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Research Paper

Estimating Time Since Deposition of Saliva Stains Using Selected Microbial Markers: A Pilot Study

Esraa Gamal Abo Elmoaty[1], Laila Ahmed Rashed[2], Basma Kamal Ibrahim[1*], Walaa Abdel hady Abdel halim[1], Neveine Ahmed El-Dessouky[1]

1. *Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Cairo University, Cairo, Egypt.*

2. *Medical Biochemistry and Molecular Biology Department, Cairo University, Cairo, Egypt.*

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ABSTRACT

Background: Forensic estimation of time since deposition (TsD) of biological evidence remains challenging in forensic investigations. Saliva is commonly found at crime scenes, and determining the age of old saliva stains using traditional methods is difficult. Recent advances in microbiome analysis suggest temporal changes in saliva-associated bacterial communities and may provide a promising tool to improve accuracy in forensic investigations. The aim of this work was to predict the time since deposition of saliva stains over a 21-day period using microbial markers.

Methods: Saliva stains were collected from ten healthy volunteers. Samples were spotted onto sterilized glass slides and kept at room temperature for molecular analysis at 0, 7, 15, and 21 days after deposition. Genomic DNA was extracted from saliva stains to detect microbial markers of selected microbes (*Enhydrobacter, Paenisporosarcina, and Firmicutes*).

Results: Linear mixed-model analysis showed that aging time significantly affected the relative abundance of *Enhydrobacter* $F(3,9.050) = 105.028, P < 0.001$, and *Paenisporosarcina* $F(3,9.576) = 90.674, P < 0.001$. In contrast, aging time had no statistically significant effect on *Firmicutes*, $F(3,19.208) = 3.381, P = 0.059$. Multiple linear regression demonstrated that *Enhydrobacter* and *Firmicutes* significantly predicted time since deposition, $F(2,21) = 64.062, P < 0.001$, explaining 85.9% of the variance ($R^2 = 0.859$). The predictive equation was: Time since deposition $= 23.036 - 1.122(\textit{Enhydrobacter}) - 0.001(\textit{Firmicutes})$.

Conclusion: Salivary bacterial markers undergo significant time-dependent succession over 21 days under ambient conditions. *Enhydrobacter* and *Paenisporosarcina* demonstrated marked decay post-deposition, whereas *Firmicutes* remained relatively stable. Combining candidate bacterial markers within a predictive linear framework yielded strong explanatory power ($R^2 = 0.859$), establishing microbial profiling of saliva stains as a viable quantitative approach for estimating time since deposition in forensic investigations.

*** Corresponding Author:**

Basma Kamal Ibrahim, MD

Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Cairo University, Cairo, Egypt.

E-mail: *basma.kamal@kasralainy.edu.eg*

Introduction

Reconstructing a crime is a crucial part of the prosecution's approach in a criminal trial. It is necessary to answer common questions such as what happened, where it happened, how it happened, and who caused it. Determining these basic issues can be greatly aided by biological evidence found at crime scenes. When is one of the trickier puzzles to figure out? A person's presence at a crime scene can be established by a trace found at the scene [1].

For criminal investigations, time since deposition (TsD) is an essential piece of information. Investigators can learn when a crime was committed by knowing when biological material was left at the scene. On the other hand, samples that don't match the period the crime is thought to have happened can be disregarded [2].

According to newly discovered data, microbial traits may be a useful method for identifying and estimating the TsD of bodily fluid stains [3].

The possibility of using deoxyribonucleic acid (DNA) profiling of human commensal bacteria to quantify saliva stain TsD in forensic contexts is demonstrated by our proof-of-principle investigation, which may be extended to other forensically important tissues [4].

In addition to being present throughout the human body, the microbiome has been shown, using 16S ribosomal RNA (16S rRNA) sequencing, to vary across body sites [5].

This study aimed to evaluate selected salivary microbial markers (*Enhydrobacter*, *Paenisporosarcina*, and *Firmicutes*) to develop a quantitative predictive model to estimate the time since deposition of forensic saliva stains over a 21-day period.

