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A Preliminary Study on the Venous and Menstrual Blood Identification by Methylation-Specific PCR

Kuo-Lan Liu [1,2], M.S. ; Li-Chin Tsai [1], Ph.D. ; Yu-Chih Lin [3], Ph.D. ; Chih-Wen Su [4], Ph.D. ; Lih-Jing Yang [4], M.S. ; James Chun-I Lee [5], Ph.D. ; Hsing-Mei Hsieh [1*], Ph.D.

[1] Department of Forensic Science, Central Police University, 56 Shu-Jen Road, Takang Vil, Kueishan District, Taoyuan 33304, Taiwan, ROC.
[2] Forensic Examination Division, Criminal Investigation Bureau, National Police Agency, 5 Lane 553, Chung Hsiao East Road Section 4, Xinyi District, Taipei 11072, Taiwan, ROC.
[3] Taichung City Government Police Department, 588 Wenxin Road Section 2, Xitun District, Taichung City 40758, Taiwan, ROC.
[4] Forensic Biology Division, Criminal Investigation Bureau, National Police Agency, 5 Lane 553, Chung Hsiao East Road Section 4, Xinyi District, Taipei 11072, Taiwan, ROC.
[5] Department of Forensic Medicine, College of Medicine, National Taiwan University, 1 Jen-Ai Road Section 1, Taipei 10051, Taiwan, ROC.

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Abstract

This study reported the development of methylation-specific PCR (MSP) to discriminate the venous blood and menstrual blood for the forensic purpose. Initially, a total of 37 candidate markers were evaluated. Six markers of them were obtained by reviewing the references, and the other 31 markers were from the Infinium Illumina Human Methylation 450 BeadChip analysis. DNA sequencing results showed that 11 out of 37 markers contained differentially methylated regions between venous blood and menstrual blood. However, only four markers (CD93, EXD3, ELOVL5 and FLJ42875) comprised the intensive methylated CpG regions which were suitable for MSP analysis. A 4-plex MSP amplification system was established and used on identification of 9 biofluids samples (3 venous blood, 3 menstrual blood and 3 semen samples). The results showed that the methylation percentages were 6.6±3.4% (CD93), 5.7±9.9% (EXD3), 0.0±0.0% (ELOVL5) and 100.0±0.0% (FLJ42875) respectively in venous blood, which were hyper-methylated or hypo-methylated. However, the methylation percentages were 38.4±6.7% (CD93), 62.9±15.3% (EXD3), 21.9±5.7% (ELOVL5) and 64.7±11.8% (FLJ42875) respectively in menstrual blood, which were partially methylated. The preliminary results showed that the methylation patterns of this system were with the potential to discriminate the venous blood and menstrual blood. This 4-plex MSP system offered the alternative method to discriminate venous blood and menstrual blood for forensic purpose.

Keywords: methylation-specific PCR (MSP), venous blood, menstrual blood

*Corresponding author: Hsing-Mei Hsieh, Department of Forensic Science, Central Police University, 56 Shujen Rd., Takang Vil., Kueishan District, Taoyuan City, Taiwan 33304 R.O.C. Tel: 886-3-3282321 ext 5119, Fax: 886-3-3275907
E-mail: mei@mail.cpu.edu.tw

Introduction

Body fluids are frequently encountered at crime scenes especially for the sexual assault and violent crime cases. These biological stains can be used not only to link the suspect via DNA-STR typing but also to provide important clues by identifying the type of body fluids. Many techniques have been developed to identify the type of body fluids, such as microscopic examination and traditional chemical, catalytical and immunological tests [1]. These techniques are usually classified as presumptive tests and confirmatory tests. In forensic practical work, the commonly used presumptive tests include Kastle-Meyer test for the detection of blood [2], AP (acid phosphatase) and PSA (prostate specific antigen) tests for the detection of semen [3], Phadebas and RSID-saliva tests for the detection of saliva [4,5]; the commonly used confirmatory tests include microscopic examination for the detection of spermatozoa [6], RSID-semen test for the detection of semen [7], and ABA card® Hematrace and HemaTrace® tests for identifying human blood [2] (Although these methods may also cause a positive result in primate blood, these animals can hardly be seen at a crime scene). In recent years, other methods for DNA methylation [8], mRNA [9,10] and microRNA [11,12] analysis have also been developed for biofluid identification.

