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A Comparative Study on Forensic Tissue Specimen Preserved in Formalin and Sodium Chloride

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Abstract

Forensic specimens serve as a vital clue in criminal investigation and in efficient administration of justice. The forensic specimens which are highly limited by its quality and quantity require a high throughput analysis to obtain a reproducible result for further action. Preservation of tissue specimen in sodium chloride is suggested for routine forensic DNA analysis. Occasionally, unknowingly the tissue specimens are preserved in formalin in hospitals and forwarded to the laboratory for DNA analysis. The present study demonstrates the effect of formalin and sodium chloride on tissue specimens and their influence on DNA profiling. A comparative study between forensic tissue specimen preserved in formalin and sodium chloride demonstrated that formalin has a negative effect on tissue specimen and significantly affects the outcome of DNA profiling results.

Keywords: DNA analysis; DNA profiling; Forensic science; Formalin; Preservative; Tissue specimen

Introduction

Forensic science which begins at the crime scene is the application of scientific knowledge of various branches of science in the investigation of crime. Whenever there is a contact between two things at a crime scene or elsewhere, there is a mutual cross transfer of evidence [1]. Therefore any type of evidence collected from the scene of crime serve as an important clue in crime investigation by linking the criminal with the crime. The evidence available must be properly collected, and preserved for analysis otherwise no amount of sophisticated laboratory instrumentation and technical expertise can rescue from potential loss or adverse circumstances.

Forensic tissue specimen is a type of biological evidence that requires to be preserved preferably in sodium chloride for further analysis in DNA division of Forensic sciences laboratory. Human tissue specimen of any type, source, and nature are subjected to various steps of protocols for DNA isolation. The isolated DNA is then quantified, amplified, and analyzed by Genetic analyzer to obtain a typeable DNA profile. Obtaining a reproducible, well balanced and good quality DNA profile is the ultimatum in DNA analysis. Formalin is used as a tissue fixative playing significant role in histopathology. As formalin is a strong disinfectant and tissue hardener, it is also used to preserve biological specimens. Occasionally, instead of sodium chloride, formalin is added as a preservative for tissue specimens that are forwarded to the laboratory for DNA analysis.

In the present study, the results of DNA profiling are compared between forensic tissue specimen preserved in formalin and sodium chloride. The mechanism behind the effect of formalin and sodium chloride on tissue specimen and thereby the outcome of profiling result is also discussed.

Materials and Methods

Study samples

Clinical sample of products of conception was used in this study. A case of sexual assault was registered u/s sec 6 of protection of child from sexual offences (POCSO) act 2012. The victim aged 16 years who has subsequently become pregnant by the assault was subjected to medical termination of pregnancy by the court by the medical termination of pregnancy (MTP) Act, 1971 taking her age and health into consideration. The procedure of incomplete abortion was performed by the method of manual vacuum aspiration (MVA) and portions of products of conception were preserved separately both in formalin and sodium chloride and were forwarded to forensic sciences laboratory for DNA analysis. These two samples served as the source of study samples.

Isolation of DNA

DNA was isolated from study samples using tissue protocol by BIOROBOT EZ1 DSP Workstation (Qiagen). Briefly, 50 mg of tissue sample preserved in formalin/sodium chloride was mixed with 200 µL ATL buffer and incubated at 85°C for 10 minutes. 20 µL proteinase K was added, vortexed and incubated at 56°C for 1 hour. The DNA extracted using EZ1 DNA Investigator Kit (Qiagen) was then stored at -20°C.

DNA quantification

The DNA quantity of the samples was determined by Real time - polymerase chain reaction (PCR) using Quantifiler Duo DNA Quantification kit (Applied Biosystems). Briefly, 2 µL study sample was mixed with 23 µL master mix containing 12.5 µL reaction mix and 10.5 µL primer and analyzed on ABI PRISM 7500 Sequence Detection Systems (Applied Biosystems) along with controls and standards; about 1 ng DNA was used for further analysis.

PCR amplification and DNA denaturation

Following DNA isolation, specific short tandem repeat (STR) regions of DNA useful in forensic investigation are amplified by PCR using AmpFlSTR Identifiler Plus PCR Amplification kit for amelogenin sex locus and also 15 autosomal STR loci namely D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818 and FGA. In brief, 1 ng (5 µL) of quantified DNA was added to 7.5 µL of PCR amplification reaction mixture containing 5 µL of AmpFlSTR Identifiler plus master mix and 2.5 µL of AmpFlSTR Identifiler plus primer.

