

Effect of Room Temperature and Length of Exposure to recover quality DNA from Earphone Swab-Derived 126bp and 143 Mitochondrial DNA at D- loop region

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Effect of Room Temperature and Length of Exposure to recover quality DNA from Earphone Swab-Derived 126bp and 143bp Mitochondrial DNA at D-loop region

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ABSTRACT :

Introduction: Exploration of crime scene devices left over and its surrounding is of significance in forensic identification. The significance is due to its association and/or potential harbor of biological evidence; ie., blood, blood spots, semen patches, vaginal swabs, buccal swabs, bones, or trace evidence. Such harboring of evidence from humankind proposes traceability under Locard's principle of contact.

Materials and Methods : Processing of cerumen expelled or traces logged from the 30 used earphones were subjected to 0, 1, 7, 14 and 20 days room temperature exposure.

Results : Length of exposure has a significant effect on decreasing the level of DNA steadily. Visualization of the results of PCR on 143bp (HVS I, nt 16268-16410) mtDNA D-loop region showed a positive (+) detection only for day 1 (4 [66.67%] samples) and day 7 (3[50%] samples). Visualization of the results of PCR on the 126bp (HVS II, nt 34-159) mtDNA D-loop region showed a positive (+) detection only for day 1 (2 [33.37%] of samples) and day 7 (6 [100%] of samples).

Conclusion: In conclusion, the length of exposure to room temperature has an effect on the quality of earphone swab-derived DNA. Decreased levels of earphone swab-derived DNA showed a significance value ($p < 0.005$) with regard to the effect of room temperature exposure

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INTRODUCTION :

Identification by means of Deoxyribonucleic Acid (DNA) analysis is modern and referred as primary independent preferred technical method^[1,2]. The profile accuracy is by its stability inside biological cells of samples like blood, semen, hairs and even organ hence making it suitable for isolation of the inquired^[3]. Traces of this biological evidence is either free disperse of the criminal environment or found logged and harbored in objects/devices lastly used and left at the scene by the perpetrator or victim^[4-6]. Such objects and devices include headsets or earphones. The association to the identification of crime perpetrator or victim and exoneration of innocent through these audio electronic devices is due to a user-contact theory supported by Locard's principle of material exchange^[7]. During its use in mobile phones, player voices or as hearing aid implants, the devices are attached to the outer ear skin, leading

to contact with expelled cerumen or degrading body cells which may yield DNA profile^[8-9]. The devices have gained innovative progress in forensic to yield biological sample and/or traces for generation of genetic profiles during investigative tasks of the reality. However, the results of DNA analysis quality on the generated profile are interfered by a number of environmental factors including room temperature^[10-11]. One alternative taken by forensic DNA experts to deal with this condition is the use of a mini- primer set^[12-13]. The technique is carried out by using a method of reducing the size of Short Tandem Repeat (STR) assays in the examination of the locus of the nuclear DNA^[14] and reducing the size of hypervariable segment [HVS] 1 or 2 of mitochondrial DNA (mtDNA), which is used as amplification products^[15]. The use and sequencing of mtDNA in forensic identification are based on ability to determine the genetic relationship, thus

expediting the process of individualization especially in cases involving a large number of individuals such as mass disaster [16]. Specificity of mtDNA in genetic relation is by a contained non-coding region, the displacement loop (D-Loop) at positions 16024-576 of nucleotides (nt). This region is also called the control region since it is a segment that contains the elements of the replication origin, transcription initiation, and regulators [17]. While acknowledging the techniques to handle samples impacted by environment, there remains a need to understand such effect on samples logged to earphone. This study, therefore, experimented association of room temperature on the effect of room temperature on the quality of earphones swab-derived mitochondrial DNA.

MATERIALS AND METHODS :

A total of 30 samples of earphone swab derived DNA was taken from respondents who agreed and signed a written informed consent. This consent was of a voluntary participation in the scholarly study aimed at knowledge exploration and contribution to the scientific community through findings sharing and publication. This scholarly aim with the purpose in understanding the effect of room temperature on samples logged to earphones as evidence that would be found at crime scene. As also, the study didn't fragment harmful content rather than the quality and trend of mtDNA on D-loop from the earphone swab derived sample. Hence, therefore, the intent preserved and refrained from profiling for personal and individualized identification.