Materials and Methods

Study design and ethical considerations

This prospective analytical pilot study was conducted on 10 healthy Egyptian adult volunteers at Kasr Al-Ainy, Faculty of Medicine, Cairo University, from November 2025 to February 2026, in collaboration with the Department of Medical Biochemistry. The study was approved by the Institutional Review Board of the Faculty of Medicine, Cairo University, Cairo, Egypt (Approval code: MD-269-2024). All participants provided written informed consent after receiving an explanation of the study objectives and procedures in accordance with the approved ethical guidelines.

Participants

Participants were aged 19–65 years and included both sexes. Individuals who had received antibiotic therapy within the preceding month were excluded. Participants were also excluded if they had symptoms or signs of systemic or local bacterial infection, including fever, oral ulceration, oral pain, redness, tongue swelling, or erosions of the oral cavity.

Before saliva collection, participants were instructed to refrain from brushing their teeth, using mouthwash, eating, drinking, smoking, and chewing gum for at least 1 h. Before collection, they rinsed their mouths thoroughly with water to remove food debris. Saliva samples were collected between 9:00 and 10:00 a.m. in sterile 15-mL Falcon tubes. Participants were seated comfortably, with their heads slightly tilted forward, and were allowed to rest for approximately 5 minutes. Saliva was allowed to accumulate on the floor of the mouth and was then expectorated directly into the sterile collection tube.

Sample preparation and experimental conditions

Samples were collected, and 50 µL of each sample was spotted onto four pieces of sterilized glass slides and kept at room temperature for molecular analysis at 0, 7, 15, and 21 days after deposition. The materials and reagents included the EasyPure® Genomic DNA Kit (Cat. No. EE101, Version 2.0; TransGen Biotech), primers designed by Biotech Serve Company, SensiFAST™ SYBR® Hi-ROX qPCR reagents (Bioline, UK), sterile glass slides, sterile 15-mL Falcon tubes, 1.5-mL microcentrifuge tubes, and PCR tubes.

DNA extraction

Genomic DNA was extracted from the saliva stains using the EasyPure® Genomic DNA Kit according to the manufacturer's instructions. For each sample, 100 µL of Lysis Buffer 2 (LB2) and 20 µL of Proteinase K were added, then incubated at 55°C until complete lysis was achieved. Subsequently, 500 µL of Binding Buffer 2 (BB2) was added, and the mixture was vortexed for 5 s and incubated at room temperature for 10 min. The entire lysate was transferred to a genomic spin column and centrifuged at 12,000 × g for 30 s at room temperature, after which the flow-through was discarded.

Next, 500 µL of Column Buffer 2 (CB2), after adding ethanol according to the manufacturer's

instructions, was added to the column and centrifuged at 12,000 × g for 30 s at room temperature. After discarding the flow-through, 500 µL of Wash Buffer 2 (WB2) was added, followed by ethanol, and the sample was centrifuged under the same conditions. The WB2 washing step was repeated once. The column was subsequently centrifuged at 12,000 × g for 2 min at room temperature to remove residual wash buffer.

For DNA elution, the spin column was transferred to a sterile 1.5-mL microcentrifuge tube, and 50–200 µL of Elution Buffer preheated to 60–70°C, or sterile deionized water with pH >7.0, was added to the center of the membrane. The column was incubated at room temperature for 1 min and centrifuged at 12,000 × g for 1 min to elute the DNA. The elution step was repeated once to improve DNA recovery. The isolated DNA was stored at −20°C until molecular analysis.

Real-time PCR and microbial marker detection

Microbial DNA was detected and quantified using real-time quantitative PCR with SYBR Green I chemistry on an Applied Biosystems StepOne™ Real-Time PCR System using software version 3.1 (USA). Each 25-µL reaction contained 12.5 µL of SYBR Green PCR Master Mix, 1 µL of forward primer, 1 µL of reverse primer, 5 µL of DNA template, and 5.5 µL of RNase-free water. The primer sequences used to detect universal 16S rRNA, *Enhydrobacter*, *Paenisporosarcina*, and *Firmicutes* are shown in Table 1.

PCR amplification consisted of an initial hold at 50°C for 2 min, followed by denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min for 40 cycles. Thus, the annealing temperature used in the assay was 60°C.