Blood is a common evidence observed at the crime scene. Venous blood could be used to substantiate the scenario that somebody has been injured dramatically, especially in a murder, violent assault or sexual assault case; however, menstrual blood could not support the assumption of injury. It indicates that the accurate discrimination between venous blood and menstrual blood could provide valuable information in criminal investigation. In early years, the identification of the menstrual blood from blood samples was usually performed by microscopic examination of endometrial stromal cells and vaginal epithelial cells; however, the results of microscopic examination are usually ambiguous [13] even though it is accompanied with the staining method [14]. Other methods focus on the detection of specific peptides or proteins in menstrual blood, such as comparing the patterns of lactate dehydrogenase (LDH) isozymes [15]; however, the specificity and reliability are insufficient for these methods [16]. Therefore, it needs to provide the more convincing methods for menstrual blood identification for forensic practical use.

Recently, the D-dimer immunochromatographic test was developed for the discrimination of venous and menstrual blood [17,18]. In other studies, microRNA (miRNA) was reported to be one of the potential candidates for menstrual blood identification [19,20]. A novel hexaplex assay using the multiplex reverse transcription-PCR (RT-PCR) for amplification of three menstrual blood specific genes (HBD1, MUC4 and MMP-11), 1 housekeeping gene (G6PDH) and the 16S rRNA-23S rRNA IGS for two bacteria *(Lactobacillus crispatus and Lactobacillus gasseri)* was confirmed to be effective for identification of vaginal secretion and menstrual blood [21]. The same strategy was used previously to develop a 11-plex assay for identification of biofluids including vaginal secretions [22]. However, in consideration of the degradation nature for RNA and protein, the methylation-based method for the more stable molecule of DNA could be the better choice for application on the old cases where only DNA extracts are available [23].

DNA methylation plays a crucial role in cell differentiation [24–26]. Many tissue-specific differentially methylated regions (tDMRs) are screened by the Infinium HumanMethylation450 BeadChip and applied on the studies of forensic body fluid identification. The techniques for analysis of the methylation status mainly include the pyrosequencing [27], single-base extension [28,29], and methylation-sensitive restriction enzyme-PCR (MSRE-PCR) [30]. In our previous study, the methylation-specific PCR (MSP) was adopted and the specific primers were used to recognize the tissue-specific differentially methylated CpG sites of the bisulfite converted DNA [29]. Although the MSP is occasionally challenging due to the efficacy of the methylated and un-methylated specific primers, the most important advantage for this method is that it could be performed by using the same equipment with STR typing in forensic DNA laboratory. Therefore, in this study, it was used to discriminate the venous blood and menstrual blood for the forensic purpose by establishing a multiplex MSP system.

Materials and Methods

Sample Collection

A total of 9 biofluid samples (3 venous blood, 3 menstrual blood and 3 semen samples) were collected

from 8 volunteers (4 males and 4 females) with informed consent and following the procedures approved by the Institutional Review Board (IRB) of Tao-Yuan General Hospital in Taiwan (IRB No. TYGH102011). Semen evidences are frequently encountered in sexual assault cases, therefore, semen samples were also collected for comparison in this study. Menstrual blood was collected from the feminine pad. An appropriate 1 cm^2 of menstrual blood stain or 20 μL of venous blood was placed into a 1.5 mL micro-tube containing 1 mL of PBS. The freshly ejaculated semen was collected and 20 μL of them was placed into a 1.5 mL micro-tube. These samples are used in the following DNA extraction.

DNA Extraction

Genomic DNA was extracted from the collected samples with the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacture's procedures, and eluted with 100 μL of sterile water at the final step. Human DNA was quantified using the Quantifiler Human DNA quantification kit (Life Technologies, NY, USA) and a 7500 Real-Time PCR machine (Life Technologies).

Bisulfite Conversion

The isolated genomic DNA was converted with sodium bisulfite by the Zymo EZ DNA Methylation Kit (Zymo Research, CA, USA) according to the manufacturer's suggestions and then quantified by the NanoDrop^{TM} 2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA).

Marker Selection

The markers used in this study for discrimination of venous blood and menstrual blood were initially searched from the references [31-33]. Furthermore, to search for the new tDMRs in this study, the difference of methylation status between the venous blood and menstrual blood was compared by analyzing the DNA of the two biofluids from the same individual using the Infinium Illumina Human Methylation 450 BeadChip (Illumina, San Diego, CA, USA) provided by the commercial services. In consideration of the following MSP amplifications, the markers are selected with two criteria: one is with a high beta-value (β, the calibration ratio of methylation) for venous blood or menstrual blood and a near zero value for the other; and the other criteria is with intensive CpG regions which include at least 2 informative cytosines (in CpG) at the 3' end of the primer annealing sites for discriminating the methylation and un-methylation.

