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Nuclei DNA Damage Due to Extreme High-Temperature Exposure during Forensic Identification Examination

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Abstract

Accurate personal identification is important in investigations because an error in the identification process may bring fatal consequences during trial. The most common identification process is the Deoxyribonucleic acid [DNA] analysis. Degraded DNA sample due to extremely high-temperature exposure may limit DNA analysis. This study aimed to analyze DNA damage patterns caused by an extremely high temperature using short tandem repeat (STR) CODIS marker. This study was conducted at the Forensic and Medicolegal Department, *Laboratorium Balai Besar Kesehatan Surabaya*, Ministry of Health of the Republic of Indonesia, Human Genetic Study Group of Universitas Airlangga, and Faculty of Science and Technology of Universitas Brawijaya Malang from July until October 2009. Results of PCR visualization using STR CODIS for costae demonstrated that the THO1 detection (+) in 1,250°C-40': 25% and the TPOX detection (+) in 1,000°C - 30': 50% whereas the results from molar teeth showed that the THOI locus detection (+) in 1,250°C-30': 25% and TPOX in 1,000°C-40': 50%. Results for PCR visualization using mini-STR CODIS for the costae presented that the mini-THOI in 1,250°C-20': 50% (+) while for the molar tooth the mini-THOI in 1,250°C-30': 25% (+) and mini-TPOX in 1,000°C-40': 50% (+). All loci were detected on costae and second molar teeth samples of the control group. Thus, extreme high-temperature exposure significantly decreased the DNA level of second costae and second molar tooth. Sequence patterns of STR loci successfully detected were TPOX, THO1, and CSF1PO.

Keywords: Costae, DNA level, extreme high temperature, molar tooth, short tandem repeat combined DNA index system

Kerusakan DNA Inti Karena Paparan Suhu Tinggi Selama Proses Identifikasi Forensik

Abstrak

Identifikasi melalui DNA secara tepat penting dalam penyelidikan karena kesalahan akan berakibat fatal selama proses persidangan. Salah satu keterbatasan adalah DNA yang terdegradasi karena paparan suhu yang sangat tinggi. Penelitian ini bertujuan menganalisis pola kerusakan DNA akibat suhu sangat tinggi menggunakan penanda CODIS *short tandem repeat* (STR). Penelitian dilakukan di Instalasi Kedokteran Forensik, Laboratorium Kemenkes, *Human Genetic Study Group*, dan Universitas Brawijaya Malang pada periode Juli sampai Oktober 2009. Visualisasi PCR menggunakan STR CODIS untuk *costae* adalah sebagai berikut: deteksi THO1 (+) pada 1250°C-40': 25%, deteksi TPOX (+) pada 1000°C-30': 50%, sedangkan hasil dari gigi molar adalah sebagai berikut: THOI locus detection (+) di 1250°C-30': 25% dan TPOX di 1000°C-40': 50%. Hasil visualisasi PCR menggunakan mini-STR CODIS untuk *costae* adalah sebagai berikut: mini-THOI pada 1250°C- 20': 50% (+) dan untuk gigi molar: mini-THOI pada 1250°C-30': 25% (+) dan mini-TPOX di 1000°C-40': 50% (+). Semua lokus terdeteksi pada kelompok kontrol pada sampel *costae* dan gigi molar kedua. Simpulan, paparan suhu tinggi yang ekstrim secara signifikan menurunkan tingkat DNA kosta dan gigi molar kedua.

Kata kunci: *Costae*, gigi molar, suhu sangat tinggi, s*hort tandem repeat combined* DNA *index system,* tingkat DNA

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Introduction

Forensic experts play an important role in forensic science, specifically during the identification of mass disaster victims. Identification through DNA analysis is an accurate and stable diagnostic tool. The genetic information of a person has similarities throughout his/her body cells and remains unchanged even after the person dies. During its development, the DNA analysis method has brought new limitations for its use. One of its limitations that might bring a serious problem for forensic DNA experts or other DNA experts is degraded DNA.[1-3]

Muscle tissue is an organ with abundant contents of the cell, where DNA can be found in its nucleus. Human muscle tissue is estimated to weigh half of the human's weight and consists of 600 types of tissue. Among the most commonly used or most recommended types of muscle tissue for DNA analysis are the psoas and masseter muscle. Psoas muscle is located in the bottom part of the abdominal posterior wall. Psoas muscle tissue is located behind the diaphragm and corresponds with quadratus lumbrorum muscle in which we can find artery, vein, and lymph node inside. Meanwhile, the masseter muscle is a masticating muscle with vertical fibers to help strengthen biting/chewing movements.[1,3]

