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Identification of body fluids—menstrual blood, saliva, and nasal secretions—over different periods of time, using mRNA

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Abstract

Background: Forensic examination of biological samples started at the beginning of the twentieth century by applying the ABO blood group system in evidence related to crimes or human identification. In the present study, real-time PCR multiplex was used to identify dried and stored swabs (saliva, nasal secretions, and menstrual blood) through the target genes of saliva (histatin 3 and statherin), nasal secretions (statherin and BPIFA1), and menstrual blood (metalloproteinases 10 and 7).

Results: The expressions of histatin 3 and statherin in the dried saliva decreased over days of storage with a significant *p* value of <0.001. BPIFA1 was highly expressed in nasal secretions, and the expression level significantly decreased throughout the study with a significant *p* value of <0.001. The MMP7 and MMP10 genes were highly expressed in the menstrual blood, and the expression level decreased over days of storage with a significant *p* value of $p<0.001$.

Conclusions: Dried swabs of the saliva, Nasal secretions, Menstrual blood can be identified over the storage duration of the study using mRNA profiling of specific markers.

Keywords: Real-time PCR multiplex, Nasal secretions, Saliva, Menstrual blood

Background

RNA and DNA can be used in various forensic applications, as quantification of RNA degradation can determine the postmortem interval and the age of some stains, wounds age by detecting gene expression reactive changes and to determine the cause of death. In order to characterize specific body fluids, many methods can be used, which involve the study of miRNA, mRNA, DNA methylation, and microbial determination (Harbison and Fleming 2016). mRNA profiling is usually used in body fluid identification that may be found in crime scenes like the saliva, vaginal mucosa, blood, semen, and menstrual secretion (van den Berge et al. 2016). This study demonstrated that histatin 3 (HTN3)-specific target gene for saliva was stable for at least 365 days at room temperature when the samples were kept in envelops, wet and dry. Another study showed that in samples treated with ultraviolet light, HTN3 was detectable for up to 180 days (Setzer et al. 2008). (Sakurada et al. 2011) reported that on dried body fluids which were stored at −80°C for 135 days the statherin STATH Ct value increased significantly ($p < 0.01$) from 1 to 69 days but was not detected at day 135.

Sirker et al. (2016) showed that the saliva markers STATH and HTN3 provided good results for dry-stored 5 μL samples and were detected until 71 weeks of storage. Concerning BPIFA1, in a study conducted by Chirnside et al. (2020), it was not detected in any non-nasal

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samples. Another marker, metalloproteinase 7 (MMP7), can be detected in dried cotton samples stored at room temperature for up to 2 years using real-time PCR multiplex (Haas et al. 2009).

Methods

This prospective cohort study was conducted at The Biochemistry Department Laboratory, Faculty of Medicine, Cairo University. The participants included in our study were healthy volunteers of both sexes aged 18–40, and their saliva, nasal, and menstrual blood swabs were obtained. All individuals with blood diseases, malignancies, and respiratory tract diseases were excluded from our study.

Consent

The participant consent was verbal and was approved by the Local Ethical Committee of Forensic Medicine and Clinical Toxicology Department (ethics reference: I-070318). The swabs were considered a non-invasive procedure, and consent can be expressed verbally.

Design of work

The present study included 180 samples from 40 volunteers: 20 females (26–30 years old) for menstrual blood collection and 10 males plus 10 females (26–30 years old) for nasal and salivary sample collection. For each fluid, 60 samples were collected (20 samples were dried at ambient temperature for 1 day before being stored at −20°C), 20 samples were dried at room temperature for 3 days before being stored at −20°C, and 20 samples were dried at room temperature for 7 days before being stored at −20°C to be studied later. For each body fluid, we intended to detect two specific markers (for the saliva, STATCH and HTN3; for nasal secretions, STATCH and BPIFA1; and for the menstrual blood, MMP7 and MMP10) and a housekeeping gene (B-actin) as an internal control.