Methylation-Specific PCR (MSP)

The MSP amplification was performed in a total volume of 25 μL which consisted of 0.75 unit of EpiTaq^{TM} HS (5U/μL Takara Bio Inc., Shiga, Japan), 2.5 μL of 10X Epi Taq PCR buffer (Mg^{2+} free), 2 μL of 2.5 mM dNTP mixture, 1.5 μL of 25 mM $MgCl_2$, optimal primer mix and approximately 5 ng of bisulfite-converted DNA as the template. The reaction was conducted in a 9700 Thermal Cycler (Life Technologies) at 95 ºC for 10 min, then 30 cycles of 94 ºC for 30 s, 60 ºC for 1 min and 72 ºC for 1 min, with a final extension at 72 ºC for 7 min. The products were then separated on a 3130xL Genetic Analyzer (Life Technology) and the GeneMapper ID 3.2.1 software was used for signal analysis. The threshold for a positive signal was 50 RFU (Relative Fluorescence Unit).

Results and Discussion

Marker Selection

The tissue-specific differentially methylated markers for discriminating the venous blood and menstrual blood were searched from two ways. Totally there were 37 candidate markers acquired: 6 markers of them were obtained from the references which had been published for biofluid identification (Table 1), whereas the other 31 markers shown in Table 2 were obtained from the Infinium Illumina Human Methylation 450 BeadChip analysis. For the latter analysis, the β values for more than 450,000 CpG sites were obtained. The 31 candidate markers were selected while the methylation difference between menstrual and venous blood was at least 0.5 ($|\beta_{MB}-\beta_B| \geq 0.5$, β_{MB} and β_B represent the β value of menstrual blood and venous blood respectively). The DNA sequencing result showed that 11 out of 37 markers contained differentially methylated regions between venous blood and menstrual blood, however, only four markers (CD93, EXD3, ELOVL5 and FLJ42875) showed intensive methylation CpG regions and were suitable for MSP analysis (Table 1 and 2).

Table 1 Candidate markers extracted from the references.

Candidate marker	CpG island location	Body fluid	Function	Reference	DNA sequencing[a]
CD93	chr20: 3067606-23067872	Venousl Blood	CD93 molecule	[31]	○
SPTB	chr14: 5299845-65300075	Venous Blood	Spectrin, beta, erythrocytic	[32]	●
MMP7	chr11: 02392055-102392259	Menstrual Blood	Matrix Metallopeptidase 7	[31]	●
MMP11	chr22: 24112901-24113283	Menstrual Blood	Matrix Metallopeptidase 11	[31]	●
FGF7	chr15: 49712966-49713171	Semen	Fibroblast Growth Factor 7	[33]	●
ZC3H12D	chr6: 149813204-149813937	Semen	Zinc Finger CCCH-Type Containing 12D	[33]	●

[a] The symbol of "○" represents the marker which shows the tissue-specific differentially methylated regions between venous and menstrual blood by DNA sequencing and meanwhile comprises intensive methylation CpGs; "●" represents the marker which doesn't show the tissue-specific differentially methylated regions between venous and menstrual blood by DNA sequencing.

Table 2 Candidate markers selected from the Illumina Human Methylation 450 BeadChip analysis.

Candidate markers	CpG island location	β_{MB}-β_{B}	DNA sequencing[a]
OPLAH	chr8:145114550-145115828	-0.701	♦
TC21B	chr2:166809970-166810826	-0.654	●
CT45A6	chrX: 135794687-135812012	-0.643	●
RUNX3	chr1:25255527-25259005	-0.601	●
CALR	chr19:13049038-13049809	-0.591	●
FLJ42875	chr1:2979275-2980758	-0.557	○
RNF220	chr1:45082739-45083285	-0.548	●
SNCAIP	chr5: 122311558-122464099	-0.544	●
FLJ42875	chr1:2979275-2980758	-0.543	●
PPT2	chr6:32121829-32122529	-0.542	●
CCDC42B	chr12:113590806-113591304	-0.532	♦
RUNX3	chr1:25255527-25259005	-0.512	●