Experimentation protocol :

The materials for DNA extraction were DNazol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and 70% ethanol (EMSURE®, Merck KGaA 64271 Darmstadt, Germany). The material for Polymerase Chain Reactions (PCR) were PCR mix -12.5µl (Promega Corporation, Madison, USA) comprising of dNTPs (ATP, CTP, TTP, GTP), MgCl₂, Taq polymerase and Nuclease-free water; mtDNA primers - 143bp (HVS I, nt 16268-16410) A F D I L 5' C A C T A G G A T A C C A A C A A A C C 3' and 5'GAGGATGGTCAAGGGAC3'^[18], 126bp (HVS II, nt 34-AFDIL 5'TCTCCAAGCGGGTGGTGCATTTA3' and 5'AAATAATAGGCAGGAGGATATC3'^[15].

Then extraction and isolation of DNA were performed. Laboratory processing was conducted at 26.5°C. Positive control (K+) was used to control findings. Other measures included the use of sterile supply, material, and equipment, assigning a code to every sample, use of protective gears to prevent contamination, calibrated of machines together laboratory room sterilization and disinfection by Ultraviolet lamps. Room Temperature exposure on the level of earphone swab derived DNA Exposition of earphone samples was

conducted at 28.5°C to 30°C of room temperature from 0, 1, 7, 14 to 20 days.

Polymerase Chain Reaction amplification and electrophoresis visualization

Cell lysed and isolated during extraction produced DNA template for PCR processing. The template under this study was polymerized and amplified using two hypervariable segments: (HVS) II (126bp, nt 34-159) and HVS I (143bp, nt 16268-16410) of D-Loop region of the mtDNA as shown in Figure 2 to Figure 5.

Results of the DNA sample extracted were measured for DNA levels and purity by using Ultraviolet-visible Spectrophotometer (UV-1601, PC, Shimadzu, Japan).

RESULTS :

The measured average concentration levels of DNA are presented in Table 1.

Table 1: Amount of mtDNA isolated from earphone samples at varied exposure interval.

Earphone (samples)	room temperature	(µg ml) ± SD	Temperature range (°C)
30	Day 0	152.89 ± 85.71	28.5 - 30
30	Day 1	11.19 ± 5.58	28.5 - 30
30	Day 7	2.15 ± 0.81	28.5 - 30
30	Day 14	0.93 ± 0.79	28.5 - 30
30	Day 20	0.16 ± 0.42	28.5 - 30

Table 1 measurements portray a decreasing effect of DNA concentration from day 1 to the twentieth day as referred to a non-exposed sample of day zero. A reference at day zero measured levels of DNA at 152.89 then decreased to 11.19 after a twenty-four hours room temperature exposure, on seventh-day measurement dropped to 2.15, then to 0.93 and lastly to 0.16 on fourteenth and twentieth respectively. Decreasing trend of DNA levels is appreciated and visualized in Figure 1, in which a sharp drop is experienced on the first day then a gradual decrease is observed steadily to twentieth.

143bp and 126bp mtDNA amplicon profiling at day 1 and 7

Profile analysis using 143bp and 126bp resulted in a band contrast formed and presented in Figure 2. Amplification and visualization of samples exposed by day one and seven became positively detected under the two primers (143bp and 126bp). Though both positively detected, band of two samples appeared with faded contrast under 126bp after seven days of exposure as observed in Figure 2.

