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Extraction of High Thoughtput DNA from Heat Degradaded Skeletal Remains – An Analysis

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A B S T R A C T

Victim identification is necessary due to many logical reasons such as to provide a closure to the grieving family, to settle dispute over the culture and religion to which the person belongs to and, to perform forensic analysis of the scene of crime. However, most natural and man-made disasters are accompanied by heat, fire, water and heavy mechanical force, all of which could cause degradation of biological samples. Skeletal sample when exposed to a temperature more than 200 degree Celsius for a significant period of time results in extreme to severe debasement of organic matrix present in it. As a result, the DNA degradation index is a key component in determining whether or not to proceed with amplification. This paper reviews the effect of temperature and time duration exposure to the temperature on the quantity and quality of DNA. it also demonstrates the amount of STRs that can be amplified when the remains are exposed to such environment. The study concludes that even with high efficiency automated DNA extraction kit chances of recovering unfragmented DNA is almost null. The degradation Index was reported to be higher in such as samples. High CT values indicate increased inhibition due to extreme contamination and comingling of the samples. However, some studies also concluded that partial identification is possible when efficiency of amplification is increased using short amplicons and shorter primers. Therefore, it is safe to conclude that even though identification using nuclear DNA for such samples proven dead end, with advancements in techniques such as development of mini-STRs for identity matching and the development of more efficient PCR kits, the challenge of profiling such DNAs will be overcome**.**

Keywords: Heat degradation, skeletal remains, DNA, DNA fingerprinting, Mini-STRs, STRs.

Introduction

The functionality and importance of bones and the skeletal system in the human body is arguably unmatched, Since, it comprises the entire framework of human body. Human skeletal system is that basic structure which provides the shapes to each morphological characteristics that a person possesses. Human skeletal system, therefore without any doubt generates the features that we currently apply for the anthropometric identification of a human being. While Bones are the most important component required for anthropometric identification, The challenge is to determine the identity of a person, when his/her entire skeleton has been severely charred and fragmented rendering anthropometric identification impossible and futile. While there are several other alternatives of anthropometry discovered with the advent of latest technology, none of them proved more effective than the technique of DNA fingerprinting.

However, the technique of DNA fingerprinting has it's own limitations when it comes to the identification of mass disaster victims as exposure to extreme heat, light and force upon the organic part of the body causes severe degradation of the biological matrix. The only organic(living) part of the body to survive such extremities are the ones which are protected by hard inorganic matrix. Human skeletal system consists of bones and cartilages of different shapes and sizes. Bone tissue is a complex material made up of three basic components: a substantial inorganic proportion (about 70%), a much lesser organic fraction (approximately 20%), and water (about 10%w/w). These component's relative proportions vary depending on the kind of bone (cortical or trabecular) and can change with development and pathology. (Kuhn et al., 2008; Pasteris, 2014; Weiner, 2010, 102, 105; White and Folkens, 2005, 33).

When a human body is exposed to fire of sufficient intensity, sufficient temperature and for a sufficient time duration Charring results in deformation and fragmentation of the tissue therefore the tissue loses its characteristic appearance. Charring starts when the temperature of fire reaches more than 500 degrees Celsius. While an extreme pressure exerted over a significant period of time tend to cause similar effects. Phenomena such as fragmentation, comingling and contamination results in loss of viability of biological samples. In most of the cases only structural components are harmed and extraction of DNA would still be possible. However, cases such as world trade Centre Attack 2001, Given the remains were fragmented and it was nearly impossible to identify someone solely by their teeth, DNA analysis was seen as essential for identification. It will be easier to successfully identify people even when the bones are substantially destroyed if there is a better knowledge of the transformations that a burnt bone undergoes and how genetically analyse them in order to conduct a successful identification study.

However, identification through DNA profiling is limited to cases where large autosomal fragments are generated as the result of amplification. Since a thermal disaster may cause heat degradation of the skeletal remains, the objective of this study is to analyse the effect of temperature and related factors on the quantity and quality DNA yield. This paper also discusses the limitation of STR profiling for such an extreme case. Finally, we will discuss the future considerations in order to generate identity profile of the victims.