Decomposition of a corpse is one of the challenges for the DNA analysis method. Although degraded DNA might provide great obstacles for forensic DNA experts, the experts have conducted research and studies to find a way for DNA analysis in certain conditions. Currently, the correlation between decomposition and fragmented DNA during forensic identification is still unclear.[4,5,6,7]

In DNA analysis, the unit for repetition length that is less than 1 kb (kilobase pair) is called short tandem repeat (STR). STR is popular because it has a short allele length (less than 1 kb), therefore making it easier to amplify through PCR and analyze degraded samples. FBI recommends 13 STR-CODIS loci for identification. Several studies showed that 3 STR loci [CSF1PO, THO1, TPOX] had high discriminant power for the Asian population.[1,3]

The purpose of the study is to investigate nuclei DNA damage after exposure to high temperatures using the STR-CODIS technique in forensic identification.

Methods

This was a laboratory experimental study to analyze DNA damage from costae and tooth DNA samples due to extremely high-temperature exposure through STR CODIS loci [CSF1PO, TPOX, THO1, miniCSF1PO, miniTHO1, miniTPOX]. This study used a randomized post-test only control group design.

The samples of this study were the second costae and molar tooth DNA from corpses. The reason why we chose the second costae and the second molar tooth was they contained more cells than the other parts of the body. All corpses that were included were bodies that remained unidentified until 48 hours [according to article 133[3] of KUHAP and PP no.81/1981]. The number of samples was 24-second costae and 24-second molar teeth and each of them was replicated 6 times so that the number of n (replication) was 6 from molar teeth and 6 from costaes. All of the costae and molar teeth were taken whole from the corpses without being cut and wrapped with aluminum foil in each sample. All of the samples were heated using muffle furnace Naberthemtool up to 1400^0C, the treatment for all samples was heated in 1000^0C for 20 minutes, 30 minutes, 40 minutes, in 1250^0C for 20 minutes, 30 minutes, 40 minutes, and in 1400^0C for 20 minutes, 30 minutes, 40 minutes, and control group. [1,8,9]

DNA samples (from the bone and molar tooth samples) were exposed to extremely high temperatures were extracted and isolated. These samples were collected from the person who is being autopsied process after 2x24 hours stayed at forensic installation. They did not have any houses so that they were usually called "*Tempat Tinggal Tidak Tetap*" (T4) or residency unknown. This research was approved by Dr. Soetomo Ethics Committee stated in Letter Number48/ panke.KKE/VI/2009. This research was conducted at Institute Tropical Disease Human Genetic, Biology Molecular Laboratorium Faculty of Science and Technology Universitas Brawijaya Malang. Materials for DNA extraction and isolation were DNAzol Reagent, Ethanol 100%, and 70 % solution. Afterward, DNA samples were amplified using the Polymerase Chain Reaction [PCR]. Materials for PCR were: PCR Mix [dNTP (ATP,CTP,TTP GTP), MgCl2, Taq Polimerase], nuclease-free water, primer-primer and mini primer Short Tandem Repeats [STR] Combined DNA Index System [CODIS] locus-locus, consisted of: (1) CSF1PO [Gen Bank Accession

X14720]: 5'-AACCTGAGTCTGCCAAGGACTAGC-3'&5'TTCCACACACCACTGGCCATCTTC-3'; (2) THO1 [Gen Bank Accession D00269]:5'-CTGGGCACGTGAGGGCAGCGTCT-3'&5'-TGCCGGAAGTCCATCCTCACAGTC-3'; (3) TPOX [Gen Bank Accession M68651]: 5'-ACTGGCACAGAACAGGCATCTAGG-3'&5'-GGAGGAACTGGGAACCACACAGGT-3'.

Instead of using primer, we also use some mini primers, such as: (1) miniCSF1PO5'-ACAGTAACTGCCTTCATAGATAG-3'&5'-GTGTCAGACCCTGTTCTAAGTA-3'; (2) mini TH01 5'-CCTGTTCCTCCCTTATTTCCC-3'&5'-GGGAACACAGACTCCATGGTG-3'; (3) mini TPOX 5'-CTTAGGGAACCCTCACTGAATG-3'&5'-GTCCTTGTCAGCGTTTATTTGC-3' (22).

