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Research paper

Tissue preservation in extreme temperatures for rapid response to military deaths

Jasmine Connell[a], Janet Chaseling[a], Mark Page[b], Kirsty Wright[a,c,*]

[a] School of Environment and Science, Griffith University, 170 Kessels Road, Nathan, Queensland 4111, Australia
[b] Royal Australian Navy, Joint Health Unit Central NSW, 276 Pitt St Sydney, New South Wales, 2000 Australia
[c] Royal Australian Air Force (RAAF), No 2 Expeditionary Health Squadron, Williamtown, New South Wales 2318, Australia

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ABSTRACT

Many deployable forensic capabilities, including those used by the Australian Defense Force (ADF), employ mobile battery-operated fridge/freezers for DNA sample preservation that are not suitable for rapid response application due to their size and weight. These fridge/freezers are expensive, require regular specialised maintenance, and have a set payload. A variety of transport media are successful preservatives for DNA samples, however, there is no research specifically targeted to their suitability for operational environments where temperatures exceed 50 °C.

This research examined whether sodium chloride (NaCl), ethanol, and dimethyl sulfoxide (DMSO) could preserve muscle and bone samples (fresh and early decomposition) as effectively as refrigeration, when stored at 21 °C, 45 °C, 55 °C, and 65 °C for at least one week. A total of 78 muscle and 78 bone samples were collected from an unknown deceased individual. Half of each tissue type was stored at 30 °C for 48 h to induce early decomposition. Following this, samples were stored in the transport media for one week at the above temperatures, and a control set of samples were refrigerated (−4 °C) without any transport media. Preserved samples would need to provide DNA profiles comparable to the refrigerated samples for the transport media to be considered a successful replacement method.

NaCl and 70% ethanol preserved muscle samples (fresh and decomposed) up to 65 °C, as well as 70% ethanol and 20% DMSO for fresh bone samples. These results were comparable with refrigeration and therefore, these preservatives could be used in rapid response operations by the military and for disaster victim identification. Conversely, under the conditions of this study, 20% DMSO and 70% ethanol failed to consistently produce full DNA profiles from decomposed bone, and NaCl performed poorly at preserving DNA from fresh and decomposed bone samples.

1. Introduction

The identification of human remains by many government agencies worldwide generally follow disaster victim identification (DVI) principals espoused by the International Criminal Police Organisation (INTERPOL) DVI guide [1]. In a military context, a designated forensic response team and associated equipment are transported from the home country to a site often remote from the disaster site itself, where a temporary mortuary is established in order to gather and examine remains. Depending on the conditions of the remains, DNA is often a key method of identification, particularly in circumstances where fingerprints or dental identification is not possible, however, many developing countries and those subject to civil and military conflict lack the ability to type DNA. In these situations, tissue samples need to be transported to other countries for DNA analysis. It is often desirable to sample tissue near the disaster site or prior to transportation of the remains elsewhere in order to speed the identification process, particularly where local agencies retain jurisdiction of the body, there is difficulty relocating remains due to zones of conflict, or there are legal / coronial processes that result in delays in releasing bodies to a foreign entity. Several options exist for sample preservation; however, a common scenario is the utilisation of mobile battery-operated blood or tissue fridges and freezers. Samples are stored in these appliances until they can be relocated to the home country, often ahead of the remains.

There are a number of limitations associated with using fridge/freezers to preserve and transport DNA samples, which make it unsuitable for a rapid response DVI application for the military. A typical fridge/freezer used by the military weighs approximately 66 kg

* Corresponding author at: School of Natural Sciences, Griffith University, 170 Kessels Road, Nathan, Queensland 4111, Australia.
E-mail address: k.wright@griffith.edu.au (K. Wright).

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Table 1
Summary of the proposed preservatives and their constituents.

Preservative	Constituents
NaCl	Solid NaCl (Analytical Reagent) (Chem-Supply)
Ethanol	100% Undenatured ethanol (Chem-supply) and sterile double distilled water (ddH_2O) (Invitrogen by Life Technologies) (3:1)[a]
DMSO	100% DMSO (Chem-Supply), 0.5 M EDTA (pH 8.0) (Chem-Supply) and ddH_2O (2:5:3), saturated with NaCl[a]

[a] The stated concentrations are for those of the liquids before mixing of the final solution.

(empty), which consumes a large proportion of the rapid response kit weight limit. They require specialised regular maintenance, the payload lacks scalability, and relies on the availability of electricity or working batteries up to 48 h. The focus of this research is evaluating preservation of muscle and bone samples under conditions that replicate transport from a military field mortuary to another country (a period of up to one week in ambient temperatures over 50 °C). Such scenarios would be encountered where agencies are required to mount a DVI response overseas, as either aid to the civilian community, or for military deaths.

This research examines three transport media to determine whether they can preserve bone and muscle samples under temperature conditions consistent with potential operational environments experienced by modern military forces (45 °C, 55 °C and 65 °C for at least one week) (Table 1). The three chemical media chosen are, sodium chloride (NaCl), 70% ethanol, and 20% dimethyl sulfoxide (DMSO). Replacing fridge/freezers with transport media would improve military and government capability for responding to deaths in combat and disaster scenarios by reducing the cost and weight of the response kits, and enabling a more efficient and reliable method to transport DNA samples.

