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PCR-Based DNA Typing of Saliva Stains Recovered from Human Skin*

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ABSTRACT: Human bites in cases of homicide, sexual assault, and abuse are often distorted due to the elasticity and curvature of the skin. Physical comparison of a bite mark to a suspect's teeth is sometimes difficult. Saliva, which is usually deposited during biting, can be collected and analyzed to identify the perpetrator. Using simulated bite mark situations in two experimental series, three samples of 40 μL of whole saliva were deposited on the skin of 27 cadavers (at 33 sites) and three samples of 100 μL of whole saliva were deposited on the skin of 5 cadavers (at 12 sites). Saliva was collected using the double swab technique at t = 5 min, t = 24 h, and t = 48 h. DNA was extracted using the modified Chelex method and submitted to PCR-based typing at two short tandem repeat loci. Results indicate that the concentration of DNA in saliva recovered from skin varies as a function of time since deposition. There is a significant decrease in concentration in the first 24 h but the concentration remains stable from 24 to 48 h. The success of PCR amplification is independent of the time since deposition or the concentration of DNA in the saliva sample. Contamination from the DNA of the cadaver was not found in any of the cases studied.

KEYWORDS: forensic science, DNA typing, forensic odontology, saliva, polymerase chain reaction, HLA-DQA1, HUMTH01, HUMVWA, short tandem repeats

The usual methods of analyzing human bite mark evidence involve systematic physical comparison of the pattern of the injury in life-sized photographs or tracings to models of the suspect's teeth (1–4). These comparisons are often subjective and depend on the experience and procedures used by the odontologist. Saliva is normally deposited on human skin during biting, sucking, licking, and kissing so the authors anticipate the potential use of the DNA present in saliva stains on skin to reach conclusions about any role a suspect may have played in causing a given bite mark, or in other cases involving saliva deposition on skin. Guidelines established by the American Board of Forensic Odontology, or ABFO, (1995) for the collection of bite mark evidence advocate swabbing of the skin to collect saliva as part of the standard operating procedure. These swabs can be tested for amylase, a component of saliva. A positive test result confirms the presence of saliva and that the observed injury is in fact a bite mark. Odontologists follow these guidelines closely when collecting and preserving bite mark evidence because the procedures outlined in the Guidelines are accepted as the professional standard of practice in bite mark cases.

It is believed that in the majority of bite mark cases, and where saliva is deposited by one person or exchanged between two persons, the situation involves a perpetrator and a victim in the context of a sexually motivated crime, most often in association with violent behavior. If the victim survives the attack, they may or may not report the incident to authorities. The evidence remains undetected if the incident is not reported. But, in most jurisdictions when a victim reports a crime and seeks treatment, the odontologist is called to collect the evidence. A saliva swab is collected along with other typical physical evidence according to the ABFO Guidelines.

In a situation in which a victim does not survive the attack, the evidence deposited at the time of contact between the perpetrator and the victim will remain in place until it is discovered. Although it may be subjected to a series of changes, such as contamination, degradation, and putrefaction, depending upon the circumstances it may be possible to recover forensically significant salivary evidence.

Salivary forensic evidence has also been studied using a variety of conventional marker systems (5–8). These methods are not highly sensitive, and their limited detectability due to the low concentrations of the polymorphic antigens, isoenzymes, and proteins of interest is an inherent problem. Blood group testing is the most common conventional saliva typing method, but this is only possible if the sample is from a secretor individual (9).

Recovery of saliva deposited on the victim's skin is a difficult problem because nucleated cells containing DNA of sufficient quality and quantity for analysis must be collected while avoiding contamination by DNA from the victim. The *double swab technique* which uses an initial wet cotton swab followed by a dry cotton swab has been shown to increase the amount of DNA recovered from dried saliva on skin (10).

Following collection of the sample from the surface of the skin, an efficient method of extracting the DNA from the cotton swabs is required. The Chelex extraction method has been shown to be useful when a sample containing saliva is submitted directly into extraction solutions. DNA can be extracted from saliva deposited on postage stamps (11), cigarette butts (12), and other objects. We

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found that extracting DNA from swabs containing saliva recovered from skin is more difficult. The DNA yield from these swabs was subsequently improved by modifying the classical Chelex technique (13,14).

The polymerase chain reaction (PCR) technique provides two important benefits to the analysis of DNA extracted from biological evidence: a) Amplification is possible from very small amounts of DNA (15–17) which allows genetic information to be obtained from evidence samples such as a single hair (18), an invisible semen stain (19,20), and similar minute biological samples, and b) Amplification is possible from very old material or from partially degraded DNA (21).

