TECHNICAL NOTE

David Sweet,[1] D.M.D, Ph.D.; Miguel Lorente,[2] M.D., Ph.D.; José A. Lorente,[2] M.D., Ph.D.; Aurora Valenzuela,[2] M.D., Ph.D., B.D.S.; and Enrique Villanueva,[2] M.D., Ph.D.

An Improved Method to Recover Saliva from Human Skin: The Double Swab Technique

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ABSTRACT: Human bite mark evidence is often found in violent crimes. Due to the difficulties of physically comparing an injury site on elastic and curved skin surfaces to the teeth of a suspect, the authors have considered using salivary DNA evidence to identify the bite perpetrator. Several techniques were evaluated to determine the best method of recovering saliva from human skin before extracting genomic DNA from the collection substrate. A classical stain recovery technique using a wet cotton swab was tested against one utilizing a wet filter paper. Additionally, a new method, referred to as the double swab technique, using a wet cotton swab followed by a dry cotton swab was also evaluated. After recovering a dried saliva stain, DNA was extracted using the modified Chelex method, quantified using the slot-blot procedure, and amplified at three polymorphic loci. The double swab technique showed the highest percentage recovery of saliva from human skin among the three methods studied. This technique is suggested as an improvement over the classical single wet cotton swab technique.

KEYWORDS: forensic science, saliva, bite marks, odontology, serology, forensic evidence

Teeth are often used as a weapon in violent crimes when one person attacks another, or in self-defense against an attacker (1). Human bite marks are found in cases of homicide, sexual assault, and crimes of family violence including child and spousal abuse (2). Conventional analysis strategies attempt to physically compare the injury pattern found on the victim's skin to the teeth of the suspected biter to reach conclusions regarding relative concordance (3).

In addition to considering bite marks as examples of physical evidence, the authors have also focused their attention on bite marks as biological evidence. It is assumed that forensically significant amounts of saliva are deposited during biting, sucking, or licking and that traces of salivary evidence can be recovered for identity testing.

Determination of the origin of a saliva stain using conventional markers is not highly sensitive nor discriminatory. The limited detectability of blood group antigens and polymorphic proteins due to their low concentrations is an inherent problem (4). Genomic DNA testing has increased sensitivity compared with conventional saliva testing methods (5). Recently, several studies have attempted to analyze DNA from salivary epithelial cells and leukocytes deposited on objects (6,7).

The amount of saliva deposited on the skin is usually very small in bite mark cases. It is necessary to use collection methods which result in recovery of the maximum possible quantity of salivary cells and which minimize any potential contamination from the cells of the victim's skin.

In the present study, several techniques were evaluated to determine the best method of collecting saliva from human skin and extracting DNA from the substrate. The classical technique using a single wet cotton swab (8,9) was tested as well as use of a section of wet filter paper laid passively on the skin to reduce potential contamination. A technique using a wet cotton swab (similar to the classical method) followed by a dry cotton swab, referred to as the *double swab technique*, was also tested.

Material and Methods

Saliva was collected from a single donor and deposited on the skin of living subjects. Attempts were made to collect and extract DNA from the resulting stains. To ensure reproducibility, a specific protocol for obtaining saliva from a single donor under repeatable conditions was followed. Approximately 1.5 mL of saliva were collected in a 1.5 mL polypropylene tube. The tube was gently vortexed to mix the contents. Using a sterile micropipette, 100 μL aliquots of saliva were removed from this solution.

Attempts were made to collect salivary DNA evidence from dried stains on the surface of the skin. It is suspected that most of the desquamated epithelial cells and leukocytes in the saliva are dehydrated following exposure to the air when the stain dries on the skin. Experimental protocols were established to determine the best method of collecting these dehydrated cells.