PCR amplification cycles for CSF1PO, THOI, TPOX locus were as followed: initial denaturation 96°C-2 minutes, for 10 cycles [subsequent denaturation 94°C-1 minute, annealing 64°C-1 minute, extension 70°C–1 minute 30 seconds], for 30 cycles [denaturation 90°C-1 minute, annealing 64°C- 1 minute, extension 70^0C – 1 minute 30 seconds]. Whereas PCR amplification cycles for miniCSF1PO, miniTHOI, miniTPOX locus were as followed: Initial denaturation 96^0C-10 minutes, for 30 cycles [denaturation 94^0C -1minute, annealing 55°C-1 minute, extension 72°C–1 minute], final extension 65°C-45 minutes. PCR amplification product were visualized using Polyacrylamide agarose gel electrophorese [PAGE] and silver nitrate straining.[1]

The statistical analysis was conducted using both parametric and non-parametric statistics depending on the normality of the data. The parametric statistics consisted of an independent sample t-test while the non-parametric statistics comprised of the chi-square test, pearson chi-square, and fisher's exact test.

Results

This study began with extremely high-temperature exposure to the second costae and molar tooth samples in 1000°C for 20 minutes, 30 minutes, 40 minutes, 1250°C for 20 minutes, 30 minutes, 40 minutes, and 1400°C for 20 minutes, 30 minutes, 40 minutes. The measurement of DNA contents and purity from the extracted samples were conducted using UV-Visible Spectrophotometer. The result of DNA level measurement is presented in the following Table:

Table 1 showed the mean DNA level (µg/mL) From all the groups containing costae and second molar teeth samples, it was revealed that the mean DNA level both from Costae and second molar tooth in the control group (without exposure) was 275.50±10.35 and 220.25±10.25. This table was represented in Figure 2.

Figure 1 showed a significant decrease in DNA level from the second costae and molar tooth due to extremely high-temperature exposure. An increased temperature was inversely proportional to the DNA level from samples. Duration of exposure also affected the DNA level of the samples. It was also inversely proportional to the DNA level of the samples. A T-test revealed significant results ($p<0.05$) for a decrease of DNA level due to extremely high-temperature exposure.

Table 1 DNA Mean Level from Samples[1]

Exposure	DNA Level (µg/mL)	
	Costae	Second Molar Tooth
Without exposure	275.50±10.35	220.25±10.25
1000°C-20 minutes	68.73±05.98	78.75±07.25
1000°C-30 minutes	60.50±13.43	63.07±09.25
1000°C-40 minutes	57.34±01.33	60.98±11.25
1250°C-20 minutes	37.84±05.89	47.25±13.85
1250°C-30 minutes	30.78±10.86	29.23±09.25
1250°C-40 minutes	10.73±11.35	10.59±04.25
1400°C-20 minutes	25.73±11.50	15.56±10.85
1400°C-30 minutes	10.76±1.,87	16.36±10.76
1400°C-40 minutes	05.55±10.65	05.17±10.25

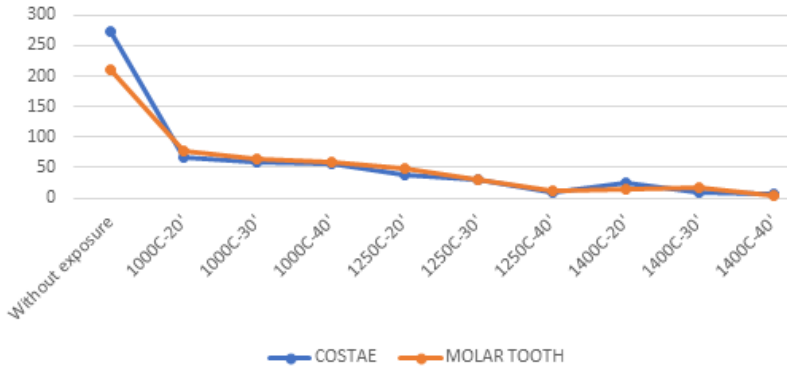

Figure 1 DNA Condition of Exposure Effects Samples[1]

This study result showed that extreme high-temperature exposure affected DNA level measurement. UV spectrophotometry revealed a significant decrease in DNA samples from costae and molar tooth samples.[8,9]

Figure 2 above shows that there are two variables among the PCR visualization results, namely: the temperature and time. Temperature consisted of 1,000°C, 1,250°C, 1,400 °C, and the time consist of 20 minutes, 30 minutes, and 40 minutes.

