Comparison of Two Tissue Preservation Methods for Non-cold Storage of Forensic Tissue Sample

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ABSTRACT

Cold-storage of forensic biological samples would help to reduce sample degradation prior to DNA analysis. However, accessibility and availability of storage facilities and space could be limited. In this study, the use of DMSO buffer and Longmire's buffer for tissue preservation at different ambient temperatures were investigated. Pieces of 0.3-g porcine tissues were cut and preserved in each buffer and stored at 25°C, 40°C, 60°C and 80°C, with different storage periods for 1 to 4 weeks. DNA analysis of the stored samples by PCR showed that PCR products could be amplified from all tissue samples when preserved up to 1 week. However, no amplification product was obtained from tissue samples stored in the 2 buffers at 80°C for 2-4 weeks. Results suggested that both buffer solutions could be used for tissue storage at ambient temperature not exceeding 60°C for 2-3 weeks. Therefore, findings of this study supported the collection, preservation and transfer of forensic tissue samples in either DMSO or Longmire's buffer without cold-storage for DNA analysis, under the limited conditions.

Keywords: Tissue preservation, DMSO, Longmire

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Introduction

DNA profiling is widely used in personal identification, disputed paternity and criminal cases such as rape, murder and robbery. However, sufficient amount of DNA must be recovered in order to proceed subsequent DNA profiling and analysis. All steps in the bio-evidence collecion, preservation and transportation of the samples must be properly performed to avoid or reduce risk of degradation of bio-evidence prior to analysis.

Cryopreservation or storage of sample by deep freezing is recommended to ensure long-term storage of tissue samples. However, deterioration of tissue sample can occur as ice crystal formation inside the cell can cause cell damage and loss of DNA material. Moreover, freezer space and the facility can become limited. A practical approach to solve those problems is to adopt a tissue preservation method that tissue samples can be appropriately stored at ambient temperature.

Among the forensic biological samples, blood, urine and tissue samples are the most collective biological samples from autopsy. Blood and tissue samples are used for DNA profiling. In case, the notification of death may be late, the blood in the body settles down after circulation ceases. Therefore, it is inconvenient to take the blood sample. Moreover, the blood is the good medium for the organisms to grow for subsequent putrefaction. Urine, sometimes, cannot be collected if the bladder of the corpse is emptied (Dimaio and Dimaio, 2001; Knight, 2016). For the tissue sample, it is possible to collect deep muscle tissue after death. Therefore, in this study, the preservation for tissue samples was preferable.

In this study, tissue samples were stored in 2 types of solutions for comparison, i.e, DMSO buffer solution and Longmire's buffer solution. DMSO solution is composed of dimethyl sulfoxide, ethylenediamine tetra acetic acid and sodium chloride. DMSO inactivates nucleases that will degrade DNA. When the sample is added to the solution, DMSO permeates tissues and cells, taking low molecular weight chemicals (EDTA and NaCl) together along with it. In the cell, the EDTA binds divalent cations, which are needed for nucleases to activate and degrade DNA. The Na+ ions from the NaCl will surround the negatively charged DNA phosphate backbone preventing water molecules from coming in contact with the hydrogen bonds of the base pairs and denaturing the double strands. By that mechanism, the DMSO solution can preserve DNA (Kilpatrick, 2002; Seutin, 1991). Longmire's buffer solution is composed of sodium dodecyl sulfate (SDS), Tris-HCl and EDTA. Sodium dodecyl sulfate is a strong anionic detergent that can solubilize the proteins and lipids. EDTA binds divalent cations, which are needed to activate nuclease and degrade DNA (Nagy, 2010).

Objectives of the study

1. To investigate the use of preservative methods for tissue storage temperature for subsequent DNA analysis, and determine the limitation of storage period.
2. To investigate the effect of high temperature storage of tissue sample on DNA analysis.

Materials and Methods

Tissue preservation methods

Porcine tissues were used in this study. Fresh pork meats (muscle tissue) were bought from the market and cut into pieces, approximately 0.3 g each for treatment. Two preservation methods were applied in this study. Details of the preservation methods used are described in the followings.