Relative quantification of microbial markers

Comparative analysis of microbial DNA in the saliva stains was performed using the comparative ΔCt method. Universal 16S rRNA was used as the reference target [6], and the Ct values for Enhydrobacter, Paenisporosarcina, and Firmicutes were normalized to their respective 16S rRNA Ct values. The ΔCt was calculated as:

$$\Delta Ct = Ct\_target - Ct\_reference$$

Where Ct_target represents the Ct value of the microbial marker, and Ct_reference represents the Ct value of universal 16S rRNA. Relative quantification (RQ) was subsequently calculated from the ΔCt values to assess changes in the relative abundance of microbial markers across post-deposition intervals and storage conditions.

The primary molecular outcome was the relative abundance of the selected microbial markers in saliva stains.

Sample size calculation

This was a pilot, proof-of-concept study; based on previous studies showing substantial time-dependent changes in bacterial abundance in saliva stains [4,7], a sensitivity analysis using G Power 3.1.9.4 with four time points (0, 7, 15, and 21 days), $\alpha = 0.05$, 80% power, and a large effect size ($f = 0.56$) indicated that 10 participants could detect large temporal changes. Therefore, 10 participants were considered appropriate for this exploratory study. Larger studies with independent validation samples are required to confirm these findings and develop a reliable TsD prediction model.

Statistical analysis

Data were coded and entered using the Statistical Package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA). Linear regression analysis was performed to predict time since deposition using bacterial abundance and was validated via split validation, but the present analysis should be considered exploratory/pilot rather than a validated forensic prediction model [8]. For comparison of serial measurements, a linear mixed-effects model was used with volunteer ID as a random effect and repeated measures of time (0, 7, 15, 21 days) as a fixed within-subject effect, with post hoc pairwise comparisons adjusted with a Bonferroni correction [9]. P-values less than 0.05 were considered statistically significant.

Table 1. Primer sequences used to detect universal 16S rRNA, Enhydrobacter, Paenisporosarcina and Firmicutes.

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)
Universal 16S rRNA	GATTAGATACCCTGGTAG	GGGTTGCGCTCGTTG
Enhydrobacter	CCTACGGGAGGCAGCAGTAG	CAACAGAGCTTTACGATCCGAAA
Paenisporosarcina	GCCTAGCTTGCTAGGTGGAT	GCAGGTACCGTCAATCTCTC
Firmicutes	GGAGYATGTGGTTTAATTCGAAGCA	AGCTGACGACAACCATGCAC

Results

Table 2 shows the relative abundance of the studied bacterial taxa on days 0, 7, 15, and 21, as mean ± SD. At Day 0, *Firmicutes* was the predominant taxon (5788.49 ± 1972.70), followed by *Paenisporosarcina* (28.680 ± 6.782) and *Enhydrobacter* (12.49 ± 3.89). Over time, *Enhydrobacter* steadily declined to 0.05 ± 0.01 by Day 21. *Paenisporosarcina* dropped sharply by Day 7 (2.128 ± 0.724) before flattening near zero (0.008 ± 0.003 at Day 21). *Firmicutes* showed a gradual decrease to 3796.44 ± 1287.66 by Day 21.

Linear mixed-model analysis confirmed that aging time significantly affected the relative abundance of *Enhydrobacter*, $F(3,9.050) = 105.028, P < 0.001$, and *Paenisporosarcina*, $F(3,9.576) = 90.674, P < 0.001$. In contrast, there was no statistically significant overall effect of aging time on *Firmicutes* $F(3,19.208) = 3.381, P = 0.059$ (Table 3).

Bonferroni-adjusted pairwise comparisons showed that all aging-time comparisons for *Enhydrobacter* were statistically significant, with $P = 0.009$ for the comparison between 0 and 7 days and $P < 0.001$ for all other comparisons. For *Paenisporosarcina*, all pairwise comparisons were statistically significant ($P < 0.001$), except for the comparison between 15 and 21 days ($P = 0.114$) (Table 4).

Table 2. Relative Abundance of Enhydrobacter, Firmicutes, and Paenisporosarcina at days 0, 7, 15, and 21.

	0 day	7 days	15 days	21 days
Enhydrobacter	12.49±3.89	6.92±2.40	0.64±0.16	0.05±0.01
Paenisporosarcina	28.680±6.782	2.128 ± 0.724	0.015±0.007	0.008±0.003
Firmicutes	5788.49±1972.70	5596.03±1958.45	4861.15±1770.82	3796.44±1287.66

Table 3. Linear mixed-model results for bacterial taxa according to aging time.