143bp mtDNA amplicon electrophoresis of day 1, 7, 14 and 20

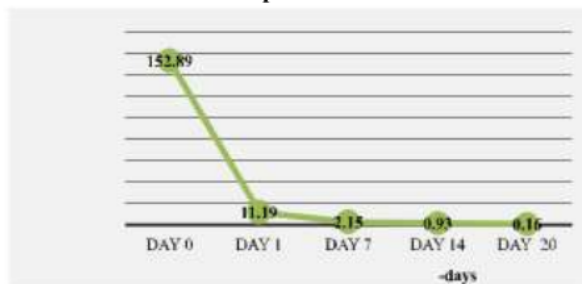
Under 2% agarose gel electrophoresis 143bp DNA amplicon, day 1 present positive [+] detection of two samples with faded bands and other 2 samples demonstrated a negative [-] detection while in day 7 samples there were 3 with negative [-]

detection (no band displayed) and there was only one sample that showed a positive [+] detection with a faint band too. Amplification and electrophoresis of samples by 143bp (HVS I, nt 16268-16410) D-loop mitochondrial DNA amplicon on day 14 and 20 using 2% agarose gel found that all samples displayed no band or negative detection as shown in **Figure 4**.

126bp mtDNA amplicon electrophoresis of day 7, 14 and 20

Amplification and electrophoretic visualization of 126bp mitochondrial DNA amplicon using 2% agarose gel on PCR swabbed earphone products exposed at 7, 14, and 20 intervals displayed variably. Day 7 exposed sample amplification gave a positive [+] contrast detection, bands were visible for the mtDNA as shown is presented in **Figure 5**. Contrary to day seven, amplification and electrophoresis of samples exposed for 14 and 20 days resulted into a no display of band contrast hence a negative [-] detection.

Figure 1: The trend of effects of the length of exposure on the mean of earphone swab derived DNA.



The measured mean levels of DNA as presented in Table 1 portray a decreasing effect of DNA. Extracted genetic material from less exposed earphone swabbed samples present before day seven evidence an elevated tabulation while a lowered graphical presentation and even gradual to those with maximized exposition. The graph confirms a sharp drop as sample get exposed to interfering factor (room temperature) – suggested by immediate cell lysis while in-vitro then the immediate destruction of primary DNA molecules hence a gradual damage continue steadily as interval persist. This trend supports the finding that the ability to profile DNA from environmental subjected sample gets diminishing with times, and in this case, the chance for identification of an individual by mtDNA D-loop profile get narrowed.

Relative extent effect of exposition on day 1, 7, 14 and 20

A representation of six samples for general percentage effect according to analysis is summarized in Table 2 and presented in Figure 6. The results show that, detection of the effects of

room temperature exposure on earphone swab-derived 143bp (HVS I, nt 16268-16410) and 126bp (HVS II, nt 34-159) mtDNA D-loop region depend also on the exposure interval and the D-loop specific region between the two (143bp and 126bp). Day one exposition analysis, 66.67% (4) of samples were positively detected at 143bp (HVS I, nt 16268)16410) D-loop region while 33.37% (2) were negatively detected similarly to those positively detected at 126bp (HVS II, nt 34-

D-Loop region hence a v-like tabulation (day one graph) in Figure 6. On day 7; 50% (3) of samples were detected at 143bp (HVS I, nt 16268-16410) D-loop region and 100% (6) of samples detected at 126bp (HVS II, nt 34–159) D-loop region. Day 14 and 20, visualized a zero percentage of DNA positive detection due to exposure effect to room temperature on all earphone swab samples (143-bp (HVS I, nt 16268 16410) and 126bp (HVS II, nt 34-159) D-loop region). Relative tabulation and data presentation are referred to in **Figure 6** and **Table 2** respectively.

Figure 2: Electrophoretic visualization of 143-bp and 126-bp PCR products of earphone swab samples (M = marker, H1& H7=samples at day 1 and 7)

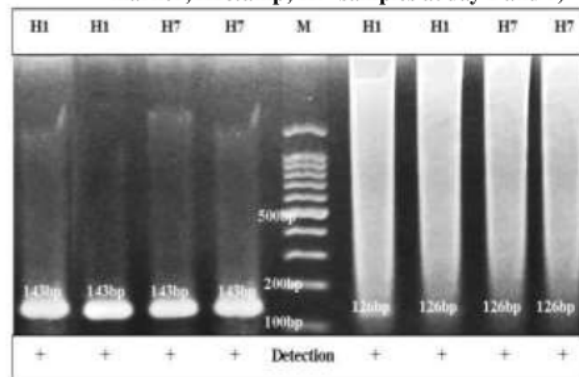


Figure 3: Electrophoretic visualization of 143-bp PCR products of arphone swab samples (M = marker, H1& H7 = samples at day 1 and 7)