Candidate markers	CpG island location	β_{MB}-β_{B}	DNA sequencing[a]
DPM2	chr9:130699759-130700266	-0.505	●
RUNX3	chr1:25255527-25259005	-0.503	●
HSPB9	chr17:40274523-40275360	-0.502	●
EXD3	chr9: 137306880-137423262	0.604	○
BIN2	chr12: 51281038-51324198	0.591	●
ELOVL5	chr6: 53267398-53349179	0.559	○
S1PR4	chr19: 3178738-3180332	0.553	●
C17orf101	chr17: 82389210-82418637	0.541	♦
SPN	chr16: 29662950-29681828	0.538	●
MYO1G	chr7: 44962661-44979105	0.535	●
RNF166	chr16: 88696495-88706421	0.534	●
JAK3	chr19:17958490-17958808	0.533	♦
ADORA2A	chr22:24819537-24820810	0.528	●
CCR4	chr3: 32951574-32954911	0.525	♦
ADORA2A	chr22:24819537-24820810	0.525	●
NEDD9	chr6: 11183298-11382348	0.519	♦
TNFRSF25	chr1: 6525996-6526228	0.518	●
C10orf79	chr10: 104129888-104232366	0.513	♦
PLCB2	chr15: 40284260-40307973	0.512	●

[a] The symbol of "○" represents the marker which shows the tissue-specific differentially methylated regions between venous and menstrual blood by DNA sequencing and meanwhile comprises intensive methylated CpGs; "♦" represents the marker which shows the tissue-specific differentially methylated regions between venous and menstrual blood by DNA sequencing but comprises scattered methylated CpGs; "●" represents the marker which doesn't show the tissue-specific differentially methylated regions between venous and menstrual blood by DNA sequencing.

MSP amplification

The bisulfite converted DNA was amplified by MSP amplification for the selected markers. Each of the four markers was preliminarily evaluated by two independent MSP amplifications, one was named methylated reaction (M) and the other was un-methylated reaction (U). The same reverse primer labelled with FAM dye was used in both reactions whereas the distinctive forward primers (MF and UF) were used in the M and U reaction respectively. The forward primer MF for the M reaction was complementary with the sequence including methylated cytosine of CpG, and the UF primer for the U

reaction was complementary with the sequence including thymine converted from the un-methylated cytosine of CpG (Table 3). The MF and UF primers allowed specifically selective amplification of the methylated and un-methylated CpG fragments respectively. The UF primer sequence was 20 nucleotides more than MF primer for each marker, therefore, the PCR products of different sizes for M and U reactions could be distinguished by using the capillary electrophoresis. The number of differentially methylated CpGs ranged from 2 to 8 for primers and 4 to 21 for amplicons respectively (Table 3). The sizes of amplicons ranged from 116 to 272 bp.

Table 3 Information of the markers used in the MSP amplification.

Marker	Location	450 ID	Primer sequence (5' → 3')	Tm (ºC)	Primer Conc. (nM) [a]	Amplicon size (bp)	CpG no. in primer	CpG no. in amplicon
CD93	chr20: 23086216-23086338	NC_000020.11	MF: CGGGAGGCGAGAAGTTTAGCGGC	62.4	40	116	4	7
			UF: GGTTTTTGTGTGGTTTTTTGTGGGAGGTGAGAAGTTTAGTGGT	66.4	40	134	4	7
			R: FAM-AACCCTCAACCTTTATATCCTTCTC	54.4	80			
EXD3	chr14: 137307071-137307207	NC_000009.12	MF: CGTCGTGAATAAATTTTTACGATACGTC	55.5	52	164	5	6
			UF: GGATGTTTTGATATAGGTTATGTTGTGAATAAATTTTTATGATATGTT	60.3	68	183	7	8
			R: FAM-AATAATTATCTCCCCTCCCCTCCTTC	58	120			
ELOVL5	chr11: 53267448-53267643	NC_000006.12	MF: GGAATTTTTTATAAAAGAAAGTATTATTCGC	52.5	200	189	2	4
			UF: TTATTTGGAGGTTGTTTTTGGGAATTTTTTATAAAAGAAAGTATTATTTGT	60.6	200	208	2	4
			R: FAM-TCCCAAAACAAAATAAAAATATATATCTCTTATAAAAAAC	55.3	400			
FLJ42875	chr22: 3060152-3060411	NC_000001.11	MF: TTATTTTACGGTTGCGGGCGCGC	58.8	80	254	5	18
			UF: GTGGGTGGTAGGAGGGATTGTTATTTTATGGTTGTGGGTGTGT	68.3	80	272	8	21
			R: FAM-CACTAAAAAAAATACAAAAAACCCCCAAC	54.1	160			

[a] The primer concentrations (Conc.) are for the multiplex amplifications. For single amplification of each marker, the primer concentrations are all the same as that of ELOVL5.

