identification of human remains, direct matches between ante-mortem and post-mortem DNA profiles provide extremely strong support for the identity of the human remains. The process of matching and calculating match probabilities is well established and within the capacity of routine forensic genetics laboratories.

However, in many cases involving human identification direct comparisons are not possible and instead the post-mortem DNA profiles generated have to be compared with ante-mortem DNA profiles from relatives. While most laboratories have the capacity to carry out relatively simple kinship testing, for example, paternity testing, the evaluation of complex kinship cases is more challenging [58]. Specialist computer software is often required to undertake comparisons of ante- and post-mortem data and perform complex kinship calculations. Both freeware and commercial software is available for his type of analysis that can compare large datasets of ante- and post-mortem DNA profiles and compute likelihood rations for any given pedigree [59–62]

4.2. Prior and posterior probabilities

In routine kinship testing incorporating prior probabilities with likelihood ratios to give a posterior probability [63] is widely practiced. In paternity testing, a value of 0.5 (i.e. 50%) is used for the prior probability; this is a figure of convenience as the scientists that carry out the analysis and produce the report typically have no knowledge of the case, other than the DNA evidence. The 50% probability of paternity based on non-genetic evidence can be altered if case specific information is considered (usually this is the role of the court). When identifying human remains following events that involve more than one individual then the prior probability has to be adjusted accordingly. In closed events, such as plane crashes, this is relatively straightforward; in its most simple form if 100 people died in an event then the prior probability for each individual would be 1/100 or 0.01. Further sub-categorization of the victims may be possible, for example, if there is a mixture of men and women then this can be taken into consideration, so taking the above example if the split of male and females was 50:50 then the prior probability for each female and each male would be 1/50 or 0.02. Depending on the availability of post-mortem data the sub-categories and therefore the prior probabilities can be further sub-divided based on features such as age, stature, and ancestry.

In open events, such as conflicts and deaths during attempted migration, obtaining accurate and detailed information can be challenging, making the estimation of prior probabilities problematic. However, gathering as much data as possible can help to refine the estimates of prior probability — for example, if eye-witness testimony provides information on the possible identity of persons in a clandestine/mass grave then the prior probability can reasonably be altered and not necessarily encompass all persons that are missing as a result of a particular conflict/event; failure to take the available information into consideration will lead to over or understatement of likelihood ratios [58].

Reducing the prior probability naturally has an impact on the posterior probability — the only way to increase the posterior probability is to increase the likelihood ratio, which can be done either by increasing the number of relatives tested, which is dependent on which relatives have already been tested and the availability of additional family members, or alternately increasing the number of loci used in testing.

5. Guidelines for undertaking the Identification of human remains and the use of DNA profiling

When forensic science and medicine are employed to provide data that will influence the outcome of legal processes, whether they are civil or criminal, there is an expectation that robust practices will be employed to ensure the quality of the data that are generated and that the data are used appropriately to inform decisions. Guidelines exist covering the technical and procedural aspects of forensic DNA profiling, some of which are international in scope whilst other are intended for domestic use, although they may have relevance in a broader context.

5.1. International Organization for Standardization (ISO) standards

The ISO is a non-governmental body that develops standards covering a wide range of industrial sectors. Two standards have a high relevance to the identification of human remains: ISO/IEC 17020:2012 ‘Conformity assessment — Requirements for the

operation of various types of bodies performing inspection' and ISO/IEC 17025:2005 'General requirements for the competence of testing and calibration laboratories'. ISO/IEC 17020:2012 is used in some jurisdictions in relation to the recovery of evidence, usually in terms of crime scene investigation, but also has applicability to the recovery of human remains. ISO/IEC 17025:2005 is widely used as a standard for forensic genetics. It is a technical standard than ensures several good practices relevant to testing and calibration laboratories and has relevance to many areas of forensic science, including genetics.

As the titles of the standards imply they are not written specifically for forensic science, but have a broad target audience. In order to bridge some of the gaps in the standard for effective application to some aspects of forensic work a supplementary publication, ILAC G19:08/2014 Modules in a Forensic Science Process, by the International Laboratory Accreditation Cooperation Organization (ILAC), has been produced for use in conjunction with the ISO standards. While the ISO and ILAC standards deal with important aspects of forensic genetics they do not provide any guidance on the methodology or procedures that are most appropriate for either routine forensic work or complex cases involving the identification of human remains.

5.2. Disaster Victim Identification (DVI) guidelines

The International Police Organization (Interpol) is the lead international organization for DVI and has produced guidelines [1] that have been widely disseminated and incorporated into national practice in multiple countries. Elements of Interpol's DVI guidelines have direct relevance to the identification of human remains following ACOSV.