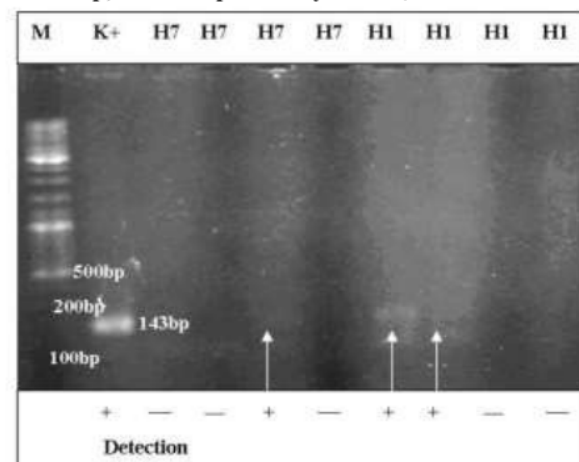


Figure 4: Electrophoretic visualization of 143-bp PCR products of earphone swab samples (M = marker, H14 & H20 = samples at day 14 and 20)

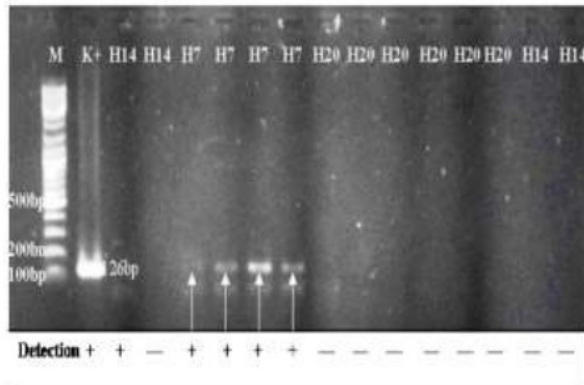
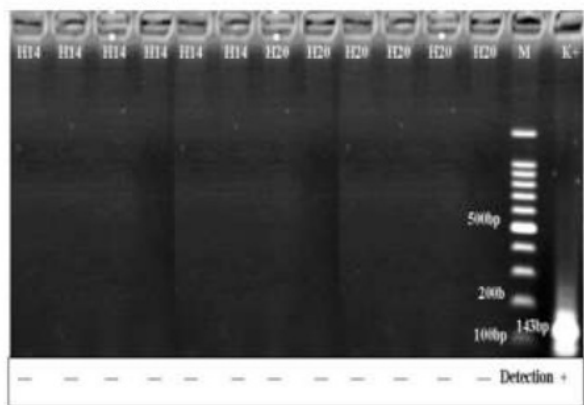


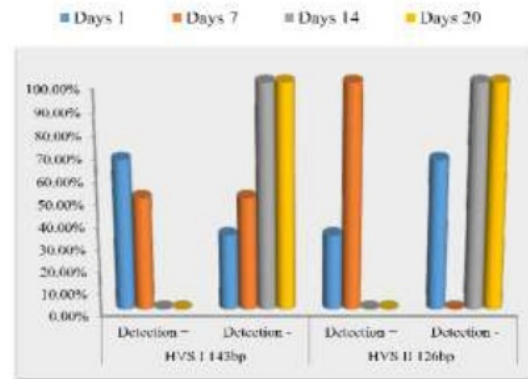
Figure 5: Visualization of 126-bp PCR products of earphone swab samples (M = marker, H7, H14 & H20 = samples at day 7, 14 and 20)