Table 2 explained the detection of all of the loci from primer CSF1PO, THO1, TPOX from costae, and molar tooth samples on both the without exposure group and the treatment group. The number 100, 75, 50, 25, and 0 reflected the percentage of locus primer. The + and - signs indicated whether the specified locus was detected or not. For example, all of the locus primer CSF1PO, THO1, and TPOX without exposure group from costae and the molar tooth was detected as indicated by numbers 100 in the column (+) and 0 in the column (-). All of the locus THO1 from costae samples on 1,000°C-20' exposure was still detected as much as 75% and it is not being detected as much as 25%.

Table 3 explained the detection of all the loci of the locus from mini primer CSF1PO,

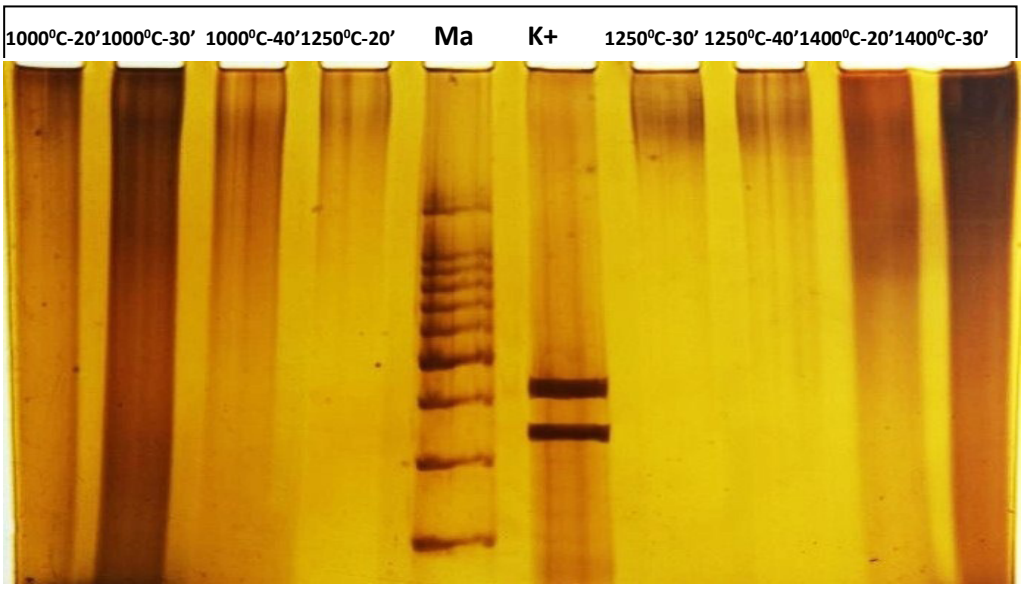

Figure 2 PCR Visualization Result of STR CODIS locus CSF1PO [321bp – 357bp], Ma [marker 100bp] in Molar Tooth Sample

Table 3 Results of Mini-CODIS Loci [CSF1PO, TPOX, THO1] Detection

Exposure	Costae [%]						Molar Tooth [%]					
	MiniCSF1PO		MiniTHOI		MiniTPOX		MiniCSF1PO		MiniTHOI		MiniTPOX	
	+	–	+	–	+	–	+	–	+	–	+	–
Without Exposure	100	0	100	0	100	0	100	0	100	0	100	0
1000°C												
20’	0	100	0	100	0	100	0	100	0	100	0	100
30’	0	100	0	100	0	100	0	100	0	100	0	100
40’	0	100	0	100	0	100	0	100	0	100	50	50
1250°C												
20’	0	100	50	50	0	100	0	100	0	100	0	100
30’	0	100	0	100	0	100	0	100	25	75	0	100
40’	0	100	0	100	0	100	0	100	0	100	0	100
1400°C												
20’	0	100	0	100	0	100	0	100	0	100	0	100
30’	0	100	0	100	0	100	0	100	0	100	0	100
40’	0	100	0	100	0	100	0	100	0	100	0	100

THO1, TPOX from costae, and molar tooth in without exposure group and treatment group. The number 100, 75, 50, 25, and 0 reflected the percentage of locus mini primer. The + and - signs indicated whether the loci were detected or not. For example All of the locus mini primer CSF1PO, THO1, and TPOX without exposure group from costae and the molar tooth is being detected, because there is a number 100 in the column (+) and 0 in the column (-). All of the locus miniTHO1 from costae samples on 1000°C - 20’ exposure is not detected because the number on column (+) is 0 and 100 is on column (-).