The DMSO buffer solution contains 20 % v/v Dimethyl Sulfoxide, 0.25 M EDTA, saturated with NaCl (pH 8.0). (Seutin, 1991; Kilpatrick, 2002; Allen-Hall, 2011; Michaud and Foran, 2011)

Longmire's buffer solution contains 2M Tris-HCl, 0.5M EDTA, 5M NaCl and 20% Sodium Dodecyl Sulfate (pH=8.0). (Longmire, 1997; Kilpatrick, 2002)

Each sample was put into 5 mL Eppendorf tube, containing 4 mL of each buffer solution, then stored at four temperatures; 25°C, 40°C, 60°C and 80°C. After storage up to 1, 2, 3 and 4 weeks, DNA was extracted from the tissue samples.

DNA extraction

DNA was extracted by Wizard® SV DNA purification kit (Promega, USA) according to manufacturer's protocol for tissue. The 20 mg tissues were incubated with digestion solution master mix (nucleic lysis solution, 0.5M EDTA with pH 8.0, Proteinase K and RNase solution) for 16-18 hours. Then, they were extracted using wizard® SV lysis buffer and column wash solution according to the protocol. Final elution volume was 100 µl.

DNA quantitation

The extracted DNA was quantified by the Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, USA) according to the manufacturer's procedure. The method included Qubit® dsDNA HS Buffer, Qubit® dsDNA HS Reagent Qubit® standard. Firstly, the Qubit® working solution was prepared by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer for the samples and 2 standards. The 2 µl DNA was added to 198 µl working solution for each sample. After calibrating the 2 standards, the samples were quantified by Qubit® 3.0 Fluorometer.

DNA amplification

Analysis of DNA fragmentation was done by PCR process using the pig *β-actin* primer set. The primer set was composed of forward and reverse primer which generated the expected sizes of PCR product of 148-bp and 366-bp (Phengon, 2008). The total volume of PCR reaction mixture is 25 µl. The reaction mixture consisted of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP, 1 unit of Taq DNA polymerase and 2.5 pmol of each forward and reverse primer. Amplification was performed in GeneAmp 9700 Thermocycler (Applied Biosystem, USA) using the following thermocycling condition; initial denaturation at 95ºC for 5 min, then 35 cycles of denaturation at 95ºC for 30 sec, annealing at 63ºC for 30 sec, extension at 72ºC for 1 min and final extension at 72ºC for 7 min.

Table 1 The sequence of forward and reverse primer of pig *β-actin* primer set

Name	Sequence (5'---3')
F1-actin	CGGAACCGCTCATTGCC
R1-actin	TAGATGGGCACAGTGTGGGT
R3-actin	AGCGCTCGTTGCCGATGGTG

DNA analysis by Gel Electrophoresis

PCR product was detected by Agarose Gel electrophoresis. The 2% (w/v) of agarose/Tris-borate-EDTA (TBE) buffer was prepared by using 1.4 g of Agarose in 70ml of TBE buffer. The 8 µl of PCR products were loaded into wells with bromophenol blue dye 2 µl. The 100bp DNA ladder was used to measure the size of PCR products.

DNA extracted from the fresh pig tissue was used as positive control and deionized water was used as negative control.

Results

In this study, total DNA yield of samples stored in DMSO and Longmire's buffer at 25°C, 40°C, 60°C and 80°C, over 1-4 week time period were compared. Results showed that up to 4000 ng of DNA could be obtained from 20 mg tissue sample. Total DNA yield of samples preserved in DMSO buffer was in the range of 21 ng to 4000 ng. For storage in Longmire buffer, total DNA yield was in the range of 17 ng to 1980 ng.

For analysis by PCR, results in figure (1) showed that the 148-bp and 366-bp of *β-actin* PCR products were obtained from 1 week samples preserved in the 2 buffer types at 25°C, 40°C, 60°C and 80°C. When storage time was increased to 2 weeks, no PCR product was detected from samples preserved in the 2 buffer solutions at 80°C. Similar PCR results were obtained when analysed samples preserved for 3 and 4 weeks. It was observed that band intensity of the 366-bp PCR products amplified from tissue samples preserved in Longmire's buffer at 60°C were slightly fainter than those amplified from samples preserved in the same buffer at lower temperatures as well as those preserved in DMSO buffer. PCR products of the correct sizes were amplified from all positive PCR control reactions. No PCR product was obtained from the negative PCR control reaction.

The results demonstrated that 148-bp and 366-bp PCR products could be obtained from tissue samples preserved in both DMSO and Longmire's buffer at 25°C, 40°C, 60°C and 80°C up to 1 week and up to 4 weeks in both preservative buffers storage at 25˚C, 40˚C and 60˚C. However, no amplification product would be obtained if the tissue stored in the 2 buffer types at 80°C for 2 weeks and longer.

