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DNA Extraction and Gene Amplification from Saliva Deposited On Skin Using Double Swab Technique

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Abstract

Saliva may be found on victims of several violent crimes and it has been shown that it can also be potentially recovered and typed from bite marks, cigarette butts, postage stamps, envelopes and other objects. The double swab technique has been shown to increase the amount of salivary DNA recovered from skin when compared to other methods.

Aim and objective: To recover DNA from saliva deposited on human skin by double swab method and amplification of microsatellites and minisatellites from the recovered DNA.

Materials and method: Saliva from five volunteers was deposited on human skin and recovered using the double swab technique. DNA extraction was done using phenol chloroform method and PCR was used for amplification of 5 genes {Amelogenin (AMG), von Willebrand factor (vWF), F13 and D4S and Apolipoprotein (APO B)}. DNA was obtained from blood and direct salivary samples of the same volunteers and was used to compare the gene amplifications obtained by double swab technique.

Results: We found that using the DNA recovered from double swab technique F13 could be amplified in all 5 samples (100%) saliva samples deposited on skin, AMG, D4S and APO B genes could be amplified in 80%, 60% and 40% samples respectively whereas vWF gene failed to amplify in any of the sample. DNA amplified from all five samples of double swab technique matched positively with DNA obtained from saliva and blood.

Conclusion: Double swab technique can be used for isolating DNA from deposited saliva samples for the purpose of forensic study though the technique needs to be refined.

Key words: Short Tandem Repeats (STR), Variable Number Tandem Repeats (VNTR), AMG, F13, VWF, D4S and APO B.

Introduction

All humans have an identity in their society when they are alive. Compassionate societies require that this identity be recognized after death. The positive identification of living or deceased persons using the unique traits and characteristics of the teeth and jaws is a cornerstone of forensics. [1]

In criminal investigations, one of the fundamental requirements is that the victim and aggressor be positively identified, and legal situations often revolve around the establishment of offender's identity. In a bite mark, tooth in combination with other mouthparts makes a mark on victims' skin or some innate object, which can be compared with the unique characteristics of suspect's dentition. Distortions can occur at different

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stages in the action of biting, such as dynamic tissue distortions, and during examination and evidence recording, making the interpretation of bite marks difficult.[2] Techniques involving DNA analysis offer a new tool when traditional identification methods fail due to the effects of heat, trauma, or autolytic processes, as well as due to distortions and difficulties in analysis.[3]

Saliva is deposited on human skin through biting, sucking, licking and kissing, and possibly through other behavior.[2] Saliva may be found on victims of several violent crimes and it has been shown that it can also be potentially recovered and typed from bite marks, cigarette butts, postage stamps, envelopes and other objects. Saliva, in contact with intact skin, maintains itself in stable conditions and can be recovered up to, at least, 60 hours after its deposition.[3]

The amount of saliva deposited on the skin is generally very little in bite mark cases, making it necessary to use methods for collecting that result in the recovery of maximum possible amount of saliva and minimize any contamination through the victim's skin cells. Stains of dried saliva are invisible, which adds to the difficulty of recognizing and collecting them. The DNA present in saliva on skin is more difficult to collect and extract than similar stains on clothing, paper or other inanimate objects. The double swab technique has been shown to increase the amount of salivary DNA recovered from skin when compared to other methods.[4]

Forensic DNA profiling methods using the polymerase chain reaction (PCR) technique to amplify small amounts of recovered DNA at specific genetic loci are sensitive techniques. Studies have shown that it is possible to discriminate one individual from all others with a high level of confidence by starting with only 1ng (1billionth of a gram) or less of target DNA. In addition to identifying the perpetrators of crimes, these same methods have been used to identify deceased victims when virtually no physical evidence remains.[1]

Our study was designed to assess the feasibility of extracting DNA from saliva deposited on human skin, to simulate the saliva deposited after bite marks in real situations, and the ability to amplify STRs and VNTR from salivary DNA for purpose of forensic individualization.

Materials and methods

This study was conducted in the Department of Oral and Maxillofacial Pathology, Saraswati Dental College and Hospital, Lucknow in co-ordination with Department of Genetics, Sanjay Gandhi Post Graduate Institute, Lucknow. Blood and saliva samples were collected from twenty unrelated, healthy volunteers with no genetic and blood disorders after taking informed consent and approval from Hospital Ethics Committee of Saraswati Dental College and Hospital.