Extraction of RNA from the saliva, nasal, and menstrual blood swabs

The extraction of intact RNA needs four steps: disruption of cells, denaturation of nucleoprotein complexes, endogenous ribonuclease (RNase) activity inactivation, and removal of DNA and proteins that contaminate the samples. The purification of RNA contaminants was performed using simple washing steps with 70% alcohol. They were then eluted by centrifuge at full speed (14,000 rpm or 10,000× for 2 min) and then stored at −20°C. Isolation reagent supported by Ambion, Inc.

Reverse transcription and PCR

The yield of the total RNA of the collected samples was determined at 260 and 280 nm using Beckman dual spectrophotometer. Gene expression was measured using One-Step Taq qRT-PCR Green Master (Ambion, Inc) in a real-time PCR instrument (StepOne, version 2.1, Applied biosystem, Foster City, USA). For qRT-PCR, we developed a triplex which would include 2 body fluid-specific markers and a housekeeping gene (B actin) as an internal control. Ten μL of the total RNA from each sample was used for RT-reaction and amplified in a total reaction volume of 25μL. The standard reaction mixture contained buffer (10 mM Tris-HCl, pH 8.3, 50mM KCl, and 1.5mM MgCl2), 0.5 mM dNTP mix, 0.4 μM of each PCR primer Table 1 and 1.25U AmpliTaq Gold® DNA polymerase. The thermal profile was as follows: 45°C for 15 min one cycle (for cDNA synthesis), 10 min

Table 1 Primer sequences

Body fluid	Gene	Primer (F and R)	Size (bp)	Reference
Nasal	BPIFA1	F:CAAGTGAATACGCCCCTGGTCG	131	van den Berge et al. 2016
		R:GAATGGGTGCAGTCACCAAGGAC		
	STATH	F:TTTGCCTTCATCTTGGCTCT]	93	Lindenbergh et al. 2012
SalivaSaliva		R:CCCATAACCGAATCTTCCAA		
	HTN3	F:ATGGGCCAGCACACAGAC.	134	
		R:GCAAAGAGACATCATGGGTA		
Menstrual Blood	MMP-7	F:TTTTCCCATGGGGAAATATAGGT	127	
		R:GAACAGGCTCAGGACTATCTC		
	MMP-10	F:GCATCTTGCATTCCTTGTGCTGTTG	107	van den Berge et al. 2016
		R:GGTATTGCTGGGCAAGATCCTTGTT		
Housekeeping Gene	B-actin (*ACTB*)	F:CTTGGGAGGGCACTTGGGGGTG		Lindenbergh et al. 2012
		R:TGACCCAGATCATGTTTGAG		

A Adenine, *C* Cytosine, *G* Guanine, *T* Thymine, *B-actin* Beta-actin, *STATH* Statherin, *HTN3* Histatin 3, *BPIFA1* Lipid-binding protein that plays a role in the innate immune responses of the upper airway, *MMP10* Metalloproteinase 10, *MMP7* Metalloproteinase 7

at 95°C for reverse transcriptase enzyme inactivation followed by 50 cycles PCR amplification were programmed. Each cycle was 10 s at 95°C for CDNA denaturation, 30 s at 60°C for primers annealing, and 30 s at 72° for Taq polymerase extension. Normalization of each target gene was calculated relative to values of β-actin housekeeping gene, and the result was analyzed with the Step One Applied Biosystem software.

Statistical analysis

Data were coded and entered using the Statistical Package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). Data were summarized using mean and standard deviation. The comparison between the different genes in the same sample was made using a paired *t* test. The comparisons between the different samples were made using an unpaired *t* test when comparing two samples, and the analysis of variance (ANOVA) with multiple-comparisons post hoc test was used when comparing more than two samples (Chan 2003). A *p* value of less than 0.05 was considered statistically significant.