[1]Forensic odontologist, UBC Faculty of Dentistry, Vancouver, BC Canada.
[2]Assistant professor, associate professors, and professor, respectively, Faculty of Medicine, University of Granada, Spain.
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Recovery of Saliva from Skin

Samples containing 100 μL of saliva were deposited on the forearm of five subjects ($n = 15$) on three consecutive days. The saliva was allowed to air dry at room temperature for 10 min. Stains were recovered on the first day using wet sterile filter papers (25 mm diameter) laid passively on the skin over the stain. On the second day, stains were deposited and then recovered using a sterile cotton swab previously immersed in sterile distilled water. On the third day, stains were collected using the double swab technique.

In the double swab method, the first swab is immersed in sterile distilled water to wet the cotton tip completely. The tip is then rolled over the surface of the skin using moderate pressure and circular motions. Rotating the swab on its long axis ensures maximum contact between the swab and the skin to wash the dried saliva from the surface and collect as much evidence as possible. The swab is then set aside to air dry completely (≥30 min).

The second swab is not moistened. Using similar pressure and movements as with the first swab, the dry tip is rotated over the skin to recover the moisture remaining on the skin's surface from the wet swab. The dry swab is rolled over the entire area to ensure all of the moisture is recovered and set aside to air dry completely (≥30 min). Because the swabs are collected from the same site, they were pooled together into a single sample.

After collection, the filter paper discs and the single and double swab samples were adequately labeled and stored at 4°C pending DNA extraction and quantitation.

DNA Extraction

Considering the small quantity of DNA anticipated from a sample of saliva, the Chelex extraction method (10) was modified to improve the yield of extraction product. When all the samples were collected, the swabs were thoroughly washed in 1.5 mL of sterile distilled water and Proteinase K (1 μg/μL). The tubes were agitated for 1 min to loosen cells from the cotton fibers and then incubated at 56°C for 60 min and 100°C for 8 min (11) to recover as many cells and DNA molecules from the cotton as possible. The swab heads were compressed in a sterile syringe to recover the solution contained in them. This solution and the remainder of the wash solution were transferred to a new polypropylene tube. This was centrifuged at 10,000 xg for 5 min. The tube containing the pellet was set aside and the supernatant was microconcentrated using Microcon-100 tubes (AMICON, Beverly, MA). The concentrated solution was resuspended in 75 μL of Tris buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) and transferred back into the original tube containing the pellet of cells. The tube was gently agitated to mix the pellet. This sample was submitted to the classic Chelex extraction method. After adding 200 μL of 5% Chelex (CHELEX-100, Bio-Rad Laboratories, Richmond, CA), the tube is agitated and incubated in a water bath at 56°C for 30 min followed by incubation in boiling water for 8 min. Extraction is completed by centrifugation at 2,500 xg for 3 min. DNA quantitation was performed according to the slot-blot procedure of Waye et al. (12).

DNA Amplification

Extracts containing 3 ng of DNA were amplified at two short tandem repeat (STR) loci and one sequence polymorphism locus to determine if the DNA was of adequate quality and quantity for analysis. Amplification reactions were carried out using a Perkin-Elmer 9600 thermocycler (Perkin-Elmer Corporation, Foster City, CA). STR locus HUMTH01 amplification characteristics included 27 cycles of denaturation at 95°C for 45 s, primer annealing at 60°c for 30 s and extension at 72°C for 30 s (13). For locus HUMVWA, amplification was completed using 30 cycles of denaturation at 94°C for 10 s, annealing at 63°C for 10 s and extension at 72°C for 10 s (14). Locus HLA-DQA1 amplification included 32 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. The STR amplification products are visualized by electrophoresis on polyacrylamide gels followed by silver staining (15). The HLA-DQA1 amplification product was treated according to the manufacturer's recommended protocol (Perkin-Elmer Corporation, Foster City, CA).

Statistical Analysis

Analyses of variance and t-tests were performed, and linear regression techniques were applied to establish the relationship between variables.

