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RESEARCH ARTICLE

PERIODIC QUALITY EVALUATION OF DNA FROM SALIVA SWABS OBTAINED FROM BITE IMPRINTS AND PRESERVED IN IDEAL AND TROPICAL ROOM TEMPERATURE – A COMPARATIVE FORENSIC STUDY

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ABSTRACT

Background: Salivary Evidence in a crime scene is scaling revolutionary heights in Forensics. Saliva identification on a victim proves malicious contact, thus carrying more evidentiary weight. Salivary DNA is also a promising tool for epidemiological genomic studies. This study was conducted to investigate state of DNA in scenarios of delayed evidence collection in tropical climatic temperatures.
Aim: To evaluate quality of genomic DNA extracted from saliva swabs obtained from bite imprints and to assess the genomic fidelity when the DNA from the dry evidence is preserved in favorable and unfavorable temperatures for a period of 60 days.
Materials and Methods: 25 participants were recruited and 2 samples, the first comprising of 5ml of unstimulated saliva and second, bite imprints recorded on putty impression material were obtained. Swabs were taken from the bite imprint on the 1st day simulating early evidence collection and 3rd day simulating delayed evidence collection respectively; the 3 day interval during which bite imprints were left unprotected at room temperature. Single swabbing technique was employed in swab collection. DNA extraction was done from the collected saliva and the swabs to compare quantity and check quality. Extracted Trace DNA was preserved in a range of tropical temperatures and inspected for degradation by PCR once in 20 days, for 60 days. On the 60th day, DNA quality comparison was done.
Results: The comparison between quantity of DNA in saliva and swab A showed statistical significance with P=0.0005 <0.001 with the mean±S.D of saliva (87.44±42.05) and Quantity of DNA from swab A being (1.53±0.72). Similarly the comparison of Quantity of DNA from saliva and swab B showed significance with P=0.0005<0.001 with swab B (0.65±0.30) and the comparison between swab A and Swab B also statistical significant with (1.53 ±0.72) and (0.65±0.30) respectively. The Trace genomic DNA which was obtained by portioning Swab B DNA into 3 parts and preserved in unfavorable temperatures exhibited reliable quality within study period which was demonstrated by DNA amplification pattern in Polymerase chain reaction.
Conclusion: In delayed Evidence collection upto 3 days, tropical climatic temperature does not seem to hamper the quality of DNA and the genomic fidelity was found to be maintained. The study also proved that Trace Genomic DNA can be preserved in normal tropical temperatures for a minimum of 60 days without the threat of complete DNA degradation.

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INTRODUCTION

Salivary diagnostics is evolving into a sophisticated science, and is a subset of the larger field of molecular diagnostics. It has become the basis for varied biomedical and clinical areas. Analysis of saliva also proves to be cost-effective for the screening of large populations (Malamud and Rodriguez-Chav, 2011; Sharmila Devi Devaraj, 2013). Saliva is also easy and safe to handle than blood because it does not clot, thus reducing the number of manipulations required and is less likely to expose operators to blood-borne diseases. For the patients or examinees, the non-invasive collection approach could dramatically reduce anxiety anddiscomfort, and increase their willingness to undergo the proposed tests (Ahmad Naeem, *et al.,* 2014; Sanjeev Mittal *et al.,* 2011). In the field of forensics and criminology, cases of physical assault, such as sexual abuse, murders and child abuse, bite marks are frequent findings on the skin. Though bite marks are efficient for human identification, it is endowed with a potential limitation that it suffers distortion from the moment of the bite until the