INTERPOL guidelines state that chemical preservatives can be used to store soft tissue at room temperature but recommend against the use of formaldehyde or formalin because it degrades the DNA [1]. As enzymes are active in the presence of water, the elimination of water is one of the main steps in a vast majority of preservation methods. In solid form, NaCl desiccates the sample, which inactivates nucleases and reduces the growth of pathogens [2]. NaCl is a common preservative that is reported to preserve muscle tissue at room temperature for one year [3]; 35 °C for one month [4]; and 37 °C for 38 weeks [5]. Michaud [6] found that liquid preservatives performed better than NaCl, most likely because they were capable of rapidly penetrating the sample to prevent decay of the DNA.

Ethanol removes water from a sample and denatures proteins and nucleases [7–9]; as such, ethanol is an antimicrobial that can prevent bacterial degradation [7,10]. It is the most commonly used alcohol for tissue preservation, and has been used as a successful preservative at various concentrations. For example, 100% ethanol was used to successfully preserve molluscan tissue at room temperature for 26 days [11], while 95% ethanol was used successfully to store mouse liver over a two year period, although a low DNA yield was obtained compared to DMSO and an additional procedure had to be used to protect the DNA from degradation during the extraction procedure [12]. At 70%, ethanol was suitable for shorter-term storage of porcine skin and muscle tissue at room temperature (less than 6 months) [6]. Michaud [6] found that 70% ethanol was as effective as DMSO in preserving DNA, and performed as well as, or better than, other concentrations (40% and 100%). For human samples, 70% ethanol and 70% ethanol containing 1 mM EDTA was used to store muscle samples at 35 °C for one month [4]. Both performed as successful preservatives. Based on these findings, 70% ethanol was investigated.

The mechanism of action for DMSO is only partially understood. It exerts antimicrobial activity [13] by altering the ribonucleic acid structures essential for bacterial protein synthesis [14], and is known to promote the dermal absorption of chemicals with which it is mixed [15], and thus enhances the absorption of other preservatives into the substrate [12]. Compared with lysis buffer and 95% ethanol, DMSO provided the best protection from DNA degradation and provided a higher yield of DNA for mouse liver stored at room temperature for two years [12]. It was used to successfully preserve avian tissue at room temperature for 24 weeks, although no PCR was conducted [7]. DMSO has also been used to preserve avian blood stored at ambient temperature and 65 °C, with no detectable degradation of DNA, as determined from agarose gel electrophoresis of crude DNA [16]. Marine invertebrate have been successfully preserved in DMSO at ambient temperature for 26 days [11] and 28 weeks [17], while porcine skin and muscle was successfully stored 6 months [6]. These studies conducted on animal samples suggests that DMSO may be a suitable preservative for human tissue, a hypothesis that was supported by Allen-Hall and McNevin [4] who successfully stored human muscle in DMSO for one month at 35 °C.

A number of proprietary preservatives exist that have successfully preserved DNA samples. TypiFix™ containers are suitable for the preservation of soft human tissue when stored at room temperature for up to five months [18], while DNA Genotek Tissue Stabilising Kit, and DNAgard® have all successfully preserved fresh human muscle tissue at 35 °C for up to one month [4]. Sorensen et al [19] also demonstrated that DNAgard® and RNA*later*® preserved fresh and decomposed human skin and muscle for up to three months at 35 °C. RNA*later*® was also shown to be as successful as DMSO and 70% ethanol when storing porcine tissue for DNA analysis, and performed better than NaCl [6].

Military operational environments can exceed 40 °C, and after fitting military vehicles with armour kits, temperatures inside those vehicles can reach 54 °C [20], while the tarmac or concrete used on airfield aprons reflects heat and further multiplies the temperature of vehicles on the ground [21]. No previous literature has examined the capability of chemical media to preserve human DNA samples in the potentially high temperature ranges experienced by armed forces in modern combat zones. Therefore, it is necessary that preservatives are tested under these conditions, to determine their effect on DNA preservation.

The most important consideration when selecting a chemical preservative is that it consistently enables a DNA profile to be obtained. Other important considerations include cost, availability, and occupational health and safety during transportation and use in the mortuary and laboratory. Vapours from ethanol may form an explosive mixture with air, thus it is classified as a Dangerous Good of Class 3 [22] in accordance with International Air Transport Association Dangerous Goods Regulations. However, when packaged correctly, it is authorised for carriage aboard service aircraft [23], including both fixed and rotary wing aircraft. NaCl has minor health considerations that are addressed by correct personal protective equipment (PPE), storage and disposal. DMSO is identified as an irritant, causing both skin and eye irritation if correct storage and handling protocols are not adhered to [24]. Neither NaCl nor DMSO are regulated as Dangerous Goods and are suitable for transport aboard service aircraft [24,25].

Preparation of the chemicals should be a simple task in a rapid response environment. For example, pouring solid NaCl into a vial containing a tissue sample is much easier than preparing a complex mixture of constituents which requires an accurate pH measurement. NaCl and 70% ethanol have low preparation requirements, however, DMSO requires a 24 h (approximate) incubation period before the solution can be used to ensure the saturated NaCl has settled [26].

Scalability of an operational response is essential for DVI purposes. In addition to the large number of casualties, decomposition and fragmentation can be extensive in high impact disasters [27]. One of the most important issues with the refrigerators used by the military is that they are only suitable for small DVI operations. Ideally, preservation methods should be readily capable of storing large quantities of tissue.

The shelf life of each chemical should also be considered, as it is