Results

Figure 1 shows the percentage of DNA recovered in relation to the theoretical DNA quantity deposited on the skin. Salivary DNA present in the stain was calculated from the average DNA concentration in the saliva of the donor (2.71 ± 0.4 ng/μL). Significant differences were found among the three methods of recovery ($p \leq 0.001$). Although the filter paper and the single swab techniques showed the poorest results (17.4 ± 5.0% and 35.3 ± 4.8% respectively), the double swab technique showed the best percentage of saliva recovery (44.6 ± 6.4%) of the three methods studied.

Amplification results demonstrate the absence of contamination by DNA from the subject's skin (16). Positive amplification results where obtained from HUMTH01, HUMVWA, and HLA-DQA1 which indicate that salivary DNA of sufficient quality and quantity was recovered (results not shown).

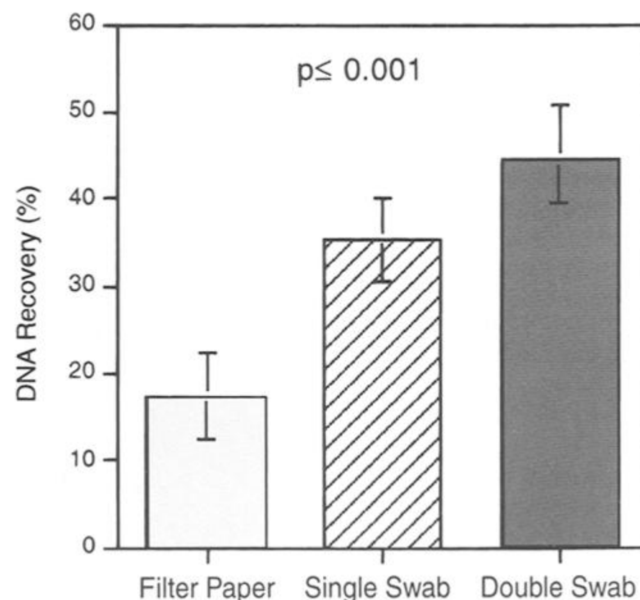

FIG. 1—*Comparison of the different methods to recover DNA from skin.*

Discussion

Current methods of recovering saliva are based on the use of a single cotton swab moistened with water (8,9). This is the method which was initially evaluated in the current study. Saliva recuperation results were relatively satisfactory (35.3 ± 4.8%) which indicates that this technique may be adequate in some cases.

Modifications to the single swab technique were undertaken due to the fear that epithelial cells (DNA from the victim) may be exfoliated as a result of rubbing the skin with the cotton swab and to improve the recovery results. A *double swab* method was devised. Using this technique, the amount of saliva collected increased to an average of 44.6 ± 6.4% of the total DNA deposited.

In cases with minimal amounts of saliva, partially degraded DNA, or where the surface of the skin is disrupted and the saliva sample may be contaminated by other DNA, use of the double swab technique is recommended. Considering that the PCR technique can be used with quantities as small as 1.0 ng of DNA, it will be possible to analyze these quantities at various loci using a multiple amplification protocol (17), or by using the sequential multiplex amplification technique (18). Therefore, the 9.3% difference between the single swab and double swab techniques is significant.

It is possible to recover a greater number of cells using the double swab procedure. It is believed that this is due to the fact that the moisture present in the first swab rehydrates and loosens the majority of the epithelial cells dried in the saliva and causes them to adhere to the cotton fibers of the swab.

When the second (dry) swab is applied to the site, the cells in the saliva are able to adhere to the fibers more easily because they are rehydrated after the application of moisture from the first swab and the time elapsed since the first swabbing.

In conclusion, the double swab technique for recovering saliva from human skin permits collection of a larger amount of DNA evidence than the classical methods studied. It is reasonable to assume that this technique may increase the amount of DNA recovered from saliva stains found on any surface at a crime scene.

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Additional information and reprint requests:
Dr. David Sweet
UBC Faculty of Dentistry
2199 Wesbrook Mall
Vancouver, BC Canada V6T 1Z3