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act of expertise, especially when the mark is left on the skin (NidhiYadav *et al.,* 2014; Verma *et al.,* 2013). Salivary DNA has emerged as a complement or even to replace bite marks, since it is a test of excellence. Human saliva stains can be found at crime scenes, alone or mixed with other biological fluids (Ramesh, 2012). The most common sites of occurrence are: the surface of objects such as envelopes, tissues, cigarette butts, cups, sites near bites and often victims of rape. It was only after the advent of polymerase chain reaction then that saliva became a great focus on looking for traces, considering the amplifiability of even minute genomic material (Rosalee *et al.,* 2011; Smita Birajdar Shrishail *et al.,* 2011). When biologic stains are found at crime scenes, they are collected; the DNA is extracted, and analyzed for the source of the contributor. However, delay in the evidence collection pose practical difficulties as in the case of rape/ sexually abused victims who are left to die. In such cases, exogenous factors like delayed discovery of the body and/or delay in interpretation and collection of evidence cannot be avoided. Significant DNA in a deceased victim can be stable and may be recovered up to 48-60 hours after deposition on the skin, depending upon environmental influences (contamination, degradation and putrefaction) (Stavrianos *et al.,* 2010). The matter of concern now is to efficiently collect the evidence, store it in feasible conditions and process it meticulously with care not to contaminate the evidence. There is a lack in studies which authorize the stability of DNA collected from evidence imprinted minimum 2 -3 days before. Studies are also found to be lacking which correlate DNA stability at tropical room temperatures and for how long they resist degradation when left at varying room temperatures. In an attempt to simulate the practical Rape Victim/ Crime setting, when the victim is left to die and there is late retrieval of body or when the evidence collection may be delayed by a few days, the time frame during which the evidence deposited on the victim's skin is left exposed to various degrading factors, the one among which is the tropical climatic condition of our country. This experiment was conducted with an aim to evaluate whether genomic DNA is available on a substrate on which bite imprints were recorded and left at tropical room temperature for 3 daysand if available, to assess and compare the quality, when the DNA is preserved in favorable and unfavorable temperatures for a period of 60 days.

MATERIALS AND MATHODS

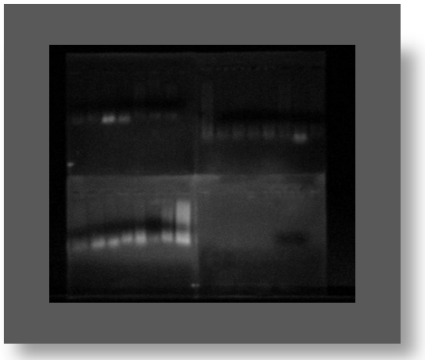
The study was conducted at Department of Oral Medicine and Radiology, Meenakshi Ammal Dental College after obtaining the Ethical Committee Board approval. The study period was between January and May 2015. Two informed consents were obtained from the participants. One explaining about the background of the study and assuring strict confidentiality and anonymity regarding participant identification and demographic details and the other to obtain consent to obtain saliva, bite imprint and to take a swab from the bite imprint for isolation of genetic material. The form also included assurance regarding appropriate disposal of the genetic material and the source, on completion of the study. 25 randomly selected volunteers who consented to participate in the study were enrolled after they understood and complied with the background of the study. The age group of the participants ranged between 22-48 years. Considering the universal applicability of the study, there were no inclusion or exclusion criteria, except for participant's known history of allergy to any of the composition of the clinical armamentarium. After obtaining consent, 25 recruited participants were requested for 2 sample types; 5ml of Unstimulated "saliva de natura" and a bite imprint using putty impression material on an anterior dual arch impression tray. Swab A was then taken from the bite imprint immediately, by single swab technique, after wetting the swab in sterile distilled water, simulating the value of the situation where evidence collection at the earliest can be beneficial. Genomic DNA was extracted immediately from the collected whole saliva and Swab A, Quantified, and the DNA from Swab A which was obtained from favorable conditions was further stored in a favorable storing temperature of -20^{o}C for 60 days. The bite imprint was then left at normal room temperature in an unprotected environment for 3 days during which the average temperature was 32^{o}C. At the end of day 3, Swab B was taken from the bite imprint and Genomic DNA was extracted and quantified.

Table 1.

Sample 1:	5ml of Unstimulated "saliva de natura" collected in sterile plastic containers.
Sample 2:	Bite imprint on a putty impression material on an anterior dual arch impression tray
Swab A	Using single swab technique from sample 2
Swab B	Taken from sample 2 by Single Swab Technique after the imprints were left for 3 days in Tropical Room Temperature (unprotected)

Genomic DNA extraction procedure revealed that the 25th sample showed no presence of DNA during Gel electrophoresis and Documentation, both in the saliva and Swab A, giving a probability that the participant selection criteria was not met, with the sample getting diluted during collection procedure/ technical errors.

