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ORIGINAL PAPER

Femur, rib, and tooth sample collection for DNA analysis in disaster victim identification (DVI)

A method to minimize contamination risk

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Abstract Although much literature is available on DNA extraction from tissue samples to obtain the best possible genotyping results, to the best of our knowledge no written recommendations exist on how to excise or extract bone and tooth samples from a victim to facilitate this. Because the possibility of cross-contamination is high, especially when excising numerous samples under disaster conditions, it is important to minimize this risk and to keep samples in optimum condition. In this paper a standard operating procedure is proposed for collection of femur, rib, and tooth samples to aid victim identification both after mass disasters and in (single) forensic investigations.

Keywords Human identification · DVI · Mass disaster · Forensic · Sample collection · DNA · Contamination

Introduction

DNA identification is a tool used in a growing number of mass disasters [1–3] and forensic investigations [4–6]. When the identity of a person cannot be established with traditional identification methods, for example facial recognition, dactyloscopy, or odontology, DNA analysis may offer a solution. This is of particular interest for people who are unrecognizable, for example as a result of fire, natural decomposition, or deliberate mutilation. The two most important requirements for DNA-based identification are collection of representative, high quality tissue samples from the victim and the availability of reference samples, either from the suspected victim or from family members, with which to compare the tissue samples [7–9]. Many publications and protocols have been presented on methodology for isolating DNA from tissue samples for genotyping purposes in the laboratory [1, 5, 10, 11]. In contrast, limited information is available about the collection of bone and tooth samples, and advice on the prevention of contamination for these samples is often contradictory [1, 3, 5, 9, 11]. Because (cross-)contamination is one of the largest pitfalls during sample collection, appropriate measures should be taken to prevent this.

The South East Asian tsunami of December 2004 was an excellent example of a mass disaster incontrovertibly showing the importance of minimizing contamination risk during tissue sample collection for DNA analysis. Forensic investigators from 31 different countries arrived in Thailand to help with disaster victim identification (DVI). The DVI teams used many different protocols. To standardize protocols and procedures, on January the 20th, the Thai tsunami victim identification (TTVI) committee was initiated. This comprised many of the scientists that were present at the scene. Most protocols were based on the Interpol Disaster Victim Identification Guide [12]. This guidance did not provide a protocol for tissue-sample collection for DNA research, however. As a result the Dutch team created guidelines for the collection of bone and tooth samples based on obvious common sense and existing theoretical knowledge. These guidelines were

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approved and recommended by the TTVI. In this paper we describe a standard operating procedure (SOP) for bone and tooth sample collection based on our experience during the aftermath of the tsunami and the guidelines we developed then. This SOP can be used for human identification both during mass disasters and in forensic investigations.

Working conditions and methods

A temporary morgue was established on the premises of a Buddhist temple in Wat Yan Yao on the peninsula Khao Lak. The tsunami victims were transported to the morgue in trucks in which the bodies were lying on top of each other, which obviously resulted in body-surface cross-contamination. Initially, the bodies were cooled with dry ice, but later all the deceased were placed in body bags, which were tagged with a tracking number and stored in containers that were cooled below 0°C. The Dutch DVI team advised placing the bodies on wooden scaffolding (in the containers) to facilitate the cooling and to prevent further putrefaction as a result of the heat of decomposition.

To identify the human remains, which were often highly putrefied and partially skeletonized, multiple methods were combined. Fingerprints and palmprints were taken and external body details, for example clothing, personal belongings, scars, and marks (e.g. tattoos) were photographed, described, and recorded [5, 13]. Autopsy was performed to expose the nature of any previous surgery. Odontology consisted of one or two periapical radiographs to assist determination of the age of children and two bitewings for adults. After exarticulation of the jaw, Polaroid images were taken of the maxillary and mandibular occlusal tables and the anterior edge-to-edge view of the incisors [14]. Also, bone and tooth samples were collected for DNA testing (as described below). All postmortem (PM) data were written on (pink Interpol) DVI forms and, after completion, entered into a database (Plass Data Software A/S, Denmark, 2003).