This relative presentation of six samples for general effect according to analysis is a summary of Table 2. The results show that, detection of the effects of room temperature exposure on earphone swab-derived 143bp (HVS I, nt 16268-16410) and 126bp (HVS II, nt 34-159) mtDNA D-loop region depend also on the exposure interval and the D-loop specific region between the two (143bp and 126bp). Day one detection relativity, 66.67% (4) of samples were positively detected at 143bp (HVS I, nt 16268-16410) D-loop region while 33.37% were negatively detected similarly to those positively detected at 126bp (HVS II, nt 34-159) D-Loop region hence a v-like tabulation (day one graphs). On day 7; 50% (3) of samples were detected at 143bp (HVS I, nt 16268-16410) D-loop region and 100% (6) of samples detected at 126bp (HVS II, nt 34-159) D-loop region. Day 14 and 20, visualized a null percentage of DNA detection from all earphone samples at

143-bp (HVS I, nt 16268-16410) and 126bp (HVS II, nt 34-159) D-loop region).

Figure 6: Relative comparison of detection of earphone swab-derived 143-bp and 126-bp mtDNA D-loop region under room temperature



This relative presentation of six samples for general effect according to analysis is a summary of Table 2. The results show that, detection of the effects of room temperature exposure on earphone swab-derived 143bp (HVS I, nt 16268)16410) and 126bp (HVS II, nt 34-159) mtDNA D-loop region depend also on the exposure interval and the D-loop specific region between the two (143bp and 126bp). Day one detection relativity, 66.67% (4) of samples were positively detected at 143bp (HVS I, nt 16268)16410) D-loop region while 33.37% (2) were negatively detected similarly to those positively detected at 126bp (HVS II, nt

159) D-Loop region hence a v-like tabulation (day one graphs). On day 7; 50% (3) of samples were detected at 143bp (HVS I, nt 16268)16410) D-loop region and 100% (6) of samples detected at 126bp (HVS II, nt 34-159) D-loop region. Day 14 and 20, visualized a null percentage of DNA detection from all earphone samples at 143-bp (HVS I, nt 16268)16410) and 126bp (HVS II, nt 34-159) D-loop region).

Table 2: swab-derived 143-bp (HVS I, nt 16268-16410) and 126-bp (HVS II, nt 34-159) mtDNA D-loop region.

Exposure Interval	HVS I 143bp nt: 16268 – 16410		HVS II 126bp nt: 34 – 159	
	Detection (+)	Detection (-)	Detection (+)	Detection (-)
Days 1	4(66.7%)	2(33.3%)	2(33.3%)	4(66.7%)
Days 7	3(50%)	3(50%)	6(100%)	0(0%)
Days 14	0(0%)	6(100%)	0(0%)	6(100%)
Days 20	0(0%)	6(100%)	0(0%)	6(100%)

RESULTS OF SAMPLE SEQUENCING :

Samples that remained to show a positive [+] detection were subjected to an analysis of sequencing for effects of exposure to room temperature. 143bp (HVS I, nt 16268-16410) and 126bp (HVS II, nt34 159) mitochondrial DNA D-loop fragments derived from earphone swab samples were sequenced using the Sanger dideoxy method by means of an Automatic DNA Sequencer based on the Dye Terminator Labeling method, protocol^[19]. The PCR product fragments sequenced were those amplified directly without cloning, a process called direct sequencing. This method was used since the process is fast and the products are the dominant nucleotide sequence of PCR-amplified DNA. Primer reaction (M1 - forward) was used to determine the nucleotide sequence of 126bp and 143bp in size in the sequencing reaction. The study was capable of determining the nucleotide sequence of the mtDNA D-loop region of the samples as shown in the below;

1st_BASE_2345711_20_143_143.ab1 for day 1

NNNNNNNGGNNNNNNNNNNNNNNNANNNGCA
TNAACCGNCAGAGCACATTA

GGGGCAGACCANNCTCGCCGCGNANACGGGNC
NCCCGGGGAGACCAAGNA

NCCCGNCGAGCCGGCANCTGNTGAGTCACGACC
CGAGAGGNGTGACGNCAGCC TGGGN

1st_BASE_397247_mtDNA_126bp1.ab1 for day 1

GTNGNNNTNNNTNCTCCTTNGNNGTCCCCCGGC
GCGCCCCCTCAAGNNNNNCTACTNGNCCTGAA
TTNATTACNGCCNCCTNNTCNCNTGANTNCTGCT
NCNNNANGNNNNNGCANNNNNNNNNACGNNNN
NNGNNNNNGTNNANNNNNNNNNNNNNNNNNN
CNTAGTCANNNGATNGNTNCTACNNNATNNNN.