Amplification was performed in MicroAmp Optical 96-well reaction plate (Applied Biosystems) in the GeneAmp PCR system 9700 with a gold-plated silver block (Applied Biosystems) using two-step PCR cycling protocol consisting of enzyme activation at 95°C for 11 min, followed by 28 cycles of denaturation at 94°C for 0.2 min and annealing/extension at 59°C for 3 min. A final extension step was performed at 60°C for 10 min, followed by a hold at 4°C.

The amplified product which is double stranded in nature is converted into single strands by performing a denaturation step at 95°C for 3 minutes followed by 4°C for 3 minutes using Hi-Di formamide in a PCR thermal cycler (Biometra).

Sample electrophoresis and data analysis

PCR products were separated and detected on the 3130 × l Genetic Analyzer using the specified G5 variable binning modules (Applied Biosystems). Samples were prepared by adding 1 µL of the PCR product or allelic ladder to 11 µL of formamide-LIZ solution (10.7 µL of deionized Hi-Di formamide and 0.3 µL of GeneScan 500 LIZ size standard; Applied Biosystems).

Capillary electrophoresis was carried out when samples were injected at 3 kV for 10 sec and electrophoresed at 15 kV for 1500 sec in performance optimized polymer-4 (POP-4) with a run temperature of 60°C. Following data collection, electrophoresis results were analyzed using GeneMapper ID-X software v1.5 (Applied Biosystems). Allele peaks were interpreted when the peak heights were ≥ 50 relative fluorescence units (RFU). Fluorescence based detection markers increased the sensitivity of measuring PCR-amplified STR alleles. After detecting the STR alleles sample genotyping was performed by determining the number of repeats in a DNA sequence.

Results

Zero DNA profile from formalin preserved tissue specimen

Preservation of tissue specimen in formalin limited the quantity of extractable DNA yielding a zero DNA profile (Figure 1A). When the peak height of the same tissue specimen was analyzed at ≤ 50 RFU, incomplete allele calls were observed at three loci alone namely D8S1179, D16S539 and vWA but with artifacts also being observed at D8S1179 and vWA (Figure 1B). On examination of the raw data, the unsuccessful DNA profiling result was confirmed with the insignificant levels of amplifiable DNA being present in the sample (Figure 1C).

Typeable DNA profile from sodium chloride preserved tissue specimen

Forensic tissue specimen preserved in sodium chloride in contrary to samples preserved in formalin, was able to yield sufficient amount of extractable DNA and hence a successful full, typeable DNA profile was generated (Figure 2A). All the allele peaks in the DNA profile were well balanced, with the allele calls being observed at all the STR loci tested. Inconsistent with this, the raw data also revealed the prevalence of amplifiable DNA in the sample, the factor responsible for typeable DNA profile (Figure 2B).

Discussion

Forensic tissue specimens serve as one of the important evidentiary sources in the investigation of crime especially in POCSO act and sexual assault cases. Tissue samples provide a robust way of analysis and can be easily processed compared to bone and other biological samples. Formalin has a wide usage for preservation of biological material. Tissue specimen that is highly preferred for histopathological studies in hospitals are routinely preserved in formalin. Following the same trend, occasionally the tissue samples that are need to be sent to Forensic Sciences laboratories for DNA examination are also preserved in formalin and forwarded for analysis.

In the present study, the outcome of the DNA profiling results was compared between the tissue specimens preserved in formalin and sodium chloride. A zero DNA profile was obtained with tissue sample preserved in formalin (Figure 1A). When the same sample was analyzed at RFU ≤ 50, allele calls, though incomplete were observed at 2-3 loci (Figure 1B). Inconsistent with this, the raw data also confirmed the DNA profiling result with the presence of insignificant levels of amplifiable DNA (Figure 1C). However tissue sample preserved in sodium chloride demonstrated a typeable, full DNA profile. The profile was well balanced, and the allele calling was observed at all the 15 STR loci tested (Figure 2A); the raw data demonstrating the presence of significant amount of amplifiable DNA (Figure 2B).