Contamination

Under chaotic and often hectic conditions, large numbers of samples from many different individuals had to be collected, nonetheless, accurately and consistently. Because it was important that samples for DNA analysis were free from contamination, great care was taken to prevent exogenous contamination by examiners or microbes and, in particular, cross-contamination with DNA from other victims. The SOP was designed to minimize (cross-)contamination during the collection of the tissue samples for DNA-based identification.

Standard operating procedure for tissue sample collection for DNA analysis

Preconditions at all times

- The site of sample collection should be clean and separate from other sites of interference, for example autopsy, dental examination, etc.
- Personal protective equipment, for example an overall or a long sleeved coat, an extra plastic apron, a hair net, and a mouth or gas mask, should be worn, both for protection of the examiner and to prevent shedding of contaminants, for example hair and saliva, from the examiner on to the samples.
- Double (surgical) gloves should be worn, so that the exterior gloves can be removed instantly if clean, dry hands are needed.
- DNA remover preparation comprises an aqueous solution of 1 mL L^{-1} liquid soap and at least 5% bleach.
- If an instrument or hand inadvertently touches an unclean area (including cleaned skin) the procedure should be stopped and the instrument again cleaned meticulously with DNA remover before proceeding further.
- If an excised tissue sample may have touched an unclean area (including cleaned skin) a new clean sample must be taken.
- The SOP should be executed with care, as if in the operating theater.

Femur

I. *Preparations for femur sample excision*

1. Prepare a large bucket and a rectangular tub containing DNA remover.
2. Fill a small container with absolute alcohol.
3. Place a fresh disposable cleaning cloth in the large bucket (for body surface cleaning) and a fresh disposable cleaning cloth and a brush in the tub (for instrument cleaning and storage).
4. Wipe the surface of the instrument table with the disposable cleaning cloth from the large bucket and then discard the cloth.
5. Clean all instruments (scalpel, surgical tweezers, small hacksaw, Freer periosteal elevator, scissors, etc.) with the disposable cleaning cloth and/or the brush from the tub and then store them at the bottom of the tub under the surface of the DNA remover.

II. *Exposure of the femur before sample excision*

6. Elevate the thigh of the victim slightly above the dissection/autopsy table, e.g. by tucking part of the body bag underneath it.
7. Clean the skin of the thigh with a fresh disposable cleaning cloth that has been soaked in the large bucket with DNA remover then discard after use.
8. Discard the outer gloves and replace with new.
9. Use a clean disposable surgical blade (a number 22 blade is ideal).
10. Make a superficial H-incision a few millimetres deep only (Fig. 1), i.e.:
    - a longitudinal incision over the topmost part of the thigh, extending from a little below the inguinal area to approximately 5 cm above the knee, plus
    - transverse incisions crossing the proximal and the distal ends of the longitudinal cut. Both transverse cuts should extend a little more than half the circumference of the thigh.
11. Clean the scalpel in the rectangular tub, paying extra attention to its neck where a skin smear may be present.
12. Deepen the H-incision with the cleaned scalpel and the surgical tweezers until the femur is touched.

Reminder: the tweezers should be cleaned if the skin is accidentally touched.

13. The femoral shaft should be freed from muscle tissue in such a way that the medial and lateral muscle compartments fold back similar to doors opening (Fig. 2). This can be facilitated by means of a few longitudinal cuts in the muscles. The object is to expose the femoral shaft in such a way that it can be approached for sawing without touching anything else.