Table 2 Standard of STR CODIS loci [CSF1PO, TPOX, THO1] Detection

Exposure	Costae [%]						Molar Tooth[%]					
	CSF1PO		THOI		TPOX		CSF1PO		THOI		TPOX	
	+	–	+	–	+	–	+	–	+	–	+	–
Without Exposure	100	0	100	0	100	0	100	0	100	0	100	0
1000°C												
20'	0	100	75	25	75	25	0	100	50	50	25	75
30’	0	100	25	75	50	50	0	100	50	50	25	75
40’	0	100	0	100	0	100	0	100	25	75	50	50
1250°C												
20’	0	100	50	50	0	100	0	100	50	50	0	100
30’	0	100	0	100	0	100	0	100	25	75	0	100
40’	0	100	25	75	0	100	0	100	0	100	0	100
1400°C												
20’	0	100	0	100	0	100	0	100	0	100	0	100
30’	0	100	0	100	0	100	0	100	0	100	0	100
40’	0	100	0	100	0	100	0	100	0	100	0	100

Discussion

The reduction of DNA level due to extremely high-temperature exposure was not a limitation for further examination. DNA level that was needed for Short Tandem Repeat (STR) test only required a minimum of 0.25-2 ng, as shown in Table 1, therefore STR test could be conducted. DNA content factor is one of the most important aspects of amplification success in DNA analysis. A decrease of DNA level up to 1 ng might bring a decrease of STR's detection ability up to 95%.[6,8,11] The effect of high-temperature exposure, as occurs in the cremation process, is divided into 4 stages: dehydration, decomposition, inversion, and fusion. These stages are reflected through color changes. Color changes represent the ongoing chemical processes in bones and teeth due to high temperature exposure.[10,11]

DNA level is correlated with the number of nucleated cells in tissues. A bone has some nucleated bone cells (osteocytes) around 20,000–45,000/mm^3, whereas a tooth has some nucleated tooth cells (odontoblast) around 20,000–26,000/mm^3. Theoretically speaking, the amount of DNA that is contained in bone is higher than the amount in a tooth. The outer structure of bones and teeth in terms of protection also plays an important role in external exposure.[5,10,11]

Bone and tooth hardness depends on the level of inorganic materials contained in the matrix, whereas its strength depends on organic materials, especially collagen fibers. Teeth enamel consists of 96% inorganic minerals, mainly calcium and phosphorus, and only 4% of enamel consists of organic matter and water. The dentin is the inner part of the tooth structure that has a harder structure compared to the bone but is softer than enamel, where 70% of dentin consists of inorganic material (mainly calcium and phosphor) and 30% of dentin consists of organic material and water. Whereas 50% of bone consists of inorganic material. A second molar, based on its pulp size, is included as a taurodont (large pulp chamber) and patterned cusp, which is a cusp that has boxes (where the second molar has 20% more boxes), therefore making it the strongest tooth compared to other types of the tooth.[8,9]

The direct effect of extremely high-temperature exposure is the loss of fluid contained in the material. In dental materials, the weight decreases from 28.6–66.7%, and in bone materials the decrease is 65.1–91.8%. Dental materials also have higher hydroxyapatite (calcium-phosphate) content compared to bone.[9]

The amplification process began with DNA template preparation through extraction and isolation process using an extraction kit (DNAzol). The PCR process used a standard primer at STR CODIS loci [CSF1PO, THO1, TPOX], one example of the visualization image can be seen below. To determine the effects of extreme high-temperature exposure and its duration to DNA level both in bone or tooth, Short Tandem Repeat (STR) examination was conducted to certain loci using the PCR method. In this study, the DNAzol extraction method was used and followed by ethanol precipitation. The advantage of ethanol precipitation is that it can separate DNA through precipitation, while its disadvantage is that it can decrease the DNA level that is obtained through extraction. Before DNA extraction from the bones and teeth, decalcification was conducted to remove calcium content. In PCR visualization of STR CODIS loci, bands could be seen. This shows that the DNA template was able to stick to primers (Figure 2).[1,11,12]