DISCUSSION :

Room Temperature exposure on the level of Earphone swab derived DNA :

DNA purity values obtained in this study were of 0.00 to 1.80 whereby the average level of DNA was 23.18±57.58 ng/µl in which the purity value of 0.00 indicates no DNA in the sample. These parameters of DNA are an important factor in DNA analysis, especially with regard to the successful amplification of DNA samples. DNA integrity is central to DNA forensic investigation. This implies that, despite the relatively high levels of obtained DNA as observed in findings, fragmentation or degradation may reduce its significance. Though the level of DNA required in DNA analysis varies depending on the need and type of investigation still adequate quality of DNA is also required, in which DNA should be in a minimally degraded condition as also elaborated by (1). Measurement of DNA in this study has shown a tendency of decreased levels of

earphone swab- derived DNA in association with the length of exposure to room temperature (Table 1). Statistical tests were performed to determine the extent to which the length of exposure to room temperature had an effect on DNA levels. Kruskal-Wallis test and t-test of the levels of earphone swab-derived DNA on average showed a significant effect of the length of exposure to room temperature (significance value of 0000, significance limit of $p < 0.05$). The t-test of earphone swab-derived DNA levels showed a significance value of 0.002 and a significance limit of $p < 0.05$. Thus, there is a significant difference in the levels of DNA for all of the length of exposure.

In addition to the levels of DNA sample, PCR based investigation also requires an adequate quality of DNA. In this case, degraded DNA inhibits analysis of information. Severely degraded DNA prevents the primer from annealing the target DNA replication (20,21). According to (22), adequate visualization requires adequate DNA purity and DNA levels for successful investigation including identification and paternity test. Exogenous factors of DNA degradation are in several types either, (23,24). Type I degradation is in which DNA is usually degraded slowly by chemical processes for a relatively long period of time. Type II degradation is in which DNA is rapidly degraded by moisture, sunlight and even extremely high temperatures. Results of the study complement with the impact suggested in type II, that the environmental effects of temperature and length of exposure had an effect on the levels of DNA. Results of DNA measurement by spectrophotometer showed a significant decrease in the levels of DNA samples. The dependence of PCR-based DNA investigation on adequate quality of DNA is explained in a number of parameters. As discussed and established in literature the success of PCR highly depends on several factors, such as deoxyribonucleotide triphosphates (dNTPs), oligonucleotide primers, DNA template, buffer solution, reaction cycle, and enzyme. Additionally, it depends on the technical and non-technical factors, such as contamination. A sufficient quality of DNA means that DNA should be in good condition and, if it is degraded, the degradation should be as minimum as possible. Severely degraded DNA prevents the primer from annealing the target replicated DNA. Thus, a good quality of DNA constitutes a fundamental prerequisite for the overall PCR success. This is according to review collected by Cavanaugh & Bathrick (2018), PCR sensitivity is a function of the number of cycles and the levels and integrity of DNA.