METHODOLOGY –

This paper will focus on the study of two major categories of extraction methods viz- Manual and Automated Extraction. In Manual Extraction method we will mainly focus on Phenol-Chloroform-isoamyl alcohol method which is the most commonly used method in the third world countries. Automated Extraction Procedure is more recent and advanced DNA extraction method which is less popular in the third world countries. In order to extract DNA from charred bones, decalcification of the bone is necessary as the Calcium and Magnesium ion found within the bone's inorganic matrix act as inhibitors of the polymerase chain reaction, therefore decreasing the DNA yield. The decalcification procedure requires chemical chelating reagents such as EDTA which chelate out calcium and magnesium ion from the solution. The decalcification procedure is necessary but time consuming. In the Decalcification procedure Each bone was then washed with a brush and EDTA for 20 minutes in boiling water before being rinsed with water and dried at 56°C for an entire night. 500 mg of the finely ground bone powder from each dried bone was utilised in one DNA extraction procedure after the bones were crushed into a powder in a sterile environment. After decalcification, the charred sample is digested with lysis buffer consisting tris base, NaCl, EDTA, SDS, Proteinase K and DTT. The chelex-based extraction method. Chelex was used to recover human DNA from burnt bones according to the procedure described by Amory et al. Chelex solution (20 g of Chelex in 200 mL). 500 mg of powder from each charred bone was mixed with 0.12 g of Tris Buffer and then incubated at 95C for 20 minutes. For three minutes, the samples were spun at 11,000 g. DNA-containing supernatant was transferred to fresh tubes. The samples were kept at 4C until they underwent additional processing (Uzair et.al; 2017). In organic extraction, a mixture of phenol chloroform and isoamyl alcohol is used to extract the DNA, extracted DNA is present in the aq. Solution while protein and lipid contamination are separated to the organic layer. Another well known procedure to extract DNA is the total demineralisation technique in which 0.50 – 0.59 g of bone powder incubated in 15 ml of lysis buffer (0.5 M EDTA [Gibco, Invitrogen Corporation, Carlsbad, CA] and 1% N-laurylsarcosinate [Fluka BioChemika, Buchs, Switzerland] and 10 mg of proteinase K [Promega, Madison, WI, USA]). The samples were incubated overnight in an incubator at 56 degrees Celsius on a thermal shaker. The next day, the tubes were centrifuged for 5 min at 1800g to pellet any particulates, the lysate was concentrated down to approximately 300 ml using an Amicon filter 100K [Millipore, Billerica, MA, USA]. The concentrated lysate was mixed with five volumes of PBI buffer (i.e., 1500 ml) [QIAGEN, Hilden, Germany] and spun through a QIAquick column (QIAGEN). Due to the large volume the samples were spun through the column in three rounds of 600 ml each. The membrane of the QIAquick column was washed three times using 750 ml of PE buffer (QIAGEN) and the DNA was eluted in 50 ml of EB buffer (QIAGEN) (Sylvain A. et.al 2012).

RESULT AND DISCUSSION –

The DNA is then Quantified using qRT-PCR before proceeding to the amplification process. The amount of PCR item in the dramatic stage is corresponded to the underlying grouping of DNA. This is finished by Choosing erratic fluorescent edge with condition that it exists in the remarkable period of the response. At the point when the fluorescence crosses this worth the cycle is named as "the limit cycle (CT or ΔCT)". Higher CT Value implies more modest beginning DNA Concentration as well as the other way around. The standard CT Values lies in scope of 0.1-0.2 where 0.2 and 0.1 shows very low and high enhancement viability respectively. To comprehend the pattern in corruption happening when the example is presented to a rising temperature for a rising time frame, we want to communicate it's degradation file. The Degradation Index (DI) of a criminological Exhibits can be determined by the accompanying process DI = concentration of small DNA target/concentration of long DNA target. The DI<1 demonstrates more noteworthy measure of enormous DNA pieces which implies less corruption while DI>1 shows huge convergence of more modest DNA sections which shows more debasement. The quantified DNA is then proceeded to Amplification process done using a thermal cycler and specific markers are amplified using different amplification kits. The amount of STR markers amplified determines the efficacy of identification process. More the number of STRs amplified more will be the match probability. According to ICMP, a profile having 11 markers amplified including Amelogenin marker is suitable for identification. The STR profiles are analysed in an electropherogram generated by Genetic analyser.