Nucleated cells that contain DNA in bone or teeth are surrounded by a matrix containing calcium-phosphate element, which is an intercellular substance, therefore an effort is needed to "extract" DNA through decalcification phases. The need for decalcification and non-decalcification before DNA extraction from bones or teeth is still debatable until now. Opinions that support decalcification before the DNA extraction process argued that calcium might affect the amplification process. DNA extraction might be conducted without a decalcification process due to practicality and effectiveness consideration for the DNA extraction process. During DNA extraction without decalcification, it is expected that the duration of DNA profiling might be shortened and the identification process might be completed quickly and precisely.[3]

The bone DNA extraction method concluded that the fastest time to decalcify bone before the DNA extraction process is around 4 hours. This relatively time-consuming process makes the non-decalcification method for bone DNA extraction becomes something to consider. However, inorganic materials that protect bone and teeth cells might become an obstacle for DNA testing if no decalcification process were conducted.[1,8]

Decalcification might be done using several methods and materials, which include the use of chelating materials. The most commonly chelating material that is used is Ethylene Diamine Tetraacetic Acid (EDTA) in the form of iodized salt. Although it is acidic, it does not act

as a mineral nor as an organic acid but rather functions to capture metal ions, specifically calcium ions. Because of its nature, which can only bind to calcium ions, this compound only works on the outer layer of bones and teeth (apatite crystals). The decalcification process with chelating materials is slow but does not affect other tissue elements, even the enzymes contained in it are still active even though the bones or teeth have undergone the decalcification process.

The non-decalcification process will give better results because less DNA is lost in the preparation process. EDTA needs to be used carefully because it is a PCR inhibitor. Residues or presence of residual EDTA in DNA extraction results is something to consider. This is important because the presence of residual EDTA in DNA isolate that will be used as DNA templates in the PCR process might reduce Mg^{2+} concentration (as a catalyst in PCR) on an optimal PCR mix. In addition to residual EDTA, it is also necessary to be aware of PO_{4-},which contaminates DNA templates and is a PCR inhibitor.[1]

Although this study reported the decrease of DNA sample level as an effect of high-temperature exposure, it did not affect DNA testing success through Short Tandem Repeat (STR). DNA level needed for examination with STR tends to be less compared to other DNA tests, therefore the failure of DNA amplification is very small. Short Tandem Repeat is a popular marker of DNA repetition in forensic DNA because various loci on STR have small allele sizes (less than 1 kb and most are 300 bp in size). This makes STR to be easily amplified using the PCR method, even though in degraded samples. The number of repetitions in the STR marker itself varies greatly between individuals, therefore making it very effective to be used for human identification. Strengths of STR include high discriminant power (>0.9), heterozygotes>70%, low mutations, low 'stutter characteristics', stronger results and reproducibility, and is located on all chromosomes.[1,6]

The results of this study found that there was almost no difference between bone and tooth components' ability to protect nuclei and mitochondrial DNA during certain conditions, including high-temperature exposure. Bones and teeth components can protect bone and tooth DNA from damage that might cause fragmented DNA.[1]

The tables below provide results of PCR detection visualization of STR CODIS loci [CSF1PO, TPOX, THO1] and mini-STR CODIS loci [CSF1PO, THOI, TPOX]

Studies on STR CODIS as a whole has not been reported, only on a few primers [Table 2], as well as mini STR CODIS [table 3]. The result of this study was related to the use of STR CODIS. Sequence patterns of STR loci that were successfully detected were TPOX, THO1, and CSF1PO. This is by their respective GC content ratio. THO1 and TPOX GC content ratios had the same relative value of 0.48, compared to CSF1PO where its GC content ratio was 0.33. These loci were successfully detected because of differences in amplification products and the presence of GC content or guanine and cytosine bonds in each locus. GC content or guanine-cytosine bonds had a high level of stability against denaturation factors compared to bonds between Adenine and Thymine.[1,3,10]

Extreme high temperatures (1,000°C-1400°C) exposure significantly ($p<0.05$) reduced the DNA level of the second costae and molar tooth material. STR CODIS and mini-STR loci detection revealed that dental materials might provide good protection, where loci were still detected (25%) regardless of high temperature (1250°C-30'). Sequence patterns of STR loci that were successfully detected were TPOX, THO1, and CSF1PO. This is by their respective GC content ratio. GC content ratio for THO1 and TPOX had the same relative value of 0.48, compared to CSF1PO where its relative value was 0.33. This limitation of the study is the samples DNA was got from the corpses in Surabaya.

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