When the PCR analysis method is used to test salivary evidence, DNA extracted from 1 μL of saliva has been shown to give the same typing result as 10 μL of whole blood (22). Other investigators, using the HLA-DQA1 typing system, have shown that 250 pg of DNA can be amplified and successfully typed from stamps and envelopes (23) and 2 to 160 ng of DNA is sufficient for typing from cigarette butts (12).

In the current study, attempts are made to evaluate the amount of DNA which can be recovered from simulated saliva stains on human skin corresponding to a bite mark, and to evaluate the presence of any contaminating DNA which may be collected from the skin of the victim during recovery of the salivary evidence. The amount of saliva deposited in a typical biting or sucking situation was carefully estimated based on the experience of one of the authors (DJS) and through preliminary studies using living donors and volunteers to quantify the saliva deposited in simulated bite marks. PCR-based typing at several short tandem repeat loci was used to study the ability to recover DNA of sufficient quantity and quality after a variety of elapsed times since saliva deposition.

Material and Methods

Sampling

Saliva samples were obtained from a single male volunteer donor. The donor's mouth was rinsed vigorously with tap water for 10 s and the water was discarded. After 5 min, the donor expectorated whole saliva which had accumulated in his mouth into a sterile 1.5 mL polypropylene tube. Approximately 1.0 mL of saliva was collected. The tubes were stored at 4°C prior to use. A control sample was separated from the donor's saliva and used to identify the donor's DNA profile.

In the first series of experiments, 33 different experimental sites were studied on the bodies of 27 cadavers with an average age of 59.7 years (10 females = 62.9 yrs., 17 males = 52.8 yrs.). A skin surface parallel to the surface supporting the body (e.g., autopsy table) and isolated from any abrasions, lacerations, or bruises (to avoid overt contamination of DNA from the cadaver) was selected. A circular area was outlined by pressing a metal ring (7.3 cm diameter) on the skin's surface for approximately 60 s using moderate force. This outline was further divided into four quadrants. The surface area of each quadrant was calculated to be approximately 10.7 cm^2 which is estimated to be the approximate area of an average adult bite mark. An aliquot of 40 μL of saliva was deposited and evenly distributed over the entire surface of each of three quadrants. The fourth quadrant was left undisturbed as a control area.

In the second series of experiments, the same protocol to divide a circular test area into four quadrants was used at 12 sites on the bodies of five additional cadavers with an average age of 59.7 years (3 females = 67.3 yrs., 2 males = 48.0 yrs.). An aliquot of 100 μL of whole saliva was distributed over the surface of each of three quadrants at each experimental site leaving the fourth quadrant undisturbed as a control area.

Five samples were collected from the cadavers in each series as follows: a) DNA reference sample (control sample from the victim to establish the victim's DNA profile) consisting of a buccal swab, whole blood, or tissue section, b) Negative control from undisturbed skin at the first quadrant, c) Positive saliva control from the second quadrant after 5 min, d) Saliva sample after 24 h from the third quadrant, and e) Saliva sample after 48 h from the fourth quadrant. This resulted in a total of 165 samples from the cadavers with 40 μL stains and 60 samples from the cadavers with 100 μL stains.

The double swab technique was used to collect the saliva samples from the skin of the cadaver (10). Using this method, a sterile cotton swab was immersed in sterile distilled water and used to wash the saliva from the skin. 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. Because the swab samples originate from the same site, the tips of the two swabs were combined into a single sample and stored at −20°C pending analysis.

DNA Extraction and Quantitation

DNA was extracted from the swabs using the Modified Chelex method previously described by the authors (14). The swabs were submitted to pre-extraction incubation in Proteinase K (1 μg/μL) at 56°C for 60 min and 100°C for 8 min to improve release of DNA and nucleated cells from the cotton fibers. Subsequently, the samples were submitted to the usual 5% Chelex extraction procedure (24) and quantified by the slot-blot procedure as described by Waye et al. (25).

DNA Amplification

Extracts containing 3 ng of DNA were amplified at two short tandem repeat (STR) polymorphic loci and one sequence polymorphism locus according to previously published protocols for HUMTH01 (26) and HUMVWA (27). In one case, locus HLA-DQA1 amplification was performed using 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 were visualized by electrophoresis on polyacrylamide gels followed by staining the gel with silver (28). The HLA-DQA1 amplification product was treated according to the manufacturer's recommended protocol (Perkin-Elmer, Foster City, CA).

Statistical Analysis

Statistical treatment of the results included use of the student *t*-test and linear regression analysis techniques.