Sylvain A. et.al 2012 Conducted an experimental study on 40 environmentally degraded skeletal samples using two protocols: ICMP protocol and full demineralisation protocol. Additionally, they purified the concentrate using PBI Buffer adjusted to 250 microlitres. They performed the DNA extraction using Qiagen's QIAcube Kit. The result shows an average CT value of 30 which was further reduced to below 30 by purification process. The average DNA yield was reported to be 18.4% for FD protocol and 10.5% for automated extraction procedure. Out of 40 Samples, 19 profiles made to the ICMP criteria while 6 samples yielded full profiles. For Full demineralisation procedure the Success percentage of STR profiling was 62%. Another Study conducted by total 10 burnt bone samples are collected and extraction of DNA was performed using organic extraction, chelex based method, total demineralisation and using Qiagen's QIAmp Investigator kit. The results showed elevated CT values up to four cycles. Out of 9, 6 samples showed complete inhibition and two samples showed no inhibition. The DNA extracted using Qiagen QIAmp investigator kit showed two samples with slight inhibition. Three samples through organic and two samples through total demineralisation showed inhibition. DNA isolated from bone sample using total demineralisation accounted for 0.284-38ng/mg and complete profiles are generated for 9 out of 10 samples which are extracted either using total demineralisation or by investigator kit. The organic method of extraction proved less effective as it produced a range of 0.08-182ng/mg of DNA out of which two samples generated no profiles and five generated full profiles. A study conducted by AM where they exposed fibula fragments to a temperature of 100-1000 degree Celsius for 5 minutes and extracted DNA from it using AMPFSTR SGM Plus Kit and AMPFSTR Minifiler kit by Applied Biosystems,

they observed almost complete degradation of nuclear DNA for temperature above 900 degrees Celsius. Similar observation was made by a study conducted by where the amount of DNA along with the STR profiles decreased significantly as the temperature and time of exposure of heat to the bone samples increased from 200-800 degree Celsius and 30-120 minutes. At 800 degrees Celsius the amount of DNA extracted was negligible and no STRs are amplified. While partial profiles are generated for two sample exposed to 200 degrees Celsius for a duration of 30 minutes. A study conducted by Jamie et al., 2021 on heat compromised bones show that the probability of STR amplification significantly decreases as the temperature increases. According to the study the MDQ probability of DNA decreases from 1/100 to 0 when temperature was raised from 180 to 200 degree Celsius. Bones compromised by temperature above 200 degrees Celsius shows no amplification while for bones exposed to temperature less than 200-degree Celsius shows partial amplification of 1 out of 100 profiles. Since average temperature in an accident scene caused by a mass fire incident is always more than 200 degrees Celsius, the data shows complete to near complete degradation of DNA samples. Studies have also shown that some STRs resists heat more than certain STRs, therefore making them more liable to be amplified. Most of the mini-STR loci belongs to this category. Since these STRs are smaller in size they are easily preserved. Therefore Mini-Filers kits proven to be more valuable in these case scenarios. Smaller Primers and the reduced gap in the flanking region results in more efficient amplification of STR loci. One such study conducted by Yudianto and Setiawan in 2020 focused on three mini-STR loci viz – FGA, CSF1PO and D2S11 revealed that even at 700 degrees Celsius a teeth sample exposed for 20-30 minutes shows significant STR amplification for all three markers except for FGA which is imperceptible at 700 degrees Celsius. D2S11 is perceptible to a temperature up-to 700 degrees Celsius for 89-129 base pair range.

In conclusion, the amount of DNA decreases significantly as we increase the temperature, the high CT values obtained for samples indicate high PCR inhibition due to presence of inhibitors such as Ca and Mg ions, phenol, proteins and lipids which were probably present

TEMPERATURE °C	TIME OF EXPOSURE(MINS)	NO. OF LOCUS AMPLIFIED
200	30	13
200	30	5
200	30	1
200	60	3
200	90	5
200	90	6
200	120	3
200	120	2
400	30	1
400	30	1
400	60	0
400	60	1
400	120	1
600	60	7
800	120	15

Table 1 NUMBER OF LOCI AMPLIFIED FOR DIFFERENT TEMPERATURE RANGE

within the DNA sample as contaminants. The high degradation Index indicates fragmentation of DNA into smaller fragments which cannot be amplified by the kits and decrease in the number of amplified loci will result in the decrease in match probability and would not be much effective in the positive identification of victims. However. Studies shown that the effectiveness of identification through DNA can be increased if we negate the root causes of lack of positive matches. The amount of STR makers for identification can be increased by protecting the samples from environmental degradation, avoid contamination as much as possible and performing methods such as total demineralisation and automated silica-based DNA extraction using investigator kits. To increase the percentage of amplification for degraded samples, the size of amplicons must be reduced. The reduction of flanking gaps will cause an increase in primer efficiency and smaller primer size, which in turn results in amplification of small sized loci. Small sized loci are not as much efficient. The studies have also shown that decalcification procedure also affects the CT values of a DNA sample solution. Since DNA in any hard tissue is protected under inorganic matrix and needed to be set free in order to be extracted.