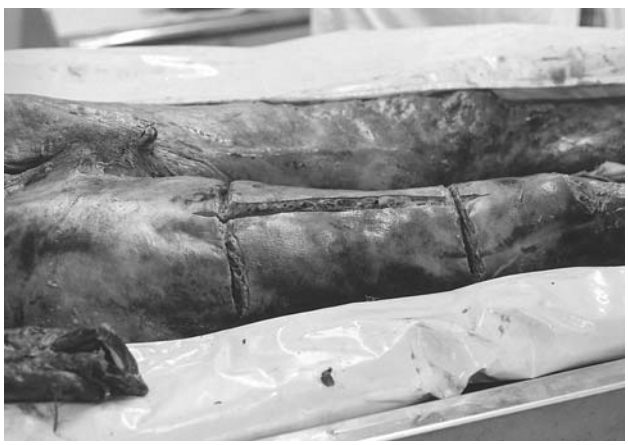

Fig. 1 H-incision of the thigh

Fig. 2 Exposing the femur before excision of the bone sample

Fig. 3 Collecting excised femur sample

III. *Processing of an excised femur wedge*

14. Remove the periosteum with the scalpel and the Freer periosteal elevator to facilitate sawing.
15. Place a clean disposable saw-blade in the hacksaw.
16. With the hacksaw saw a wedge from the midshaft of the femur (Fig. 3). If possible do NOT saw through the complete shaft, because the femur will become unstable for further sawing and transportation.
17. Lift the wedge with DNA-free tweezers.
18. Rinse the sample in the small container with absolute alcohol to accelerate the drying process. Do not put the sample down in the meantime.
19. Once “dry”, put the sample in a sample container (Fig. 3), sealing it with tape and marking it with the appropriate tracking number.
20. Store the sample container in a freezer.
21. Complete the inventory list and the chain-of-custody form.
22. Close and suture the excision wound.

23. Clean every instrument with the DNA remover in the rectangular tub and store the instruments at the bottom of the tub under the surface of the DNA remover.
24. Replace the contents of the small container with fresh absolute alcohol.

Rib

I. *Preparations for rib sample excision*

1. Repeat paragraphs 1–5 as described above.

II. *Exposure of the rib before sample excision*

2. Palpate a rib in the lower half of the chest.
3. Clean the chest area with a fresh disposable cleaning cloth that has been soaked in the large bucket with DNA remover and then discard after use.
4. Discard the outer gloves and replace with new.
5. Use a clean disposable surgical blade (a number 22 blade is ideal).
6. Make a superficial rectangular incision with a length of ca. 10 cm and only a few millimeters deep, well surrounding the osteochondral junction of the chosen rib.
7. Clean the scalpel in the rectangular tub, paying extra attention to its neck where a skin smear may be present.
8. Excise the skin and the underlying muscle tissue in one movement.
9. Clean the scalpel as described above.
10. Deepen the incision along the sides of the rib until you penetrate the thoracic cage.

Reminder: the tweezers should be cleaned if the skin is accidentally touched.

11. Expose the rib in such a way that it can be approached with scissors without touching anything else (Fig. 4).

III. *Processing of an excised rib sample*

12. Use the scissors to cut through the *bone* of the rib, approximately 3 cm from the osteochondral junction.
13. While holding the bone end with DNA-free tweezers, cut with the scissors through the *cartilaginous part* of the rib, also ca. 3 cm from the osteochondral junction (Fig. 5).
14. Repeat paragraphs 18–24 as described above.