DNA extraction from blood and saliva was done by salting out method using phenol-chloroform as described by Coomey *et al.* [6] and was purified by ethanol precipitation. DNA extraction from saliva deposited on human skin was done by using double swab technique. 0.25ml of saliva was taken from 5 samples randomly selected from the study group. It was spread on the researcher's forearm and allowed to dry for ten minutes. A swab immersed in sterile distilled water was used to wipe the saliva from the skin. The surface was swabbed for about 15 seconds using moderately strong pressure and circular motions. The swabs were rotated along its long axis allowing each side of the swab to come into contact with the target surface. This was followed by a second, dry sterile cotton swab to absorb the water left behind on the skin by the initial swab and to collect additional salivary cells. The swabs were completely air dried and were transferred to micro centrifuge tubes. 160 µL of lysis buffer was added followed by 30 µL proteinase K and it was incubated at 56°C for 2 hrs. It was then washed repeatedly with sterile water for

recovering the DNA and the cotton swabs were removed from the wash solution. To this wash solution, 400 µL of phenol- chloroform was added and it was centrifuged at 12000 rpm for 10 minutes. The supernatant was transferred to a fresh micro centrifuge tube to which 1 ml of ethanol was added and centrifuge again 12000 rpm for 10 minutes. Supernatant solution was again decanted and the pellet was rinsed in 70% alcohol followed by drying the pellet at 56°C for 4 hours. Quality and quantity analysis of the DNA thus obtained will be performed by gel electrophoresis and spectrophotometry respectively. This resultant DNA solution was stored at -20°C until PCR analysis. [2, 6, 7]

The integrity of high molecular weight DNA is an important factor, which should be considered during extraction steps. Integrity was checked by electrophoresis on 0.8% Agarose for STRs and 8% Polyacylamide Gel Electrophoresis (PAGE) for VNTR.

Selection criteria of the STR markers

Selection of the STR markers was based on the global surveys carried out by *Lezaun P A et al.1997* [8] and *Destro- Bisol 2000*[9] which suggested that these STRs are one of the most polymorphic markers. Furthermore, studies by *Agarwal et al.* on nine endogamous groups of Uttar Pradesh also suggested that these markers can be employed for individualization purposes based on their high heterozygosity estimates.[10] Table 1 shows primer sequences for all the four STRs and a VNTR.

Table 1: Primer sequences for STRs and VNTR loci

S. No.	Locus	Primer sequences
1	AMG	Amel A-5'CCCTGGGCTCTGTAAAGAATAGTG3' Amel B- 5' ATCAGAGCTTAAACTGGGAAGCTG3'
2	Vwf	5' CCCTAGTGGATGATAAGAATAATC 3' 5' GGACAGATGATAAATACATAGGATGGATGG 3'
3	F13	5′ GAGGTTGCACTCCAGCCTTT 3′ 5′ ATGCCATGCAGATTAGAAA 3′
4	D4S	5' TCAGTCTCTCTCTTTCTCCTTGCA 3' 5' TAGGAGCCTGTGGTCCTGTT 3'
5	ApoB′ HVR	5' TCTCTATTTCCATCTCTGTCTCC 3' 5' TCACCCCTGTCTATGGTCTCG 3'

Results

Analysis of the quantity of isolated DNA was done using spectrophotometery by calculating concentration of DNA in the isolates from optical density readings at 260nm (OD260). The DNA concentration in blood samples ranged from 100 mg/ml to 175 mg/ml with mean of 126 mg/ml. Concentration of DNA isolated from direct salivary samples ranged from 45 mg/ml to 61.5 mg/ml with mean of 54.3mg/ml while those from five double swab samples ranged from 5mg/ml to 10 mg/ml, with mean of 8.2 mg/ml (Table 2).

Quality of DNA obtained was assessed by calculating the ratio of optical density readings at 260nm and 280nm. As shown in the table 3, OD260/OD280 ratio of DNA samples from blood ranged from 1.6 to 1.8 with a mean of 1.7. In saliva OD260/OD280 ratio ranged from 1.4 to 1.6 with mean of 1.5 while that of double swab samples it ranged from 1.3 to 1.5 with mean 1.4. In saliva 1 sample (20%) was in optimal OD range of 1.6 to 2 whereas in blood all 5 samples were in optimal range (100%). Quality of DNA extracted through double swab in all 5 samples was below optimal range.

Table 4 shows results of DNA typing of the four STRs (AMG, vWF, F13, and DS4) and one VNTR (APO-B) that were used for typing the DNA amplified from the salivary samples deposited on skin using PCR.

We found that using double swab technique AMG gene could be amplified in four out of five (80%) of saliva samples deposited on skin. In one sample where AMG could not be amplified the corresponding direct saliva sample was able to amplify the gene. All the amplified samples of double swab matched positively with corresponding blood and saliva

PCR amplification

STR and VNTR were genotyped by using single locus PCR.

samples, giving 100% matching and overall success of 80% (figure 1). None of the salivary samples deposited on skin showed vWF amplification by double swab technique as compared to their corresponding blood and saliva samples which amplified. Further F13 could be amplified in all five (100%) of saliva samples deposited on skin. All the amplified samples of double swab matched positively with corresponding blood and saliva samples, giving 100% matching and overall success of 100% (figure 3). D4S could be amplified in three out of five (60%) of saliva samples deposited on skin. In two samples where D4S could not be amplified the corresponding direct saliva sample was also not able to amplify the gene. All the amplified samples of double swab matched positively with corresponding blood and saliva samples, giving 100% matching and overall success of 60% (Figure 4).