Results

The expression of the genes, STATH and BPIFA1, in nasal secretions decreased during different periods of storage, but was still detectable after 7 days, HTN3 and STATH expression in the saliva decreased during different periods of storage but still detected after 7 days and MMP7 expression in menstrual blood decreased during different periods of storage but still detected after 7 days (results shown in Table 2). The STATH and BPIFA1 gene expression levels in the nasal swabs on the 1st, 3rd, and 7th day were significantly higher than the B-actin expression levels. The BPIFA1 expression levels on the first, third, and seventh days were significantly higher than the STATH expression levels (Table 3). The HTN3 and STATH gene expression level in the saliva was considerably higher than the B-actin gene expression levels on the first, third, and seventh days, and no statistically significant difference was observed between the HTN3 and STATH gene expression levels on the first, third, and seventh days (Table 4). The STATH expression level in the saliva on the first day was significantly higher than that in nasal secretions (Table 5). The MMP7 and MMP10 expression levels on the first, third, and seventh days were significantly higher than the B-actin expression level, and no statistically significant difference was observed between the expressions of both genes on the first, third, and seventh days (Table 6).

Discussion

Our results of dried samples of the saliva, nasal secretions, and menstrual blood are similar to those reported by Sakurada et al. (2011), in which dried body fluids (vaginal secretions, nasal secretions, semen, saliva, urine, blood, and sweat) were stored at room temperature and then at −80°C to be examined over 135 days using real-time PCR multiplex, indicating that the cycle threshold

Table 2 Gene expression levels at different storage days

Gene expression at different days	Menstrual blood genes		Saliva genes		Nasal genes	
	MMP7	MMp10	STATH	HTN3	STATH	BPIFIA
1st day	35.08 ± 1.98	34.72 ± 2.36	36.64 ± 3.33	35.31 ± 2.80	28.05 ± 2.91	33.78 ± 4.41
3rd day	29.78 ± 2.98	29.62 ± 1.17	29.21 ± 4.88	29.85 ± 2.53	25.90 ± 3.53	32.21 ± 3.02
7th day	25.97 ± 3.16	25.70 ± 2.23	23.08 ± 3.44	25.36 ± 3.61	21.81 ± 3.62	27.78 ± 4.69
p value	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**

ANOVA test

STATH Statherin, *HTN3* Histatin3, *BPIFA1* Lipid-binding protein that plays a role in the innate immune responses of the upper airway, *MMP10* Metalloproteinase 10, *MMP7* Metalloproteinase 7

**P* is significant if < 0.05, ***P* is highly significant if < 0.01

Table 3 Comparison between the gene expression levels in dried nasal swabs

Nasal genes (mean ± SD)								
STATH	B-actin	*p* value	BPIFA1	B-actin	*p* value	STATH	BPIFA1	*p* value
28.05 ± 2.91	24.99 ± 3.15	0.003**	33.78 ± 4.41	24.99 ± 3.15	< 0.001**	28.05 ± 2.91	33.78 ± 4.41	<0.001**
25.90 ± 3.53	20.15 ± 2.41	< 0.001**	32.21 ± 3.02	20.15 ± 2.41	< 0.001**	25.90 ± 3.53	32.21 ± 3.02	<0.001**
21.81 ± 3.62	18.57 ± 2.37	0.002**	27.78 ± 4.69	18.57 ± 2.37	< 0.001**	21.81 ± 3.62	27.78 ± 4.69	0.001**

B-actin Beta-actin, *STATH* Statherin, *BPIFA1* Lipid-binding protein that plays a role in the innate immune responses of the upper airway, *SD* Standard deviation

Paired *t* test (**P* is significant if < 0.05, ***P* is highly significant if < 0.01)

Table 4 Comparison between the gene expression levels in dried saliva swabs

Saliva genes (mean ± SD and *p* values)								
STATH	**B-actin**	***p* value**	**HTN3**	**B-actin**	***p* value**	**STATH**	**HTN3**	***p* value**
36.64 ± 3.33	24.78 ± 2.63	<0.001**	35.31 ± 2.80	24.78 ± 2.63	<0.001**	36.64 ± 3.33	35.31 ± 2.80	0.126
29.21 ± 4.88	20.98 ± 3.05	<0.001**	29.85 ± 2.53	20.98 ± 3.05	<0.001**	29.21 ± 4.88	29.85 ± 2.53	0.688
23.08 ± 3.44	17.85 ± 2.60	<0.001**	25.36 ± 3.61	17.85 ± 2.60	<0.001**	23.08 ± 3.44	25.36 ± 3.61	0.069