Tooth

I. *Preparations for tooth sample extraction*

1. Prepare a bowl with DNA remover and permanently keep a toothbrush and tweezers in the bowl.

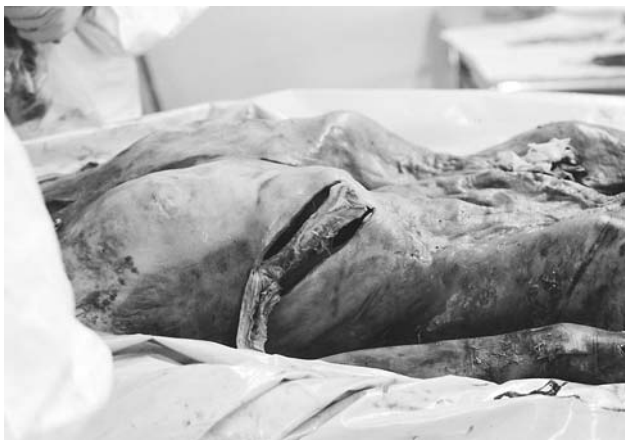

Fig. 4 Exposing the rib before excision of the bone sample

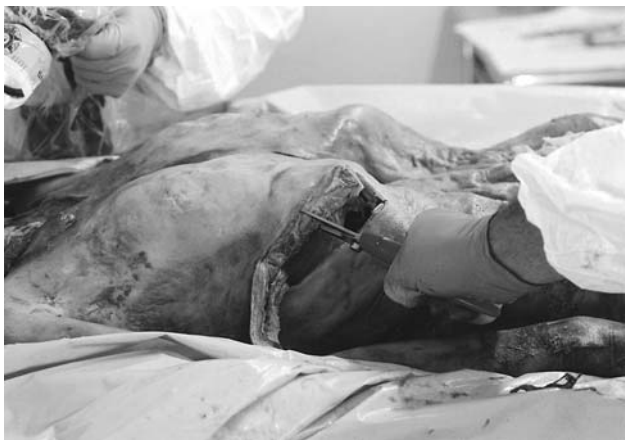

Fig. 5 Cutting the rib

2. Fill a small container with absolute alcohol and permanently keep a pair of DNA-free tweezers in this container.

II. *Processing of an extracted tooth specimen*

3. Extract a healthy intact tooth (i.e. without caries, fillings, or other artificial modifications), preferably a canine, an upper incisor, or a molar, with intact roots (see Recommendations) with extraction pliers.
4. Drop the extracted tooth, which is still dirty, in the bowl containing DNA remover.
5. Discard the outer gloves and replace with new.
6. Clean the tooth with the toothbrush from the bowl. You may use your gloved hands.
7. Lift the tooth with the tweezers from the bowl and drop it in the small container containing absolute alcohol; place the tweezers back in the bowl.
8. Lift the tooth with the tweezers from this small container after rinsing it with absolute alcohol to

facilitate the drying process. Do not put the tooth down in the meantime.
9. Repeat paragraphs 19–21 as described above.
10. Clean the toothbrush and the tweezers from the bowl with the DNA remover.
11. Replace the contents of the small container with fresh absolute alcohol.

Recommendations

Some additional recommendations are suggested with regard to this standard operating procedure. During earlier work on disaster victim identification in Kosovo [15, 16] it appeared to be difficult to keep a grip on a scalpel during autopsy of seriously decomposed corpses. Especially under disaster conditions, you do not want to "lose" the scalpel in the corpse and risk injury to yourself or others nearby. Thus, a large grip was designed that can hold a standard surgical blade (Fig. 6). The grip is ergonomically shaped to facilitate control. It is made of brass, which is bacteriocidal as a result of the regular formation of a layer of copper oxide on its surface.

Routine work at the "morgue" showed that even passive storage of amputation saws in the tub with DNA remover resulted in blunting within hours. This was probably because of erosion by the aggressive cleaning fluid solution. It appeared that a small tool shop hacksaw (Fig. 6) was of more practical use than the standard amputation saw. Sharpening of the blades was no longer needed, because they could be simply replaced by inexpensive disposable blades.

No electrical equipment, for example an electric saw, was used during the sample collection for excising bone samples. This was for two major reasons: first, the possibility of spreading aerosols or small particles of tissue that could cause contamination of other samples and, second, cleaning the blades with DNA remover causes blunting and replacing them is relatively expensive.