Following the experience of the identification program following the World Trade Center attack the National Institute of Justice (USA) published 'Lessons Learned from 9/11' [64]. The American Association of Blood Banks (AABB) has also published guidelines on using DNA analysis for mass fatality operations [65] and the Scientific Working Group on DNA Analysis Methods (USA) (SWGDAM) has produced guidelines for missing persons casework [66].

The International Society for Forensic Genetics also published a set of guidelines for the use of DNA in DVI [48], drawing on extensive experience for the forensic community involved large-scale programs that incorporated a genetic component, such as the World Trade Center identifications and the work of the International Committee for Missing Persons (ICMP) in the Balkans.

In response to challenges faced in the field, the International Committee of the Red Cross (ICRC) produced a guide to best practice for using DNA for identifications in ACOSV [67] that provides practical advice on sample collection and an overview of the potential for incorporating DNA into identification programs. In addition several publications deal with the experiences of specific identification programs that in many cases have a wider relevance.

5.3. A good practice guide for the use of forensic genetics applied to human rights and international humanitarian law investigations

Despite the wealth of guidance and case studies that have been published there is a lack of guidance that draws on the extensive experience of organizations that have been involved in identification programs related to human rights and international humanitarian law investigations. This was articulated in Resolution A/HRC/RES/10/26, adopted by the UN Human rights Council on 27th March 2009: Operative paragraph 6 "Requests the Office of the UN High Commissioner for Human Rights to request information from States, intergovernmental and non-governmental organizations on best practices in the use of forensic genetics for identifying victims of serious violations of human rights and international humanitarian law with a view to considering the possibility of drafting a manual that may serve as a guide for the application of forensic genetics, including, where appropriate, the voluntary creation and operation of genetic banks, with appropriate safeguards."

In response, Argentina's Ministry for Foreign Affairs and Worship (MREC), with the support of the ICRC coordinated experts, both legal and forensic, within Argentina (including the Argentine Forensic Anthropology Team (EAAF) and the Grandmothers of Plaza Mayo), Latin America, Spain and Portugal, and then worldwide have produced guidelines that aim to support States and Agencies that are tasked with undertaking such sensitive and complex investigations [68].

The resulting guidelines [2] comprise four sections: an overview of the forensic and ethical issues faced when undertaking this type of investigation; legal aspects of human rights (HR)/international humanitarian law (IHL) violations with reference to the identification of victims; the use of personal genetic data when identifying victims of HR/IHL abuses; and technical aspects of incorporating forensic genetics into an identification program. The guidelines consolidate the experiences of several agencies working with situations that involve abuses of human rights law and international humanitarian law, largely in South and Central America. The guidelines provide legal and ethical, as well as technical, guidelines, that it is envisaged will be a valuable reference for forensic investigations framed under international human rights and international humanitarian law provisions. The guidelines have been promoted to national agencies, regional intergovernmental organizations such as MERCOSUR in South and Central America and the 2015 to the United Nations 28th Session of the Human Rights Council.

6. Discussion and conclusions

The investigation of cases of ACOSV are nearly always complicated by a myriad of factors such as the time elapsed between the events occurring and the investigations being undertaken, the systematic destruction of evidence (including the victims), typically large numbers of victims, and the necessity for cooperation across international borders following international conflicts between parties or in some cases between parties currently in a state of conflict.

Forensic genetics, in combination with other forensic lines of evidence that can collectively contribute to robust identifications and allow the remains to be returned to the families of the missing. Several guidelines and standards are published that draw on previous experiences to improve both the processes employed to establish identities of victims and also technical aspects of the data collection and analysis.

In most cases of ACOSV, especially when large numbers of victims are involved, DNA data will be needed in order to make a robust identification. Sometimes this is possible, indeed necessary, using DNA in isolation. However, when other lines of evidence are available to either generate hypotheses of identity for victims or to test the hypotheses of identity generated by DNA analyses through a DNA-led approach then the potential for misidentifications is reduced. The importance of this cannot be underestimated; a small number of misidentifications can cause the families of the missing to lose confidence in the validity of the identification process and so increase their torment [69].

The guidelines developed through the Argentine government, who since the early 1980s pioneered the application of forensic methods, including forensic genetics, to investigations of HR/IHL violations and the search for the missing summarize the

experiences of over three decades from several agencies. The search for the missing being initiated in an increasing number of contexts and continues in others. DNA analysis continues to evolve, becoming more sensitive and powerful as more markers can be analyzed, which in turn widens the scope of cases where forensic genetics can be applied and contribute to robust identification. By drawing on the experiences from a broad range of contexts, the States, agencies and parties involved in the identification of victims of ACOSV can be more effective in their identification programs and ultimately reduce the suffering of the families of the missing.

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