HTN3 Histatin 3, *B-actin* Beta-actin, *STATH* Statherin, *SD* Standard deviation

Paired *t* test (**P* is significant if < 0.05, ***P* is highly significant if < 0.01)

Table 5 Comparison between the STATH expression levels in dried nasal and saliva swabs

STATH		**1st day**	**3rd day**	**7th day**
Nasal	**Mean ± SD**	28.05 ± 2.91	25.90 ± 3.53	21.81 ± 3.62
Saliva	**Mean ± SD**	36.64 ± 3.33	29.21 ± 4.88	23.08 ± 3.44
***p*value**		<0.001**	0.019	0.264

STATH Statherin, *SD* Standard deviation

Unpaired *t* test (**P* is significant if < 0.05, ***P* is highly significant if < 0.01)

(Ct) values of STATH and HTN3 in the saliva stains showed no statistical difference. In the nasal stains, the STATH Ct value increased significantly ($p < 0.01$) from 1 to 69 days but was not detected after 135 days. Previously, Sakurada et al. (2012) aimed to identify nasal blood by comparing the mRNA expression levels of target genes specific to blood, nasal secretion, and saliva and found that the statherin gene (STATH), which is a saliva-specific gene, was highly expressed in nasal secretions and also detected after 4 months of sample storage at room temperature. These authors reported that the histatin gene (HTN3) was only expressed at high levels in the saliva, whereas the Ct values for b-actin (ACTB), hemoglobin subunit beta (HBB), and STATH increased after 1 month of storage at room temperature. Roeder and Haas (2013) reported that the HTN3 and STATH mRNAs were abundant in the salivary samples and could not be detected in the blood, menstrual blood, semen, sweat, skin, and CSF, except for STATH that had minute amplification in the menstrual blood. Hanson et al. (2018) also reported that HTN3 in the saliva was highly expressed in a study conducted in Switzerland. Akutsu and Watanabe (2020) reported that STATH was detected only in all nasal secretion and salivary samples and not detected in the other body fluids included in the study (urine, saliva, vaginal, semen, and menstrual blood). Moreover, HTN3 was only detected in the salivary samples. In contrast, Sakurada et al. (2011) reported that no significant statistical difference was observed between the cycle threshold (Ct) values of STATH in the nasal stains and STATH in the saliva stains in 1-day-old stains. In concordance with the results of the present study, the STATH Ct value in the nasal stains was higher than the STATH and HTN3 Ct value in the saliva stains at 7 and 69 days. van den Berge et al. (2016) also reported the detection of STATH in the saliva and nasal samples and the differentiation between the nasal secretions and saliva based on the absence of HTN3. Sakurada et al. (2011) also reported that in the saliva, the STATH and HTN3 cycle threshold (Ct) values increased significantly over 1–69 days and no significant difference was observed between samples at 69 and 135 days. The study used real-time PCR multiplex on dried body fluids (nasal secretions, vaginal secretions, saliva, semen, urine, sweat, and blood), which were stored at room temperature and at −80°C and then examined over a 135-day period. Sirker et al. (2016) examined the dry-stored 5 μL of the saliva and found that STATH and HTN3 were detected until 71 weeks. Their study focused on different volumes (5, 0.5, and 0.05 μL) of the saliva, blood, and semen over

Table 6 Comparison between the gene expression levels in dried menstrual blood swabs