Table 2 DNA yield **(**ng**)** obtaining from each storage condition

Period	DMSO Buffer				Longmire's Buffer			
	25˚C	40˚C	60˚C	80˚C	25˚C	40˚C	60˚C	80˚C
Week 1	307	352	2150	60	316	910	1490	72
Week 2	310	830	3030	21	820	1310	300	17
Week 3	369	4000	1980	37	735	1530	140	30
Week 4	612	2250	1660	74	378	1980	240	215

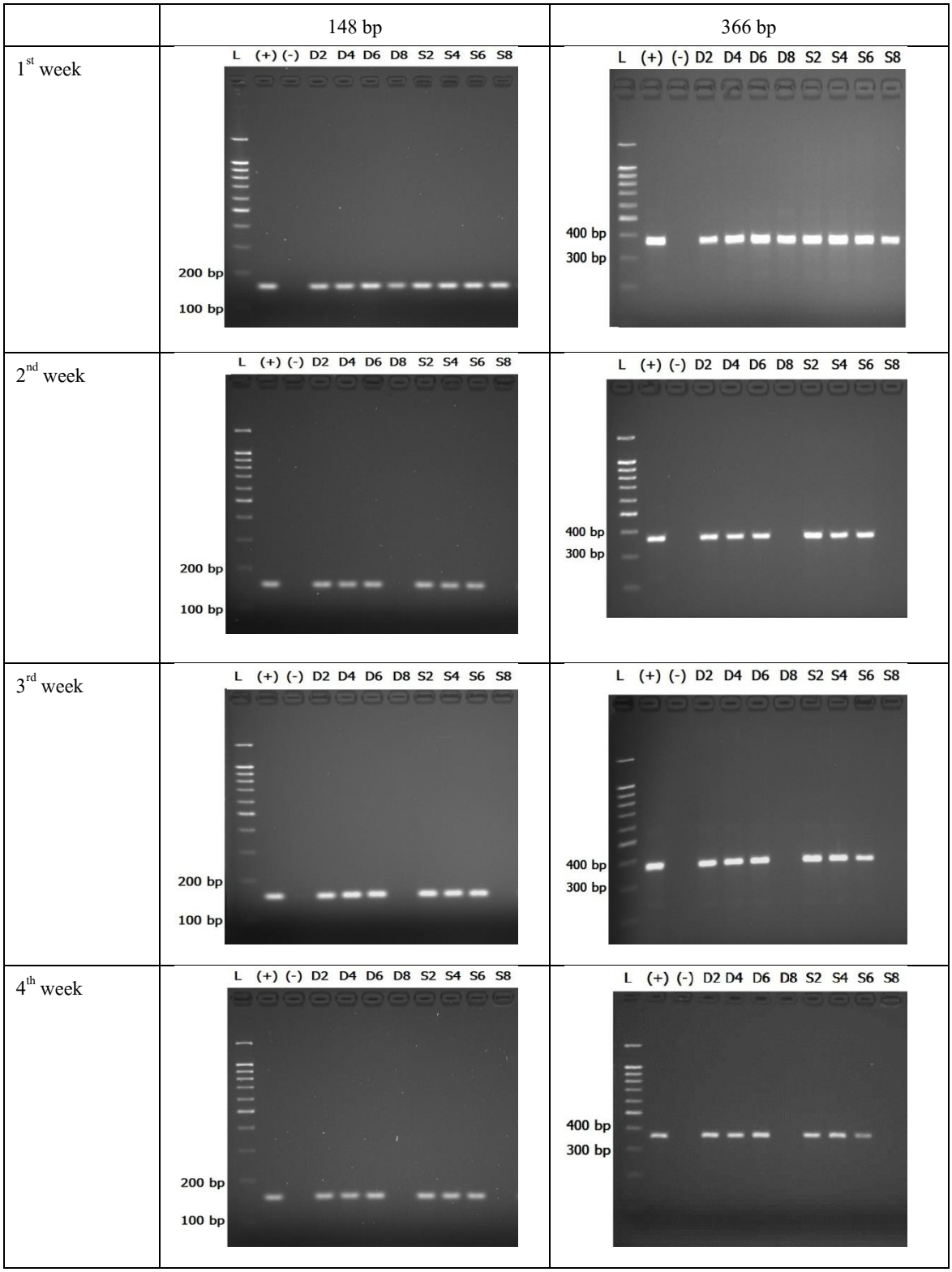

Figure 2 Photographs of 2% (w/v) agarose/TBE gels showing the 148-bp and 366-bp PCR products of the samples stored in 2 types of solution at 4 different temperatures. L= DNA Ladder, **(+)** = Positive control, **(-)** = Negative control, D = Samples stored in DMSO buffer, S = Longmire's buffer solution, 2 = 25°C, 4 = 40°C, 6 = 60°C, 8 = 80°C.