Genomic DNA Bands From Saliva Samples visualized by **Trans-Illumination** with **UV light , viewed in Gel Documentation System**



Note: *Absence of Genomic DNA band in 25th sample depicting absence of demonstrable Genetic material in the sample*

The Extracted Genomic DNA were Quantified by the UV spectrophotometer, and documented.The collected data was analysed with SPSS 16.0 version. To describe about the data descriptive statistics mean & S.D were used for continuous variables. To find the significant difference between the

bivariate samples in Paired groups the Wilcoxon signed rank test was used. In the above statistical tool the probability value .05 was considered as significant level.

DNA from Whole saliva	Swab A	Swab B
Range: Max: 155µg/mL Min: 40µg/mL Mean± S.D(87.44±42.05)	Range: Max: 2.89 µg/mL Min: 0.72 µg/mL Mean ± S.D(1.53±0.72)	Range: Max: 1.03µg/mL Min:0.22 µg/mL Mean ± S.D (0.65±0.30)

Comparison between Quantity of DNA from saliva and swab A shows P=0.0005<0.001 (Statistically significant)
Comparison between Quantity of DNA from SwabA and SwabB P=0.0005<0.001 (Statistically significant)

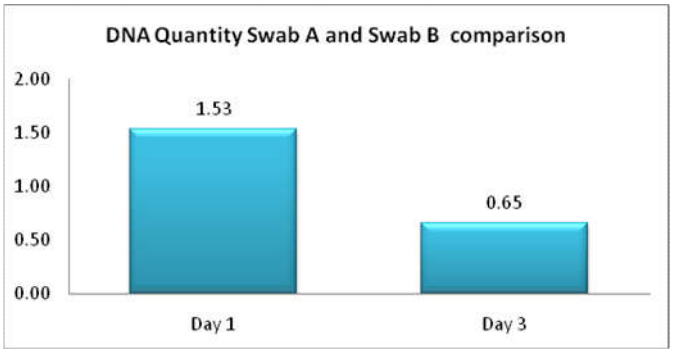
The comparison between DNA Quantity from saliva and swab A showed statistical significance with P=0.0005<0.001 with the mean±S.D of saliva (87.44±42.05) and of swab A being (1.53±0.72). Similarly the comparison of DNA quantity between saliva and swab B showed statistical significance with P=0.0005<0.001 with swab B being (0.65±0.30).Comparison between quantity of DNA obtained from swab A and Swab B was also statistical significant with (1.53 ±0.72) and (0.65±0.30) respectively.

Bivariate analysis using Wilcoxon signed rank test

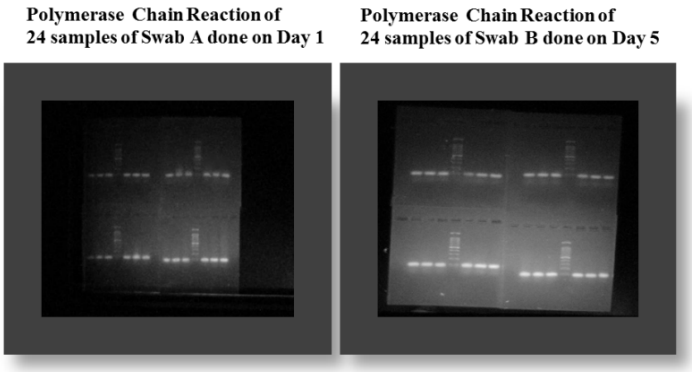
Polymerase chain reaction test was done on DNA from Swab A and Swab B to check for the amplifiability of the DNA using the B-actin gene, as the gene was strictly human. The result of the PCR product is illustrated in the picture below. To perfect the proposed analysis, DNA obtained from favorable conditions- Swab A(,i.e., Evidence collection at the earliest), was stored in ideal storing temperatures of -20^{o}C for 60 days and the DNA obtained from unfavorable conditions – Swab B (i.e., Delayed Evidence collection after subjecting the sample to unprotected environmental exposure at average room temperature of 32^{o}C for 3 days) was portioned into 3 subgroups as Portion B1, B2 and B3 and each portion was incubated at 25, 35 and 45^{o} C respectively for 60 days. In a periodic interval of 20 days a PCR was run on all samples and checked for degradation of the sample.