The consequences of tissue preservation with formalin on the integrity of the extracted DNA have been described in a number of studies [2,3]. Do and Dobrovic [4] reported that extensive fragmentation of DNA by formalin significantly reduces the amount of amplifiable templates available for PCR amplification. In accordance with this, our current study revealed that formalin inhibits amplicon production significantly affecting the outcome of DNA profiling result.

DNA is repaired with great efficiency in living cells, but this repair ceases upon death of the organism or preservation of a sample. Depending on the conditions of preservation, the DNA in such samples degrades more or less strongly over time and often becomes inaccessible to genetic studies. Formalin heavily interferes with PCR-based STR typing because of time- dependent degradation and cross-linking of DNA often leading to no results after fixation longer than 72 hours [5]. Ludyga et al. [6] have also reported that fragmentation of DNA in formalin fixed tissues was shown to be increased with longer storage time.

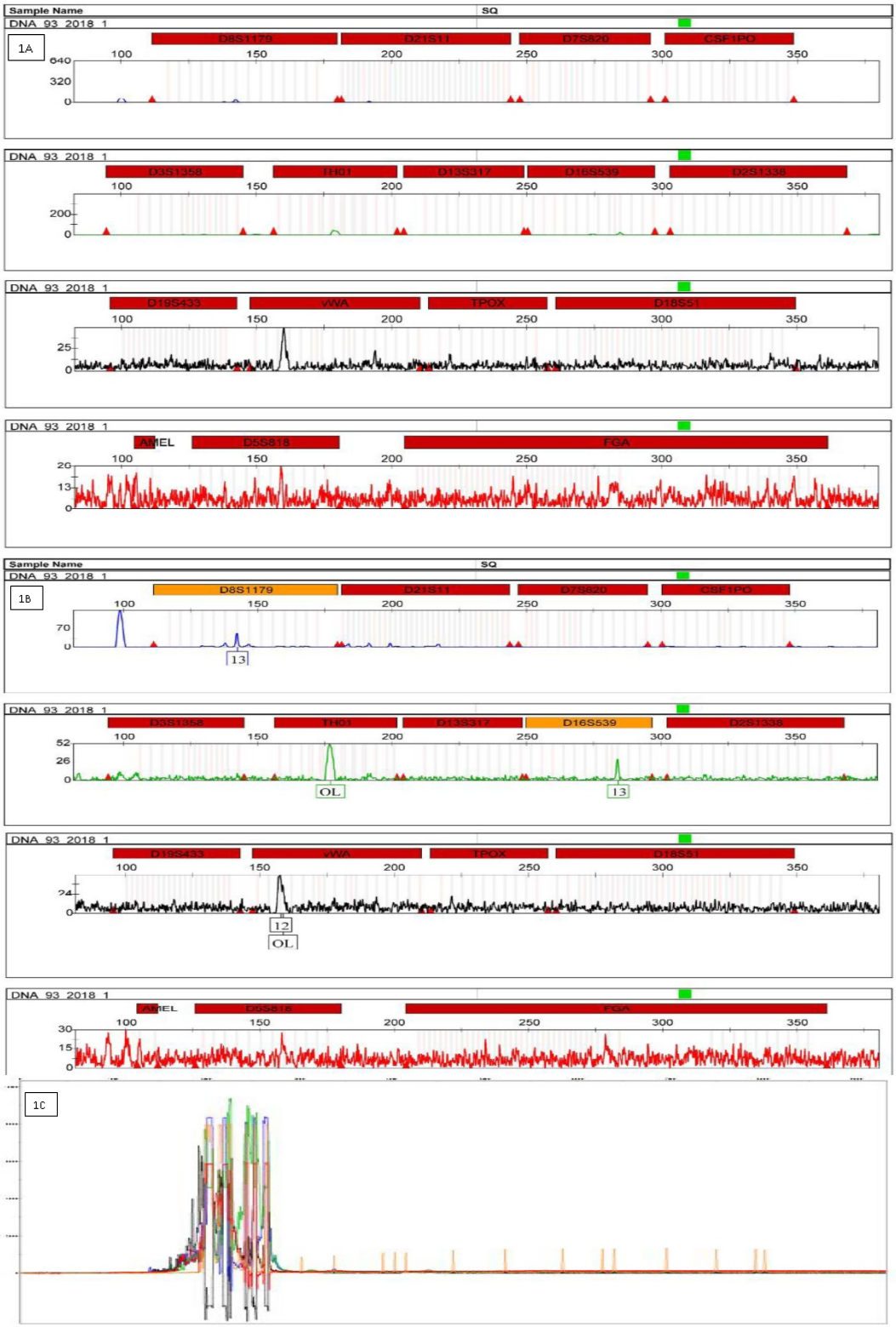

Figure 1: (A) Zero DNA profile from formalin preserved tissue specimen; (B) Incomplete allele calls observed at few loci when analysed at RFU ≤ 50; (C) Raw data revealing the absence of amplifiable DNA.

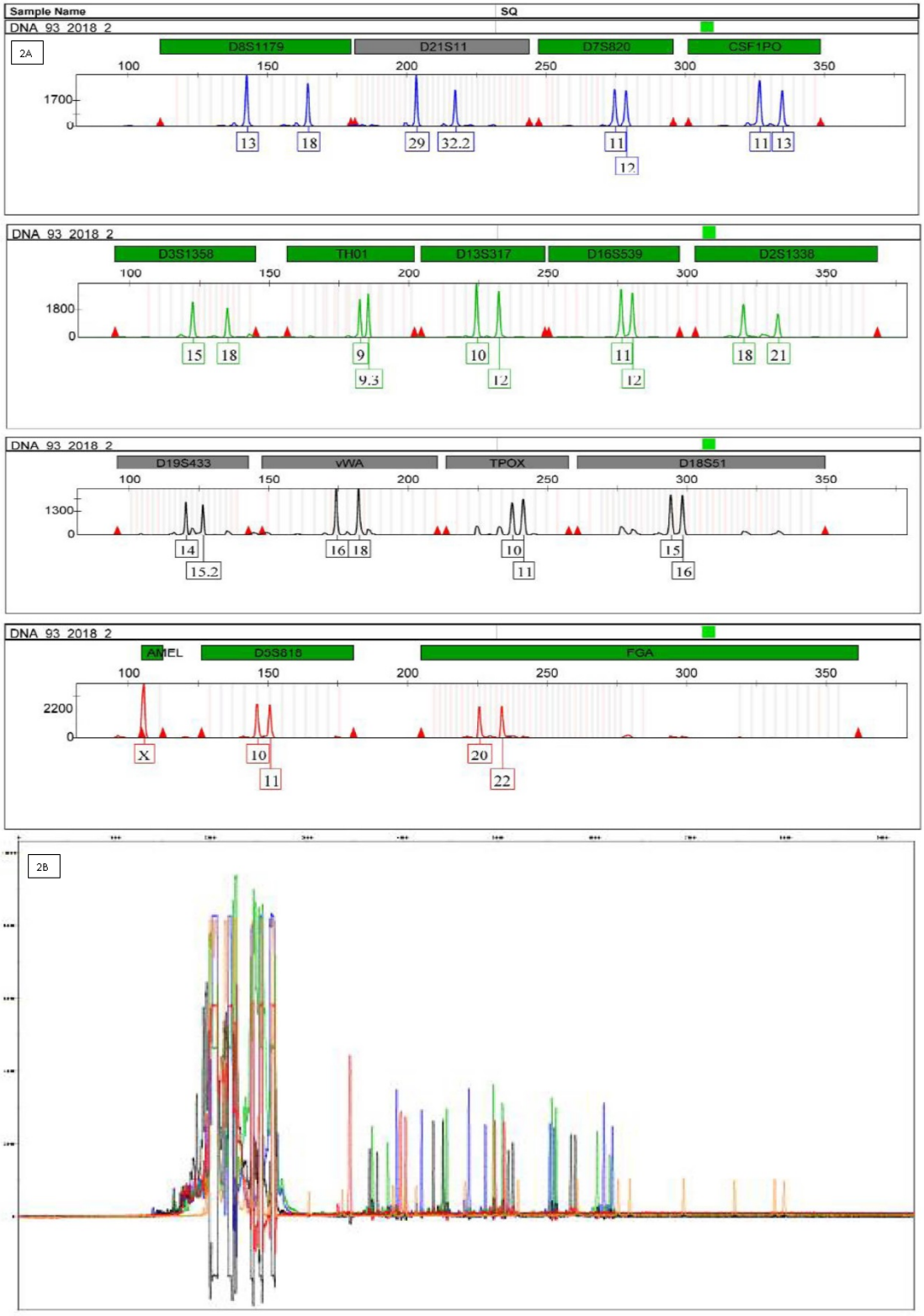

Figure 2: (A) Typeable DNA profile from sodium chloride preserved tissue specimen; (B) Raw data revealing the presence of amplifiable DNA.