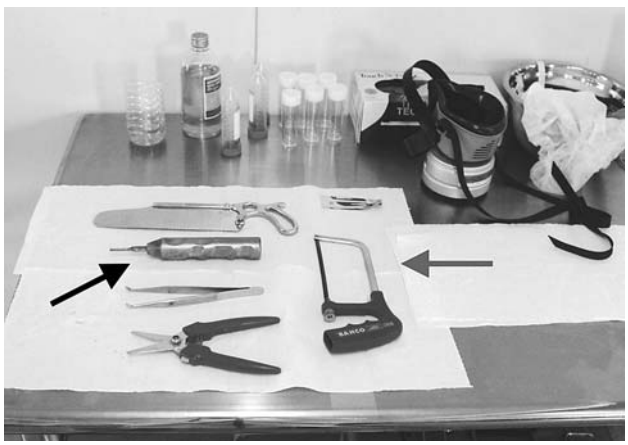

Fig. 6 Instrument table: brass grip (*black arrow*) and hacksaw (*grey arrow*)

When possible, it may be better for an odontologist to collect the tooth sample, because their training enables them to distinguish intact from artificially modified teeth and to extract without damaging the teeth. For DNA analysis the intact element with the largest pulp-cavity is preferred, because this should yield the largest amount of DNA. The dimension of the pulp-cavity depends on the size of the tooth [17] and is age-dependent as a result of secondary dentine deposition. In children, open roots make the teeth much more susceptible to contamination and to destruction of DNA by the DNA remover.

We recommend using femur wedges instead of rib samples for DNA analysis. Because ribs have a very thin cortex and tend to protrude through the skin, the risk of contamination may be greater, especially for submerged corpses. After recovery of such samples, cleaning may be difficult or even impossible without damaging the endogenous DNA. We have, nevertheless, described the SOP for excision of a rib sample, because some countries insist on using rib samples for genotyping purposes. Their choice to use rib samples is probably because spongy or cancellous bone can be rich in DNA. Prinz et al. [9], however, report that preservation of cancellous bone is not reliable and dense cortical bone should always be the first choice, preferably from the weight-bearing long bones of the legs.

Finally, ensure that directly after excision or extraction, the bone and tooth samples are frozen. If no freezer is available, cooling the sample containers in a bath of water with melting ice will be effective as long as the ice melts, the temperature thus staying at 0°C.

Discussion

Because no special record was kept of the samples excised from the tsunami victims by means of the above described SOP, it is, unfortunately, very difficult (if not impossible) to track the samples and discover whether they provided adequate DNA profiles. A similar SOP for femur and tooth sample collection is used at our laboratory at The Netherlands Forensic Institute, which provides good genotyping results. Nevertheless, the hectic situation of a mass disaster contrasts sharply with the conditions during single forensic cases. Shortly after a mass disaster has occurred, especially, neither facilities nor trained people are immediately available for identification work. The absence of a cooling/freezer facility shortly after the tsunami, for example, led to ongoing decomposition of the victims' bodies, which impeded identification. The number of victims to be

identified was, in addition, so large that the identification teams had to work in multiple shifts and train extra people on site. Because the victims' bodies could easily cross-contaminate each other during transport and storage, it was also necessary to devote special attention to preventing cross-contamination of the bone and tooth samples from the body surface or unclean instruments. This contrasts markedly with a specialized forensic laboratory in which all the necessary facilities are available, the personnel is well-trained, and the section rooms and instruments are cleaned after each autopsy.

One of the assumptions made in the above-described protocol is that the tissue samples, assuming they are excised in a correct manner, are free from contamination and ready for DNA extraction. This is not always true. For example, during the WTC disaster the body parts were highly commingled, and during excavation of the mass graves in the former Yugoslavia the bodies were grossly putrefied or even skeletonized [5, 11]. In these situations, tissue samples can easily become contaminated. It is, therefore, necessary to clean the bones and bone fragments to remove contaminating DNA and potential polymerase chain reaction (PCR) inhibitors. Multiple cleaning methods are available. For body remains from the mass graves in the former Yugoslavia, Andelinovic et al. [5] report that all bone surfaces were cleaned from remnant soft tissue and traces of soil and were also brushed in warm water with mild detergent. Complete bones were then rinsed with distilled water several times and dried in air. Bone fragments were washed with commercial bleach, three times with deionized water, twice with 70% ethanol and dried in air for 24 h. Alonso et al. [10] report that outer surfaces of tooth samples were extensively washed with distilled and sterile water before irradiation with UV light for 30 min on each side. Both Zehner [3] and Alonso et al. [10] advise physical removal of the external and internal surfaces of the bone. Several methods are described for this removal, for example rasping, sawing, or abrasion with sandpaper or a sanding machine [3, 10, 18–20]. Sanding, and grinding in a later stage of the research, can generate bone dust, which can lead to sample cross-contamination. Manual processing of single bone samples reduces the chance of sample cross-contamination compared with batch processing [8].