Menstrual blood genes (mean ± SD and *p* values)								
	MMP7	**B-actin**	***p* value**	**MMP10**	**B-actin**	***p* value**	**MMP7**	**MMP10**	***p* value**
1st day	35.08 ± 1.98	26.24 ± 2.25	<0.001**	34.72 ± 2.36	26.24 ± 2.25	<0.001**	35.08 ± 1.98	34.72 ± 2.36	0.511
3rd day	29.78 ± 2.98	26.24 ± 2.25	<0.001**	29.62 ± 1.17	26.24 ± 2.25	<0.001**	29.78 ± 2.98	29.62 ± 1.17	0.822
7th day	25.97 ± 3.16	17.44 ± 2.25	<0.001**	25.70 ± 2.23	17.44 ± 2.25	<0.001**	25.97 ± 3.16	25.70 ± 2.23	0.822

MMP10 Metalloproteinase 10, *MMP7* Metalloproteinase 7, *SD* Standard deviation

Paired *t* test (**P* is significant if < 0.05, ***P* is highly significant if < 0.01)

72 weeks using PCR. Watanabe et al. (2017) also reported that HTN3 and STATH were detected after 6 months but variable in 9 and 12 months in dried cotton saliva samples stored at room temperature and high temperature (35°C) and not detected after 6 months in wet conditions. Van den Berge et al. (2016) revealed that theoretically, we could discriminate the peripheral blood from the nasal blood using BPIFA1 (nasal mucosa marker). Sakurada et al. (2012) also reported that the STATH expression in the nasal fluid could not be detected after the sample was stored for 4 months at room temperature. Haas et al. (2009) reported that MMP7 could be detected in dried cotton samples stored at room temperature for up to 2 years. Song et al. (2015) reported that MMP10 was only detected in menstrual blood and not detected in other body fluids included in the study (semen, saliva, blood, and vaginal secretions).

Conclusions

Based on the RT-PCR results, the expression levels of saliva genes (HTN3 and STATH), nasal genes (BPIFA1 and STATH), and menstrual blood genes (MMP7 and MMP10) have decreased from day 1 to day 7 of storage at room temperature. BPIFA1 was highly expressed than STATH in nasal secretions. STATH was highly expressed in the saliva than in nasal secretions. No significant difference was observed between the HTN3 and STATH expression levels in the saliva and between the MMP7 and MMP10 expression levels in the menstrual blood from day 1 to day 7. Therefore, this study proves the usefulness of mRNA profiling in the identification of body fluids, such as the saliva, nasal secretion, and menstrual blood, at different storage durations (1, 3, and 7 days) at room temperature. We recommend the examination of body fluids at 30, 90, 180, and 365 days at different conditions (wet envelop at room temperature, luminescent, UV rays outside with and without rain) and the confirmation of our results by further investigating the relationship between such expressions.

Abbreviations

PCR: Polymerase chain reaction; B-Actin or ACTB: Beta-actin; BPIFA1: A lipid-binding protein that plays a role in the innate immune responses of the upper airway; cDNA: Complementary DNA; CT: Cycle threshold; HBB: Hemoglobin subunit beta; HTN3: Histatin 3; MMP: Metalloproteinase; SD: Standard deviation; STATH: Statherin.

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None.

Authors' contributions

NS and RF designed the study and wrote the manuscript. NS collected the data. SG, RF, and SM analyzed the data. NS and RF wrote the results. LR finalized the methodology and tools used. SG, SM, and RF revised the paper. The authors made significant contributions in finalizing the manuscript. The final manuscript has been read and approved by the authors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was approved by the local Ethical Committee of Forensic Medicine and Clinical Toxicology Department, Faculty of Medicine, Cairo University. Regarding the ethics committee reference numbers, it is not available because the study was approved by the local Ethical Committee of Forensic Medicine and Clinical Toxicology Department which gives only approval without number. Participant consent was verbal and was approved by the local Ethical Committee of Forensic Medicine and Clinical Toxicology Department (ethics reference: I-070318). The swabs were considered a non-invasive procedure, and consent can be expressed verbally.

Consent for publication

Not applicable.

Competing interests

The authors declared that they have no competing interests.

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