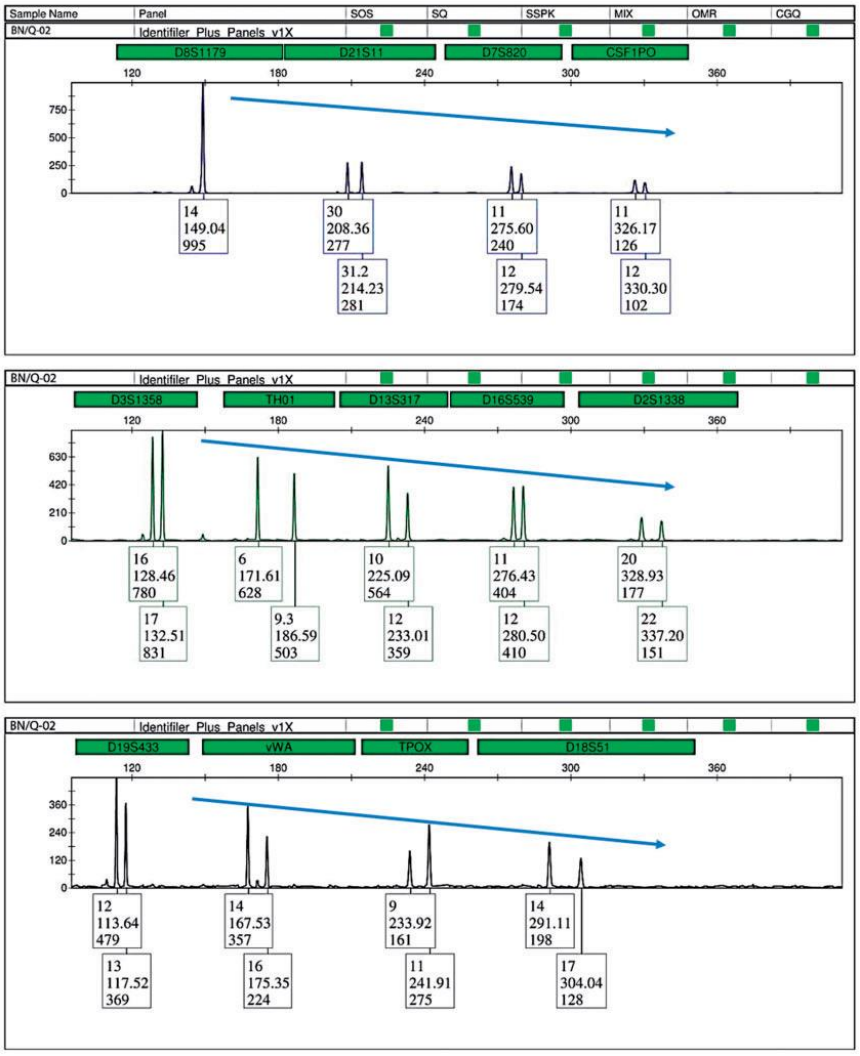

Figure 1Electropherogram showing the DNA degradation pattern

SOURCE -

Uzair, A., Rasool, N., & Wasim, M. (Year). Evaluation of different methods for DNA extraction from human burnt bones and the generation of genetic profiles for identification. J