Hence PCR success rate of DNA from older-formalin fixed tissues was shown to be decreased. Consistent with this, the zero DNA profile observed in the present study is due to tissue sample that has been preserved in formalin for a longer period. The absence of allele calls might be due to the extensive fragmentation of DNA during storage significantly lowering the amount of amplifiable DNA templates. Gino et al. [7] have also demonstrated that the DNA recovered from the samples fixed with formalin was lower and highly degraded. Further it was added that a lack of amplification of greater than 200 bp loci was observed for these samples.

Direct use of formalin preserved samples may affect the autosomal STR analysis resulting in false- negative effects [8]. Accordingly, the present study demonstrated partial DNA profile with the presence of allele calls at three loci alone namely D8S1179, D16S539, and vWA but with artifacts also being observed at D8S1179 and vWA in comparison with the result from sodium chloride preserved tissue specimen. Corroborating our study results, Williams et al. [9] have reported the prevalence of artificial mutations in the ratio of 1:500 bases in formalin fixed cancer cells. Artifacts could be the consequence of formalin damaging or cross linking cytosine nucleotides, on either strand, so that the Taq DNA polymerase would not recognize them and instead of a guanosine, an adenosine is incorporated thereby an artificial C-T or G-A mutation is created. Also, damaged DNA has been described to promote jumping between templates during enzymatic amplification. Taq DNA polymerase may insert an adenosine residue when it encounters the end of a template molecule, then jump to another template and continue the extension. As a result, an artificial mutation may be produced and amplified [10].

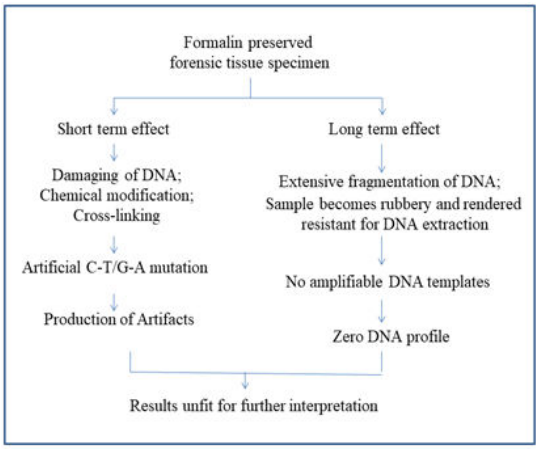

Figure 3: Effect of formalin on forensic tissue specimens.

The current study on the effect of formalin and sodium chloride on forensic tissue specimen corroborates the findings reported earlier [3,11-13]. Formaldehyde preserves tissue morphology, but it inhibits modern genetic analytical techniques such as PCR and DNA sequencing. Tissue lysis is the main obstacle in obtaining DNA from formalin exposed tissues. This is due to cross-linking or adducts formation by formalin that inhibits DNA extraction and making DNA more susceptible to structural damage and fragmentation during extraction (Figure 3). On the other hand, use of sodium chloride as a preservative plays a significant role in maintaining the integrity of the sample. Success rate was high and reproducible results were produced even under conditions of longer storage period.

Conclusion

The study reinforces the importance of use of sodium chloride as a preservative rather than formalin for tissue specimens for forensic DNA analysis. Damaging effects, chemical modification, cross-linking and adducts formation by formalin on long-term exposure makes the tissue rubbery, rendering it resistant for DNA extraction and thereby lowering the number of amplifiable DNA templates. However, a typeable, full DNA profile was obtained when sodium chloride was used as preservative. Hence tissue specimens that are forwarded for forensic DNA analysis must be preserved especially in sodium chloride for obtaining highly reproducible results which could aid in and expedite the process of criminal investigation.

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