Polymerase Chain Reaction amplification and Electrophoresis visualization

Visualization of PCR products was analyzed descriptively by

looking at the appearance of bands according to PCR product size. Positive detection [+] effects of room temperature exposure and the non-detection in the visualization of PCR products for these samples was related to DNA quality contained. DNA quality includes DNA levels, DNA purity and DNA condition state (degradation). In the present study, the average level of DNA was $0.93 \pm 0.79 \mu\text{g/ml}$ on day 14 and $0.16 \pm 0.42 \mu\text{g/ml}$ on day 20; the trend explains the degrading process. DNA degradation was among the causes of DNA detection failure in PCR-based DNA analysis in our study as well portrayed by Figure 4 and some bands in Figure 3 and 5. This is consistent with (26) with regard to several possible causes for failure detection of DNA, including a minimal amount of target DNA, DNA degradation that prevents the primer from annealing, lack of DNA polymerase and PCR cycle also presence of PCR inhibitors. The failure of amplification is characterized by the absence of bands on the electrophoresis results due to lack of DNA polymerase and PCR cycle. This can be controlled by the use of PCR master mix that contains Mg 2+ and Taq DNA polymerase, dNTPs in quantities tailored to the needs of optimal PCR reaction and having a proven reliability in the current PCR reaction (27). From the article lack of cycle in a PCR reaction is controlled by optimizing the primers used for PCR while adhering to Mg 2+ recommended concentration (should be 1.5 to 4 mM). An excessive concentration of Mg 2+ causes a non-specific reaction with the presence of smear on electrophoresis. In contrary, the inadequacy of Mg 2+ causes no amplified product or no band on the electrophoresis results as also guided in protocols (28). An excessive amount of Taq polymerase causes a smear formation (non-specific reaction) on electrophoresis, whereas inadequacy of it causes a reduced efficiency of amplification and non-appearance of bands. Tris-borate-EDTA (TBE) used in decalcification of samples may serve as a PCR inhibitor. This can be avoided by using ethanol to precipitate DNA extracted/isolated. Ethanol is a potential material to separate DNA from other components during DNA extraction (29). Some findings in this study show a failure of detection in the form of failed PCR amplification of DNA. This failure can be caused by degradation or damage to DNA from the effects of the length of exposure to room temperature. Cerumen attached to earphones contains detached epithelial cells of the outer ear canal. Those epithelial cells are not protected instead of directly exposed to external factors that cause degradation to contained genetic materials, DNA. According (30,31), DNA damage caused by external factors such as exposure to X-rays, chemical agents, spontaneous instability and extreme temperature will result in many types of damage, such as damage to DNA strands (both double and single strand), base damage (damage to DNA bases), sugar

damage and even presence of DNA crosslink and DNA-protein crosslink. Those environmental factors lead to degraded DNA. This degradation can be fast or slow, depending on the affecting factors and length of exposure. Findings of this our study shows that earphone swabs can still be used as a material for DNA analysis of mtDNA with regard to the effects of exposure to room temperature (28.5°C - 30°C) up to day 7. This is in potential consideration when applied to expedite forensic identification, especially in a mass disaster or criminal cases on the crime scene with little trace evidence.

Sample sequencing information derived

The positively [+] detected samples and analyzed for nitrogenous bases forming nucleotides sequence was in response of appreciating the damaging effect extent. Room temperature for samples exposed for day-1 and day-7 by 143bp and 126bp mitochondrial DNA D-loop fragments derived from earphone swab under Sanger dideoxy method portrayed potential effect impacted even to those still detected positive band contrast. The derived sequence of 126bp and 143bp mtDNA amplicon from earphone swab samples showed degraded nucleotides on day 1, as marked with 'N' in generated two sequences. This symbol 'N' according to the International Union of Pure and Applied Chemistry (IUPAC) is suggested to indicate the presence of ambiguities (32). Therefore a suggested room temperature effect per lapsed time interval.

CONCLUSION :

This study concludes that :

The length of exposure to room temperature has an effect on the quality of earphone swab-derived DNA.

Decreased levels of earphone swab-derived DNA showed a significance value ($p < 0.005$) with regard to the effect of exposure to room temperature.

Visualization of the results of PCR on 143bp (HVS I, nt 16268-16410) mtDNA D-loop region showed a positive (+) detection only for day 1 (4 [66.67%] samples) and day 7 (3 [50%] samples) of room temperature exposure. Meanwhile, visualization of the results of PCR on 126bp (HVS II, nt 34-159) mtDNA D-loop region showed a positive (+) detection on similar exposure interval - day 1 (2 [33.37%] samples) and day 7 (6 [100%] samples) of room temperature.

ABBREVIATIONS :

bp: base pair(s); D-loop: Displacement loop; DNA: Deoxyribonucleic Acid; nt: nucleotides; HVS: Hyper Variable Segments; mtDNA: mitochondrial DNA; PCR: Polymerase Chain Reactions; STR: Short-Tandem Repeat; TBE: Tris-borate-EDTA.