Discussion

This study was aimed to adopt tissue storage methods without freezing for forensic tissue sample. The methods can be helpful where the facility for freezing is limited or unavailable. Therefore, the different degrees of temperature; 25°C, 40°C, 60°C, 80°C, were also set up to simulate the ambient condition of tropical countries. Actually, ambient temperature may not rise to 50°C, however, it simulated the condition that the sample is accidentally near to the heating source or object, exposed to direct sunlight, and conditions such as storage in car boots. In this study, the tissues stored in all preservative solution remained in good condition at the temperatures of 25°C, 40°C and 60°C up to 4 weeks. For the samples of storage at 80°C, DNA yield from both solutions were very low, and could not be amplified by PCR.

Kilpatrick described that DMSO buffer is the best preservative solution among other two solutions – Longmire's buffer and ethanol (Kilpatrick, 2002). The result of this study is consistent with the previous work. However, in Kilpatrick's research, the storage temperature was room temperature (25°C) and the storage time was 2 years. In this study, the storage period was only up to four weeks to fit with the forensic investigation process, but the storage temperatures were increased to 40°C, 60°C and 80°C. Although the samples from storage at 80°C could not be amplified, it was found that PCR products were successfully amplified for the DNA samples stored in the 2 buffer at 40°C and 60°C. And no DNA fragmentation sign was observed when storage was up to 2 weeks. However, results also demonstrated a sign of subtle DNA fragmentation when sample was stored up to 3 and 4 weeks.

In forensic DNA profiling by STR analysis, the range of PCR products or STR alleles would be approximately 100-400 bp long. The *β-actin* nuclear DNA marker used in this study represented the small and large STR product range. It could be inferred that there would be high possibility to obtain full DNA profile from these tissue samples stored in the described conditions.

There were some limitations for this study. Firstly, the tissue samples were pig tissues that were collected fresh. However, in actual forensic case, the tissue samples may already be degraded to some extent. Second, each tissue sample in preservation was stored at a constant temperature. In actual case, the ambient temperature may be fluctuating. Third, this study was emphasized in only one month duration to simulate a delayed transportation period of sample to the laboratory for subsequent analysis. It is not aimed to store the samples for long period or permanently. The future researches are suggested to overcome these limitations.

Conclusions

DNA analysis could be performed in samples stored up to 4 weeks in DMSO buffer solution and Longmire's buffer solution at 25°C, 40°C and 60°, but not at 80°C. Therefore, preservation solutions can be used to store the tissue samples at ambient temperature not exceeding 60°C for later DNA extraction and analysis. DMSO or Longmire's buffer is suggested to apply for storage of tissue if there is lack of facility for cold storage.

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References

Allen-Hall A. Preservation of human muscle in conditions commonly associated with mass disasters: University of Canberra; 2011.

Dimaio D, Dimaio D. Forensic Pathology. 2nd ed: CRC Press; 2001.

Kilpatrick CW. Noncryogenic preservation of mammalian tissues for DNA extraction: an assessment of storage methods. Biochem Genet. 2002;40(1-2):53-62.

Knight B. Forensic Pathology. 4th ed: CRC Press; 2016.

Longmire JL, Maltbie M, Baker RJ. Use of" lysis buffer" in DNA isolation and its implication for museum collections: Museum of Texas Tech University; 1997.

Michaud CL, Foran DR. Simplified field preservation of tissues for subsequent DNA analyses. Journal of forensic sciences. 2011;56(4):846-52.

Nagy ZT. A hands-on overview of tissue preservation methods for molecular genetic analyses. Organisms Diversity & Evolution. 2010;10(1):91-105.

Phengon P, Wongwiggarn S, Panvisavas N. Analysis of DNA from degraded tissue. Forensic Science International: Genetics Supplement Series. 2008;1(1):439-41.

Seutin G, White BN, Boag PT. Preservation of avian blood and tissue samples for DNA analyses. Canadian Journal of Zoology. 1991;69(1):82-90.