The effect of the environment on victims can vary greatly among different types of forensic cases and mass disasters. The tsunami victims were exposed to sea water and warm humid air whereas the victims of the WTC disaster were exposed to intense fire, heat, and subsequent extinguishing water. In contrast, the bones of people killed during the war in the former Yugoslavia were exposed to highly acidic soil and chemical agents that were used in deliberate attempts to degrade their DNA [1]. These different effects may cause different kinds of DNA damage. It would be interesting to develop a method to determine the type and extent of this damage and, where possible, to develop corresponding protocols for DNA extraction and the subsequent DNA analysis.

It is not always clear what effect environmental factors have had on tissues and whether these tissues can still provide good genotyping results. In general, blood or intact soft tissue samples are preferred for DNA analysis but when body putrefaction precludes DNA preservation or when much commingling of soft tissue is suspected, bone and tooth samples are preferred [7, 9]. During identification of the tsunami victims, not only bone and tooth samples were used for genotyping purposes. Steinlechner et al. [21] described the use of swabs from two, as intact as possible, internal organ or muscle surfaces at the disaster site in Sri Lanka. Because the quality of DNA in soft tissue decreases rapidly with time, this method requires the swabs be taken from relatively fresh material. An advantage is that the analysis is less laborious and time-consuming than for bone and tooth samples. When the effect of precise environmental factors is uncertain, it seems sensible to collect different kinds of tissue sample from each victim. Another advantage of collecting several samples per victim from the outset is avoidance of laborious re-sampling and re-labeling efforts when no DNA profile could be obtained from the first sample. Such sampling also gives rise to the possibility of a duplication policy, in which two specimens collected from the same body or body part are tested. This could help in identifying mislabeled or switched samples or extract-to-extract contamination, which could lead to incorrect identification when based on a single extraction [9].

DNA genotyping should not be problematical if the tissue samples are of high quality at the moment they reach the laboratory. Unfortunately, as a result of post-mortem processes, the DNA in forensic (mass disaster) tissue samples is often limited in quality and/or quantity, leading to difficulties in DNA analysis. In current forensic DNA practice the number of repeats of specific DNA fragments, called short tandem repeats (STRs), is counted at different loci in the genome and plotted per locus in a DNA profile. The DNA fragments to be analyzed range in size between 114 and 353 base pairs. Degradation of the DNA may result in the inability to detect the larger DNA fragments, reducing the chance of victim identification. Although other genotyping methods are being developed, with the objective of using shorter DNA fragments, for example mini-STRs [22, 23] and single nucleotide polymorphisms (SNPs) [8, 24, 25], the results will be still determined by the quality of the tissue samples to be analyzed. It is, therefore, of the greatest importance to collect tissue samples of the highest possible quality, to minimize the risk of contamination, and to keep the samples under

optimum conditions until they can be genotyped in the laboratory.

Conclusion

This standard operating procedure for excision and extraction of bone and tooth samples to be used for genotyping purposes was developed under disaster conditions and is based on common sense, theoretical knowledge, and best practice. Because the materials used are inexpensive and easy to obtain, execution of the SOP should not cause problems. Further research and use of this SOP under controlled circumstances (e.g. in the laboratory or in single forensic cases) may reveal the possibility of improvement.

Educational message

1. Wear protective clothing and work in a clean separate area to minimize exogenous contamination risk.
2. Clean instruments thoroughly after touching the skin of a victim and before examining another victim to minimize the risk of cross-contamination.
3. Freeze, or cool when no freezer is available, the tissue samples directly after collection (and labeling) to keep the samples under optimum conditions until they can be genotyped.

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