VNTR APO- B could be amplified in two out of five (40%) of saliva samples deposited on skin. In two out of three samples where APO B could not be amplified though the corresponding direct saliva sample was able to amplify the gene. All the amplified samples of double swab matched positively with corresponding blood and saliva samples, giving 100% matching and overall success of 40% (Figure 5).

Table 2: Quantity of DNA obtained from blood, saliva and saliva deposited on skin samples

S.No	SAMPLE NUMBER	Blood DNA CONC- (g/ml)	Saliva DNA CONC- (g/ml)	Double Swab DNA CONC- (g/ml)
1	1	100.0	50.0	10.0
2	2	105.0	61.5	8.5
3	3	150.0	58.5	10.0
4	4	175.0	56.5	7.5
5	5	100.0	45.0	5.0

Table 3: OD260/OD280 ratio of the DNA isolates from blood, direct saliva, and double swab samples

S. No	Sample Number	Blood Nucliec Acid Protein Ratio	Saliva Nucliec Acid Protein Ratio	Double Swab Nucliec Acid Protein Ratio
1	1	1.6	1.5	1.3
2	2	1.7	1.5	1.4
3	3	1.7	1.5	1.4
4	4	1.8	1.4	1.5
5	5	1.6	1.6	1.3

Table 4: Shows PCR amplification results of the various STRs and the VNTR for salivary samples deposited on human skin and collected by double swab method from five randomly selected subjects

S.NO:	AMG		vWF		F13		D4S		APOB	
	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
1 S	785	953	NA		204	204	NA		NA	
B	785	953	119	123	204	204	181	181	42R	42R
DS	785	953	NA		204	204	NA		NA	
2 S	785	953	107	107	204	204	181	181	41R	41R
B	785	953	107	107	204	204	181	181	41R	41R
DS	785	953	NA		204	204	181	181	NA	
3 S	785	953	103	127	204	204	181	181	41R,	41R
B	785	953	103	127	204	204	181	181	41 R	41R
DS	785	953	NA		204	204	181	181	41 R	41R
4 S	785	953	NA		204	204	NA		43R	45R
B	785	953	NA		204	204	181	197	43R	45R
DS	785	953	NA		204	204	NA		43R	45R
5 S	785	953	107	111	204	204	181	181	NA	
B	785	953	107	111	204	204	181	181	41R	41R
DS	NA		NA		204	204	181	181	NA	

A1: Allele 1, A2: Allele 2, S: Saliva samples, B: Blood samples, DS: double swab samples, NA: Not Amplified

Figure 1: Amplification of STR AMG in double swab samples with blood and direct saliva samples

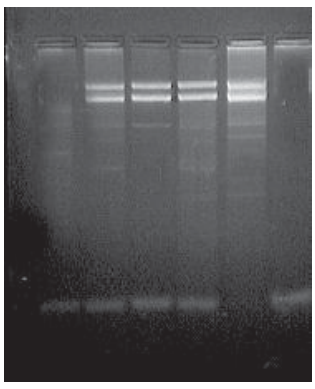


Figure 2: Amplification and matching of STR vWF in double swab with blood and saliva samples

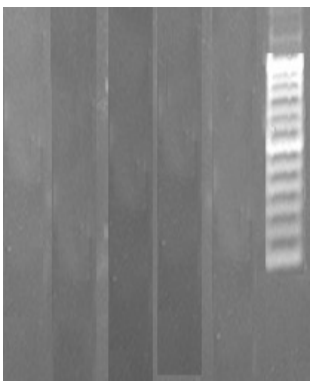


Figure 3: Amplification and matching of STR F13 in double swab with blood and saliva samples

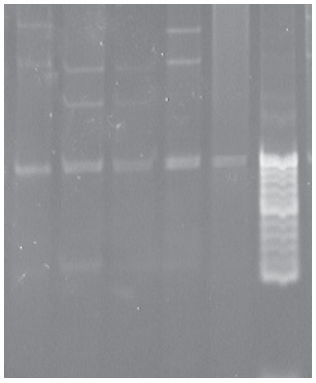


Figure 4: Amplification and matching of STR D4S in double swab with blood and saliva samples