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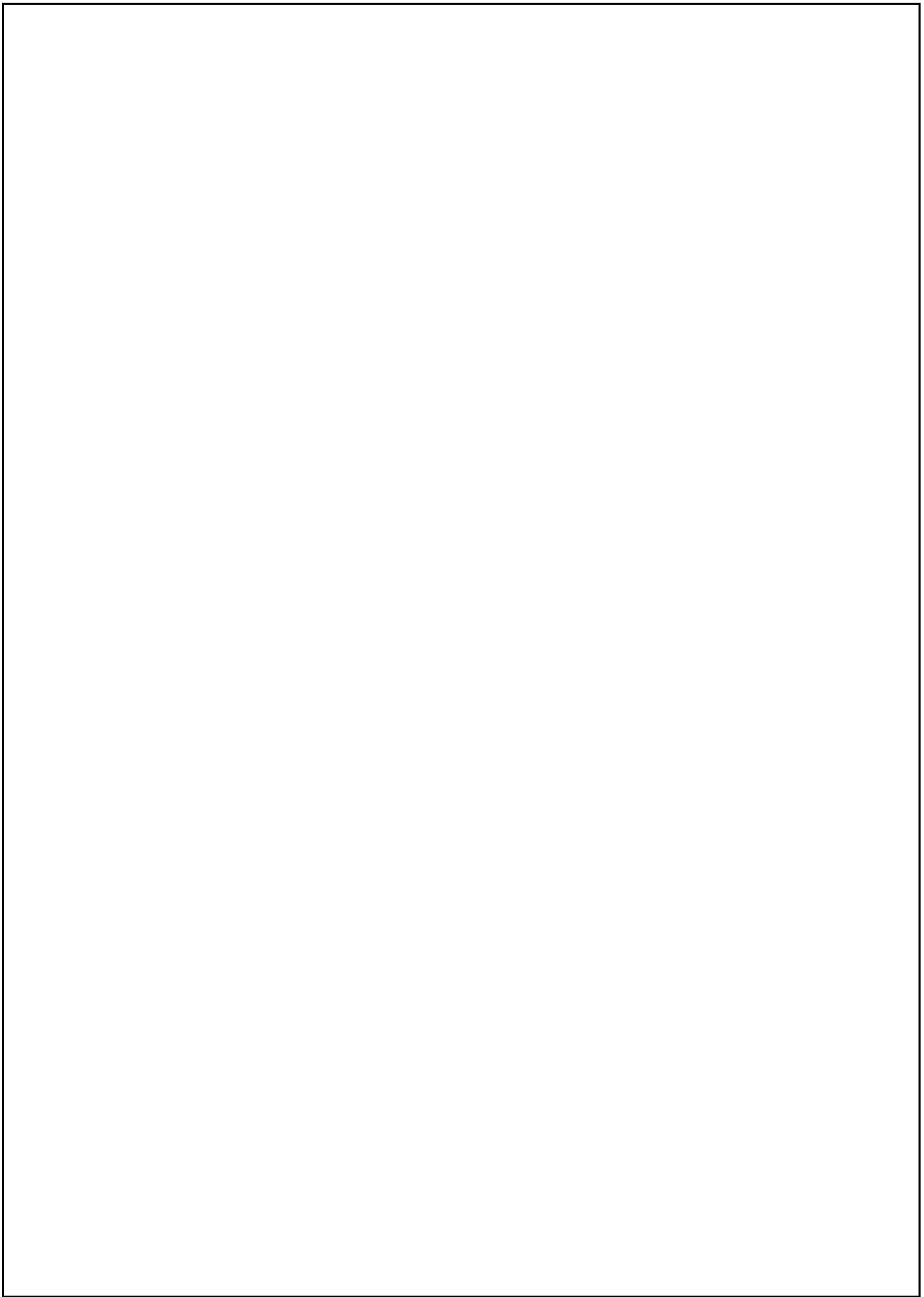
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