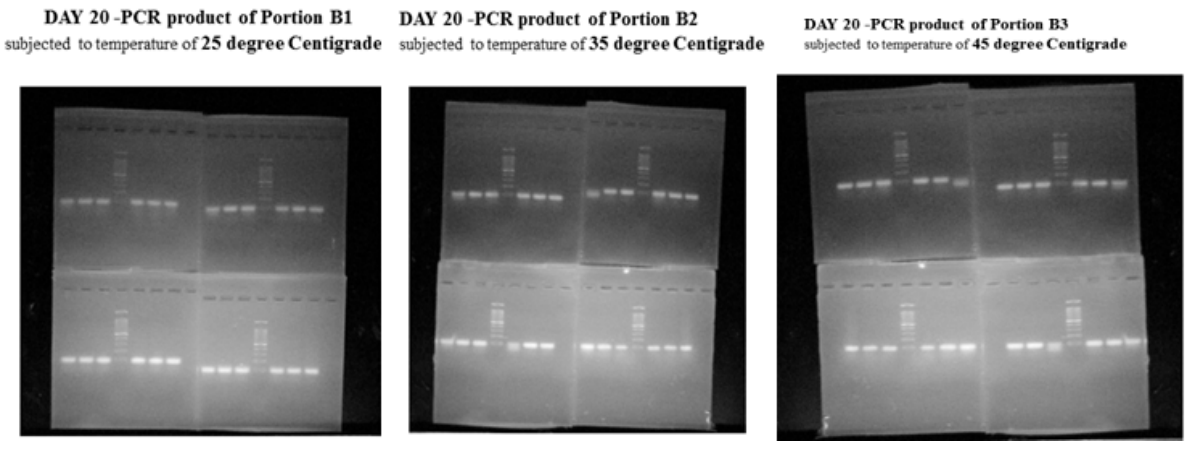
		Mean	SD	Z value	P value
Pair 1	Saliva Day 1	87.44	42.05	4.286	0.0005**
	Swab Day 1 (swab A)	1.53	0.72		
Pair 2	Saliva Day 1	87.44	42.05	4.286	0.0005**
	Swab Day 3 (swab B)	0.65	0.30		
Pair 3	Swab Day 1 (Swab A)	1.53	0.72	4.287	0.0005**
	Swab Day 3 (Swab B)	0.65	0.30		

Highly Statistical significant at P<0.001 level

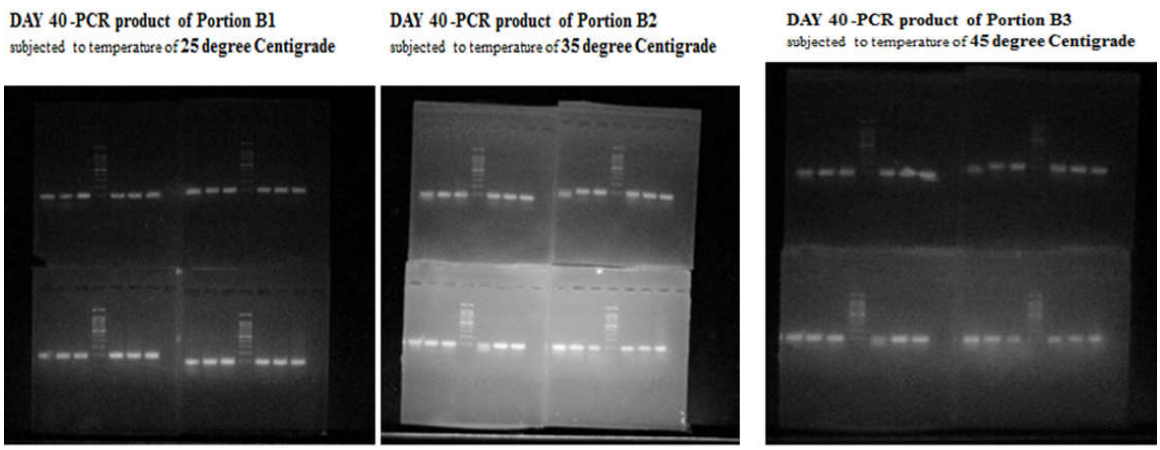


Polymerase chain Reaction was also run on the Samples from Swab A stored at -20^{o}C for 60 days for cross examination purpose which demonstrated positive results as expected.