Bacterial taxon	Effect	Numerator df	Denominator df	F	P value
Enhydrobacter	Time	3	9.050	105.028	<0.001
Paenisporosarcina	Time	3	9.576	90.674	<0.001
Firmicutes	Time	3	19.208	3.381	0.059

Note. Time was treated as a within-subject effect. P values indicate the significance of the overall effect of aging time on each bacterial taxon. Intercept effects are not shown because they are not directly relevant to the assessment of changes across aging time.

Table 4. Bonferroni-adjusted post-hoc pairwise comparisons among aging times.

Bacterial taxon	Aging-time comparison	Mean Difference (I−J)	SE	df	P value	95% CI
Enhydrobacter	0 vs day 7	5.570	1.445	14.976	0.009	1.180 to 9.960
	0 vs day 15	11.848	1.232	9.032	<0.001	7.709 to 15.987
	0 vs day 21	12.443	1.230	9.000	<0.001	8.303 to 16.583
	7 vs day 15	6.278	0.760	9.084	<0.001	3.727 to 8.829
	7 vs day 21	6.873	0.758	9.001	<0.001	4.321 to 9.425
	15 vs day 21	0.595	0.052	9.150	<0.001	0.421 to 0.769
Paenisporosarcina	0 vs day 7	26.552	2.157	9.205	<0.001	19.339 to 33.766
	0 vs day 15	28.665	2.145	9.000	<0.001	21.450 to 35.881
	0 vs day 21	28.672	2.145	9.000	<0.001	21.457 to 35.888
	7 vs day 15	2.113	0.229	9.002	<0.001	1.343 to 2.883
	7 vs day 21	2.120	0.229	9.000	<0.001	1.350 to 2.890
	15 vs day 21	0.007	0.002	11.069	0.114	-0.001 to 0.015

Note. Values are based on estimated marginal means. P values were adjusted for multiple comparisons using the Bonferroni method. Only one direction of each pairwise comparison is presented because the reverse comparison contains identical information with the opposite sign.

Table 5. Multiple linear regression model for prediction of time since deposition using Enhydrobacter and Firmicutes.

Predictor	B	SE	Standardized β	t	P value	95% CI for B
Constant	23.036	2.270	—	10.149	<0.001	18.316 to 27.756
Enhydrobacter	−1.122	0.120	−0.815	−9.377	<0.001	−1.371 to −0.874
Firmicutes	−0.001	0.000	−0.247	−2.847	<0.001	−0.002 to 0.000

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Model summary: R = 0.927; R^2 = 0.859; adjusted R^2 = 0.846; standard error of the estimate = 3.1889. ANOVA: F (2, 21) = 64.062, P < 0.001.

Multiple linear regression analysis showed that *Enhydrobacter* and *Firmicutes* significantly predicted time since deposition, $F\ open\ paren\ 2{,}21\ ,21) = 64.062, p << 0.001$. The model explained 85.9% of the variance in time since deposition ($R^2 = 0.859$; adjusted $R^2 = 0.846$). *Enhydrobacter* showed a stronger standardized association with time since deposition ($\beta = -0.815$), compared with *Firmicutes* ($\beta = -0.247$). The regression equation was: Time since deposition $= 23.036 - 1.122(Enhydrobacter) - 0.001(Firmicutes)$(Table 5).

Discussion

Forensic investigations frequently rely on biological traces left at crime scenes to reconstruct events and identify individuals involved. Among these traces, saliva stains are commonly encountered in cases involving biting, licking, or contact with objects such as cigarette butts, drinking containers, or envelopes. Saliva contains epithelial cells, proteins, nucleic acids, and a diverse microbial community, all of which can serve as valuable forensic markers. Beyond individual identification, saliva traces can also provide temporal information about when the stain was deposited, which can be crucial for establishing timelines in criminal investigations [10].