Results

All of the samples were amplified at the HUMTH01 and HUMVWA loci. If no amplification was observed during the first attempt, the procedure was repeated a second time under similar conditions to confirm this finding. It was discovered that the genotype of the saliva of the donor was identical to the cadaver at both

experimental loci in only one case (HUMTH01: 6,8; HUMVWA: 15,18). In this case, to be certain that the amplification result was from the salivary DNA and not from the cadaver's skin, amplification at HLA-DQA1 was undertaken. Different genotypes were identified (saliva: 1.1,2; cadaver: 2,4).

Average values and standard deviations of DNA concentrations for both experimental series (Series 1, stains = 40 μL of saliva, Series 2, stains = 100 μL of saliva) are presented in Fig. 1. Significant differences were found between the concentration of DNA detected in each series. The amount of DNA recovered was higher for the second series (100 μL) for the three times studied (at 5 min, t_{exp} = 12.8228 (43 d.f.); at 24 h, t_{exp} = 12.1839 (43 d.f.); at 48 h, t_{exp} = 10.8012 (43 d.f.)). Significant differences (p 0.001) were found in the concentration of DNA between the three times (40 μL, F_{exp} = 26.56 (2,96) d.f.; 100 μL, F_{exp} = 64.2278 (3,22) d.f.).

Percentages of positive amplifications for HUMTH01 and HUMVWA in both experimental series are shown in Table 1. Although both loci presented similar percentages of positive amplifications, differences were found depending on the amount of saliva deposited on the skin.

Discussion

Quantitation

Quantitation of the DNA present in the sample was completed in all cases prior to subjecting the sample to PCR analysis. This allowed refinement of the techniques to optimize the results and potentiate the satisfactory study of a large variety of biological samples, including samples containing different quantities of DNA.

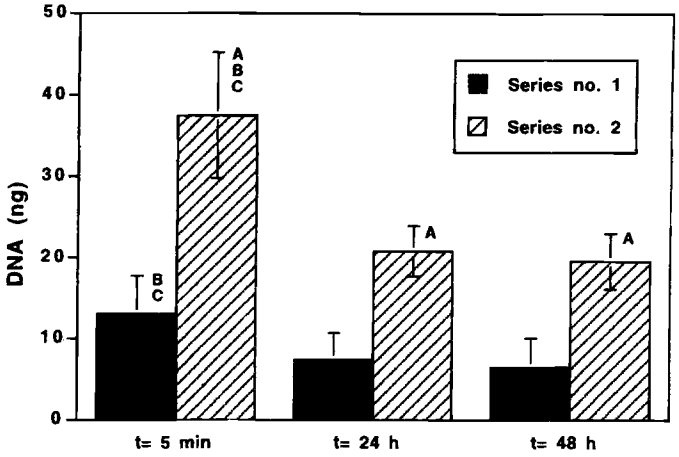

FIG. 1—*Average DNA quantity (ng) and standard deviations in both experimental series (Series no. 1: 40 μL; Series no. 2: 100 μL). A-significant differences (p ≤ 0.001) between series, B-significant differences (p ≤ 0.001) between 5 min and 24 h, and C-significant differences (p ≤ 0.001) between 5 min and 48 h.*

Considering the group of negative control samples where no saliva was deposited on the skin, no significant quantity of DNA was obtained using the double swab recovery technique. Slot-blot patterns from these samples were consistently less intense than those from the weakest DNA reference standard (0.125 ng). These patterns could neither be observed nor quantified. These results indicate that washing an area of the skin approximately 11 cm^2 in size using moderate force with the double swab technique does not produce a measurable amount of DNA from the cells of the skin (10).

The average amount of DNA recovered in most cases and at most times was sufficiently large to enable PCR amplification. As expected, the amount of DNA recovered was higher from the samples containing 100 μL than from those containing 40 μL. When 40 μL was deposited, the average of DNA recovered was 13.03 ng after 5 min, 7.42 ng after 24 h, and 6.46 ng after 48 h.

The variability between samples which is indicated by the large standard deviation is expected if it is remembered that these samples are biological in nature and were left in contact with cadaver skin over a period of 24 h. Changes in postmortem conditions such as temperature and humidity potentially have an effect on recovery. Also, changes in the skin quality of the cadaver which may result from perimortem physiological changes such as pain, cause of death, and chronological age may also be a factor. This variability of conditions is a universal problem which is common in thanatochemistry and has been previously described by many authors (29–32).

Comparison of the amount of saliva recovered after 5 min with the amount collected after 24 h revealed a significant loss of DNA ($p \leq 0.001$) in both experimental series. This was encountered by Hochmeister et al. during studies of DNA from saliva deposited on cigarette butts (12). No statistically significant differences were detected between the values at 24 h and 48 h. As a result of these findings, it was determined that the potential to recover nucleated cells from saliva, which can be directly estimated from the quantity of DNA recovered, diminishes significantly at 24 h and 48 h compared to the quantity recovered at 5 min. However, there are no practical differences between the amount recovered at these two time intervals.