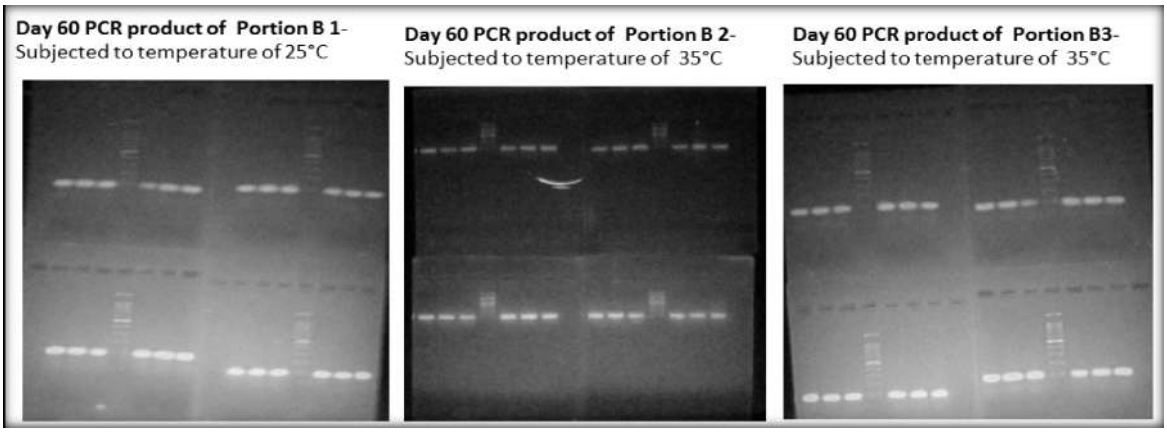




Day 20-Polymerase Chain Reaction of Portion B1, B2, B3
Day 20- PCR product of size86bp, run along with 100 bp DNA ladder



Day 40-Polymerase Chain Reaction of Portion B1, B2, B3
PCR product of size 86bp, run along with 100 bp DNA ladder



Day 60-Polymerase Chain Reaction of Portion B1, B2, B3
PCR product of size 86 bp, run along with 100 bp DNA ladder

The PCR product of Portion B1, B2, B3, incubated at temperatures of 25, 35 and 45 degrees showed DNA of amplifiable quality on the 20th, 40th and 60th day respectively. The Trace genomic DNA which was obtained by portioning Swab B DNA into 3 parts and preserved in unfavorable temperatures maintained genomic fidelity within study period which was demonstrated by DNA amplification pattern in Polymerase chain reaction.

The Quality of DNA obtained from Unfavorable Conditions and Incubated In Tropical temperature range of 25- 45°C For 60 Days, maintains genomic fidelity Favorable Conditions And Stored In Ideal Temperature Of -20°c, Based On The Amplification Pattern Of The DNA Sample In The Respective Polymerase Chain Reactions.

DISCUSSION

DNA in Forensics plays an important role in analyzing and interpreting the medical-judiciary proof of offence and help in exclusion of the suspects or to point out the culpability elements. Saliva is considered a very useful DNA source due to the fact that its composition is 99% of water, has leukocytes (25 to 650,000) and scaled off epithelial cells (6 to 600,000) which contribute to the DNA content of saliva (Ricardo Henrique Alves Silva, 2006).

A lot of literature has considered saliva as a reliable source of DNA for a wide variety of genetic studies. A study Looi *et al*., in 2012, (Looi *et al.,* 2012) attempted to do a Quantitative and qualitative assessment of DNA from saliva and blood by comparing the yield, purity, and performance of DNA using the non-invasive collection kit (Oragene DNA OG500 and OG575 kit). The study also performed a PCR-based analysis and found that the DNA yield from saliva from the manual purification method was comparable to the DNA yield from blood by the salt precipitation method. Both the salivary DNA and DNA derived from blood proved to be of high purity (A260/280 > 1.70). Genotype results (PCR-RFLP and direct sequencing) for all sets of blood-saliva DNA samples were in 100% concordance. Similar Studies by Abraham *et al*., 2012, Viltrop *et al*., 2010, Pulford *et al.,* 2013, and many others arrived at the similar conclusion that, Saliva is a viable alternative DNA source for genotyping studies. Our study also reiterates this fact with the average salivary DNA obtained from 25 samples of 5ml of saliva being, 91.84 µg/mL. Sample collection systems by Law enforcement agencies involve collection, protection and documentation of biological evidence in a format compatible to perform micro fluidic DNA analysis. The specific collection method employed depends on the state and condition of the biological evidence. The most commonly used evidence collection device