The current study demonstrated that salivary microbial composition changes over time after deposition, supporting the potential use of microbial markers to estimate TSD. Among the markers examined, *Enhydrobacter* showed the most consistent temporal pattern, with a progressive decrease throughout the 21-day aging period and significant differences across all aging intervals, consistent with findings in [7], which identified *Enhydrobacter* as a TSD-dependent marker in saliva stains and demonstrated the potential of microbial changes for estimating deposition time.

Similarly, *Paenisporosarcina* showed a significant decrease with aging, although most of the change occurred during the earlier period, with no significant difference between days 15 and 21, suggesting that it may be more useful for distinguishing earlier stages of saliva-stain aging than later intervals. Its association with TSD is also supported by [7], which identified Paenisporosarcina as a useful microbial marker for saliva-stain aging.

Firmicutes, on the other hand, showed a gradual decrease but did not demonstrate a significant overall time effect (P = 0.059); however, it contributed significantly to the multiple regression model, suggesting that it may provide complementary information when combined with *Enhydrobacter*. This observation is consistent with [11], which reported a significant decrease in *Firmicutes* during longer-term indoor aging. [12] Firmicutes were similarly identified among bacterial groups associated with time-dependent microbial changes in biological traces. The difference between the non-significant overall time effect and its significant contribution in the regression is therefore not contradictory, as the regression evaluates the combined predictive contribution of the markers rather than their individual changes across aging points.

The multiple regression model significantly predicted TSD, explaining 85.9% of its variation (R^2 = 0.859; adjusted R^2 = 0.846); *Enhydrobacter* was the strongest predictor (β = −0.815), while *Firmicutes* provided a smaller but significant contribution (β = −0.247). The negative coefficients indicate that higher abundance of these markers was associated with shorter TSD under the conditions of this study. These findings are consistent with [13], which reported a Mean Absolute Error (MAE) of 1.20 days using four bacterial genera, and [4], which found that targeted bacterial markers explained at least 86.5% of TSD variation; however, the increased error during external validation reported by [4] highlights the importance of independent validation.

Other studies further support the potential of microbial approaches for TSD estimation, with [7] achieving a 16S-based Mean Absolute Deviation (MAD) of 1.41 days and [14] reporting an MAE of 0.30 days using species-level markers over a short aging period [15]. Demonstrated the benefit of combining microbial taxonomic and functional information, whereas [14] showed that absolute microbial abundance may improve early TSD discrimination.

[16] Demonstrated additional value from integrating fungal and bacterial profiles, and [10] showed that mRNA degradation markers can also provide temporal information. These studies indicate that prediction accuracy depends strongly on the biological markers, analytical approach, and deposition duration.

Both environmental and biological variation remain important challenges for forensic application, as [17] reported that humidity and environmental exposure can substantially alter salivary microbial communities, while [18] found that dry conditions better preserve microbial community structure [12]. Also demonstrated the influence of environmental microorganisms on temporal changes in the microbiome [3]; reported substantial donor-related variation and emphasized that body-fluid identification should precede microbiome-based TSD estimation.

The present study revealed that *Enhydrobacter* is the most informative marker among those examined, while *Paenisporosarcina* provides useful information particularly during earlier aging, and *Firmicutes* contributes complementary information to the predictive model. The combination of markers with different temporal behaviors represents the main strength of the present approach. Nevertheless, the findings were obtained under controlled indoor conditions over 21 days; therefore, multi-donor, multi-substrate, and environmentally diverse validation is required before application in forensic casework.

Conclusion

The present study demonstrates that salivary bacterial markers undergo significant time-dependent shifts following deposition under indoor ambient conditions. Specifically, aging time significantly influenced the relative abundance of *Enhydrobacter* and *Paenisporosarcina* over 21 days, whereas overall *Firmicutes* levels remained relatively stable. A multiple linear regression model utilizing *Enhydrobacter* and *Firmicutes* successfully predicted time since deposition, accounting for 85.9% of the variance ($R^2 = 0.859$), with *Enhydrobacter* serving as the primary predictive marker ($\beta = -0.815$). Current findings highlight the utility of target salivary bacterial profiling as a valuable quantitative tool for estimating the time since deposition in forensic investigations.

Ethical Approval

This study was approved by the Institutional Review Board of the Faculty of Medicine, Cairo University, Cairo, Egypt (Approval code: MD-269-2024).

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Conflicts of Interest

The authors report there are no competing interests to declare.

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