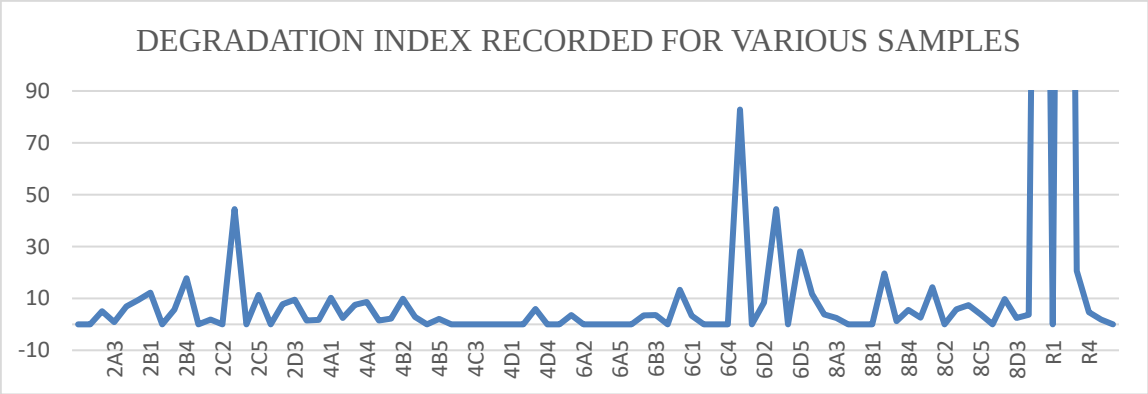

Figure 2 DEGRADATION INDEX FOR VARIOUS TEMPERATURE RANGE

Source - Chakraborty, S., & Chauhan, T. (Year). Extraction and quantification of DNA from heat induced altered bones. Masnuscript submitted for review

Figure 3 Target DNA sample for amplification

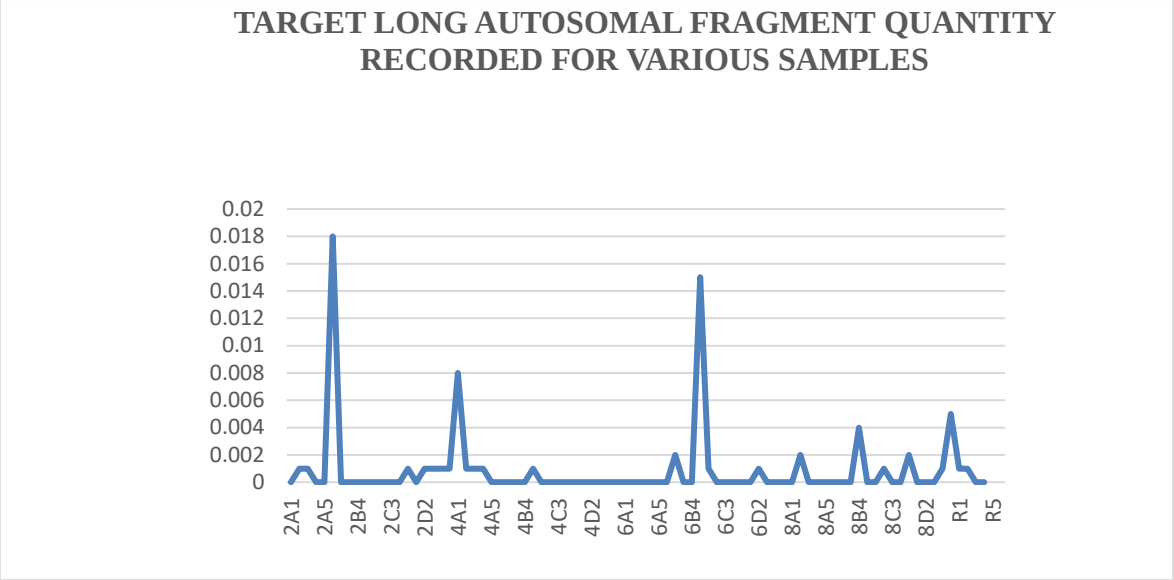

Figure 3 Target DNA sample for amplification

Source - Chakraborty, S., & Chauhan, T. (Year). Extraction and quantification of DNA from heat induced altered bones. Manuscript submitted for review

Therefore, in order to have an effective DNA extraction, the decalcification procedure need to be performed twice a day for a period of three to four days.

CONCLUSIONS –

Since the DNA of a hard tissue is trapped within its inorganic matrix, it is difficult as it to extract it out. When we add extra factors like heat, light and contamination to which the DNA is supposed to be exposed, the extraction becomes near to impossible, therefore in a mass disaster case, Nuclear DNA isn't much reliable due to its severe degradation. With the development of advances in the DNA extraction procedures and development of short amplicons Mini-STRs, the DNA extraction is becoming more effective for such extreme cases. In some cases, mitochondrial DNA typing technology and SNP based typing are being utilized for positive identification but both mitochondrial DNA and SNP based typing has its own disadvantages.

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