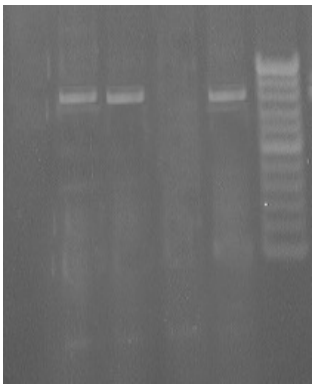
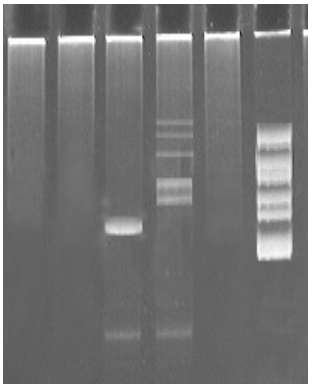


Figure 5: Amplification and matching of VNTR APO B in double swab with blood and saliva samples



Discussion

Saliva is a very useful DNA source due to the fact that it contains leukocytes and exfoliated epithelial cells from the host. PCR allows replication of thousands of copies of a specific DNA sequence in vitro, enabling the study of small amounts of DNA.

In cases where insufficient amount or when partially degraded DNA is available[6] short tandem repeats (STRs) or microsatellites specimens are potentially informative as they are amongst the most polymorphic markers reported till date. It consist of tandemly repeated DNA sequences with a core repeat of 1–5 base pairs (bp).[11] Attractiveness of STR typing includes its simplicity, rapidity, amenability to automation and capability for testing very small quantities of DNA.[12]

Variable number of tandem repeat (VNTR) are variable noncoding DNA stretches in the human genome composed of core units of a fixed nucleotide sequence that are repeated between 2 and 10000 times, depending on the type of polymorphism. The core units of these repeats are composed of hundreds of nucleotides which can be repeated hundred times. [13]

Amelogenin gene, is located on the X chromosome (Xp22.1 -p22.3) and Y chromosome (Yp11.2).[14]It is employed for human identity testing, criminal and parentage testing cases.[15] VWA is located in the von Willebrand Factor (vWF) gene; is utilized in routine forensic DNA profiling,[16] forensic analysis, paternity determination, detection of genetic disorders and cancers.[17]F13 is present on chromosome 6p24.2-p23 (F13A1) and has application in forensic analysis and paternity determination. D4S represents more than 6 percent of the total DNA in cells. Highly accurate comprehensive sequence for chromosomes 4 represents its efficiency for the Human Genome Project, in that it makes possible more detailed and conclusive analyses.[18] Apolipoprotein-B (ApoB) gene has been mapped on the short arm of chromosome 2p23 -24.[19] It is highly polymorphic is very useful in association, linkage analysis studies. [20] and for pedigree analysis.[21]

Variable success rate was found on amplification of DNA from saliva deposited

on skin (recovered by double swab method) for the STRs and VNTR. While all samples of F13 could be amplified others showed lower success rate; AMG (4/5), D4S (3/5), and APO B (2/5). None of our double swab saliva samples were able to amplify the STR vWF. Our results are in concordance with other researchers and the suboptimal purity and small quantity of DNA obtained from saliva would be the most probable explanation. Also presence of certain unknown PCR enzyme inhibitors in saliva sample may be a hindrance for amplification of more sensitive STRs.[2]

Samples of DNA isolated from saliva deposited on human skin has high probability of contamination by victim's DNA, especially if firm pressure is applied during sample collection, and hence could lead to possible error in interpretation of results. It is been suggested that if the contaminating DNA is present in comparable levels to the target DNA, its amplification could confuse the interpretation of the typing results. Whenever saliva is used as a source of DNA, such a risk of contamination by foreign DNA must be considered due to the ease of contact with other materials through the mouth.[22]

The double swab technique of collection of deposited salivary samples may afford an advantage over the traditional single swab technique in this aspect as using a wet swab in the first step loosens the salivary epithelial cells which are then collected by gentle swabbing in the second step.[23]This ensures collection of maximum cells with minimum physical pressure. On the other hand collection of sample by single swab would require a firmer pressure and vigorous swabbing to ensure recovery of sufficient material but in turn increases the risk of scraping the inner nucleated cells of the host skin.

Though the amplification of STRs from salivary samples was inconsistent, the amplified DNA samples, both from directly collected as well as deposited saliva showed excellent matching with DNA obtained from blood. Our results show high reliability of PCR technique in amplifying gene loci from salivary samples. Even though our samples showed high positive matching, the reliability of salivary DNA is not absolute.

Based on our findings we concluded that even though amplification of DNA from salivary samples is a technique sensitive procedure, requiring strict control of various factors, it has a potential to be utilized in forensics. However, the technique has to standardize by further studies on a larger and more varied sample size so as to produce predictable results.

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