The explanation of the loss is two-fold. First, we must consider the existence of an intimate association, similar to adhesion, between the nucleated cells in saliva and the epidermis of the cadaver. This makes the recuperation of these cells quite difficult. Increasing the amount of pressure utilized in the recovery operation may assist in releasing salivary cells from the surface of the skin, however, this also potentiates the possible release of epidermal cells from the cadaver (contamination) which could produce false positive results at amplification. Second, after 24 h of elapsed time, it is assumed that an indeterminate amount of degradation (presumably fairly high according to the results) of the salivary cells will have occurred. The cells may lose their integrity and

TABLE 1—*Positive amplification percentages for HUMTH01 and HUMVWA after various elapsed times since saliva deposition on human skin.*

	t = 5 min		t = 24 h		t = 48 h	
Locus	Series 1 (40 μL)	Series 2 (100 μL)	Series 1 (40 μL)	Series 2 (100 μL)	Series 1 (40 μL)	Series 2 (100 μL)
HUMTH01	78.8	100	75.8	83.3	69.7	83.3
HUMVWA	78.8	100	66.7	83.3	57.6	83.3

liberate the intracellular contents, including DNA, which is subsequently impossible to recover.

Amplification

Amplification of genetic material present in saliva and which is recovered from the surface of the skin must be considered with due caution. Despite the fact that no contamination from the skin of the cadaver was discovered in the experimental results, this possibility cannot be completely discounted.

Use of the normal number of PCR amplification cycles is recommended because it is important to amplify the minimal amount of evidence present but it is also important not to amplify any possible contamination from the skin of the victim. It is preferable to use the standard amplification reaction protocols and to adjust the concentration of reagents in the master mix in proportion to the amount of target DNA. If the results of the first amplification are negative, it is possible to repeat the process using sequential multiplex amplification (33) which permits recovery of the genomic DNA used in the analysis of the first locus after purification with Microcon-100 tubes (AMICON). Subsequent amplification at the same locus, or at different or additional loci, is possible using new primers.

It is not always the quantity of recovered DNA which will determine whether amplification will be positive. If the quality of DNA is low (34), or if there is contamination from blood products (35), systematic reduction in positive amplification results may occur.

Comparison was made of the positive and negative amplification results obtained for each of the loci analyzed (see Table 1). There were no statistically significant differences found for the amplifications at one or the other locus for each experimental series. Although it was not the intention of this project to study the improvement of amplification yield, results indicate that it may be possible to augment the number of positive amplifications through changes to the conditions and parameters within the amplification reaction.

No difference was detected in the number of positive and negative amplifications as a function of time because the percentage of amplifications are of the same magnitude at the three times studied. Therefore, if a low concentration of DNA is discovered at a given time, this should not affect the relative success of amplification. The quality and not the quantity of DNA recovered from the evidence is the most important factor (34). Some differences were found depending on the amount of DNA recovered from human skin. Average DNA concentrations in cases with positive amplifications for both loci were higher than in cases with negative amplifications for the series with 40 μL saliva.

In situations involving living victims of bite marks, forensic biological and physical evidence can usually be collected soon after deposition. In a homicide situation, the victim's body may not be discovered for some time after death and the ambient environmental conditions in which the body is deposited become significant. A standard operating procedure has been developed and recommended by the American Board of Forensic Odontology (1995) for collecting forensic bite mark evidence. The established protocol includes examination of suspicious injuries by the odontologist prior to postmortem washing or handling of the body. Following extensive photographic documentation of the site, saliva swabs are always indicated to attempt to recover amylase and DNA evidence. This is important even in cases in which skin abrasions or lacerations from teeth are present because a DNA profile from the perpetrator can be discriminated from the profile of the victim through use of adequate control samples. In the current study, using controls from the victim and the saliva donor, it was always possible to identify the source of DNA.

In conclusion, the results presented here show that saliva trace evidence contains forensically significant quantities of DNA which are stable during the postmortem interval over a reasonable period of time. Contamination from the skin of the cadaver was not found in any cases studied and a high percentage of positive PCR amplifications were obtained at the HUMTH01 and HUMVWA loci from saliva deposited on the skin of cadavers. In cases in which contamination from the victim's DNA may be present through collection of cells from abrasions, lacerations, and sloughing skin, etc., adequate internal controls should allow interpretation of the anticipated results.

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