from salivary stains is the Swab Method. In an experiment conducted by David Sweet *et al.,* 1997, several techniques were evaluated to determine the best method of recovering saliva from human skin before extracting genomic DNA from the collection substrate. It was in this article, that the double swab technique, using a wet cotton swab followed by a dry cotton swab was introduced and evaluated. The double swab technique showed the highest percentage recovery of saliva from human skin among the three methods studied. The double swab technique was undertaken as a Modification to the single swab technique due to the fear that epithelial cells (DNA from the victim) may be exfoliated due to rubbing the skin with the cotton swab and to improve the recovery results. However in a study by Williams *et al.,* 2013, which was done to compare various sampling techniques in order to identify the most effective DNA recovery method from uneven and ridged surfaces , the study was concluded stating that there was no significant difference in the amount of DNA recovery between the single swab and double swab techniques ; in fact the mean Nanospectrometer values were slightly higher from Single Swab [2.89 (1.14)] than Double Swab technique [2.00 (2.00)].Since there was only a single source of DNA in our study, and since the swabbing was done on a ridged impression surface, a single swabbing method was followed and the results complied with the study, demonstrating adequate DNA recovery on Day 1 and Trace DNA recovery on Day 3 of our experiment .

Recovered DNA and related evidence are usually stored in ideal storing temperatures of -20 degree Celsius and long term storages are sometimes done at -80 degree Celsius also. However to check the reliability of the DNA in tropical temperatures, we incubated the Trace DNA sample to a tropical temperature ranges. Our study yielded a positive amplification for upto 60 days in a temperature range of 25-45 C. This result correlated with studies done by Ng *et al.,* 2006, where conditions involved the extraction of the DNA immediately after saliva collection (condition 1) or when stored at air-conditioned room temperature (20 degrees C) for 1 month (condition 2) and 6 months (condition 3) as well as at -80 degrees C for 6 months (condition 4). The effect of incorporating an additional incubation of saliva samples at 30 degrees C for 2 weeks was also examined. Results demonstrated that DNA yield was unaffected by incubation of saliva at 30 degrees C but DNA yield under condition 3 was significantly higher compared to conditions 1 and 2 and concluded that differences in storage conditions did not impact DNA quality in real time PCR experiments and genotyping fidelity was maintained. This result was in consensus with the results obtained in our study. In another study by Dominique Quinque *et al*. in 2006115, Saliva samples stored for up to 30 days at 37 °C, was used for DNA extraction. They were able to reliably and accurately type genetic markers, making this method suitable for Weld conditions and transportation of samples back to the laboratory. Our study also complied with the results of this study showing DNA amplification even at extended time periods and temperatures. (Max: 45degrees- 60 days). Various long term studies have also been done on the storage of DNA, one among which is a study by Ivanova and Kuzmina *et al.,* 2013. They evaluated three dry-state DNA stabilization systemson 96-well panels of insect DNA stored at 56 °C and at room temperature. Unprotected samples that were stored dry at room temperature and at 56 °C, and diluted samples stored at 4 °C and at -20 °C were included as controls.PCR and selective sequencing were performed over a 4-year interval to test the condition of DNA extracts which was found to be reliable with preservants. Our study however did not involve utilization of any commercial preservation systems.

Conclusion

Our study effectively compared the Quantity of DNA that can be extracted from Whole Saliva with the quantity of DNA deposited on a Swab, in the scenario of early evidence collection. A determination of presence of DNA after exposure of evidence to our tropical climatic conditions for 3 days was also done and the variation in quantity documented. This analysis is first of its kind with no correlating studies available for the same. The second phase of the study investigated the stability of Trace DNA demonstrated within the study period, so that the crime scene / victims clothing / inanimate objects in the scene of crime like carpets, or other accessories can be investigated with renewed hope, further, extensively and elaborately, provided the crime scene has been isolated for investigatory purposes. Considering the stability of DNA evidence in room temperatures, Revisiting of DNA evidence cheaper without special equipment or preservants .Population genetic studies are also benefitted wherein the mass samples can be stored in room temperature itself until further analysis. We plan to take forward this study with a futuristic eye starting with an increased Sample size, and utilizing a Biodegradable medium to collect bite imprints in place of the relatively stable medium used in our present study. With adequate resources, the outcome of the study can also be fulfilling when a Complete DNA profiling is done for individual identification.

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