For time and labor saving, the multiplex (4-plex) MSP system was established in this study, and the optimal concentration of each primer was shown in Table 3. The methylation patterns of semen, venous and menstrual blood in the 4-plex MSP matched the results from the separate M and U reactions of each marker. It showed that the distinctive forward primers (MF and UF) in each marker could specifically recognize the methylated and un-methylated cytosine of CpG respectively for bisulfite converted DNA and successfully amplify the methylated and un-methylated products in the 4-plex MSP. As expected, the four markers were hyper-methylated or hypo-methylated in venous blood and semen, however, partially methylated in menstrual blood.

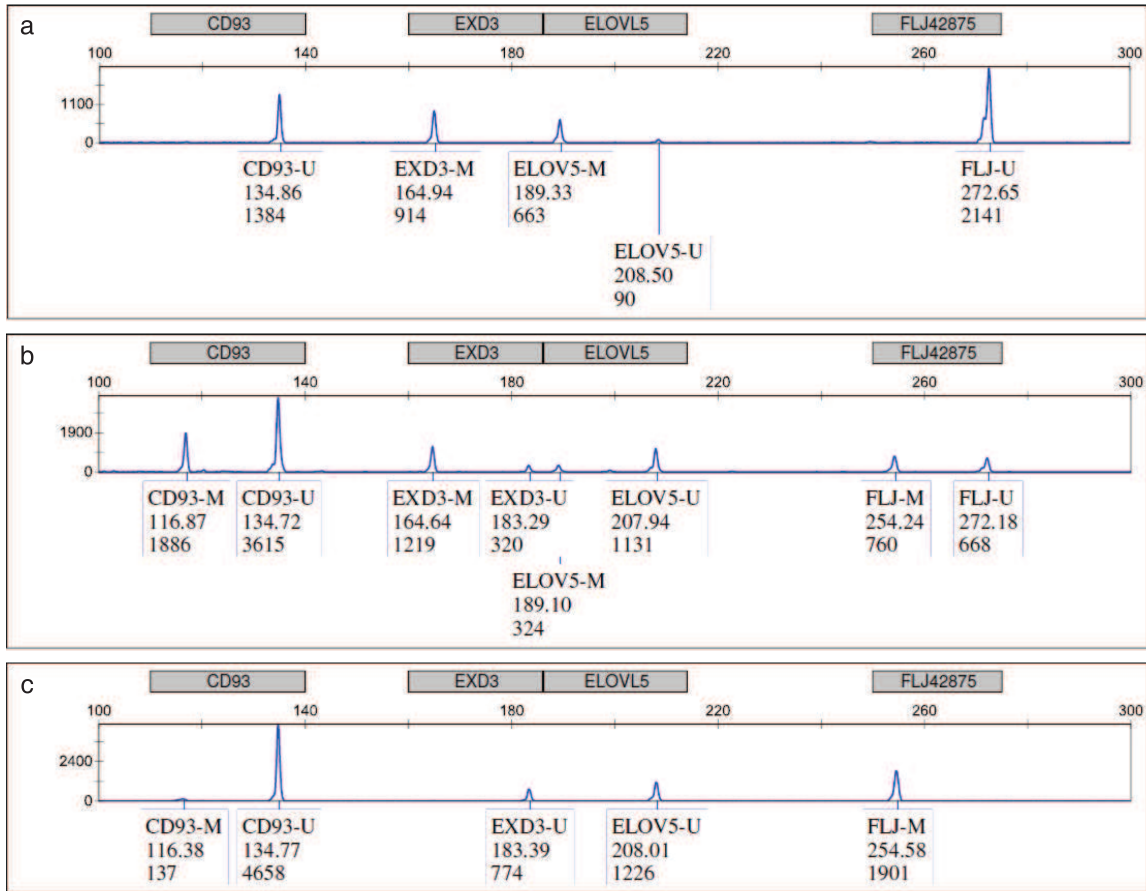

Fig. 1 Electropherogram of one example for the biofluid identified by the 4-plex MSP system. The DNA used is from venous blood (a), menstrual blood (b) and semen (c). The peaks labelled with M and U represent the products from the M reaction and U reaction respectively.

To quantify the methylation level of the samples, the methylation percentage was calculated. The methylation percentage was calculated as the peak height of the methylated product signal divided by the sum of both methylated and un-methylated peak heights. Table 4 showed the mean and standard deviation of methylation percentage for venous blood (n=3), menstrual blood (n=3) and semen (n=3) in the 4-plex MSP. For venous blood, the mean and standard deviation (SD) of methylation percentage for CD93, EXD3, ELOVL5 and FLJ42875 were 6.6±3.4%, 5.7±9.9%, 0.0±0.0% and 100.0±0.0% respectively. It showed hypo-methylation in CD93, EXD3 and ELOVL5 but hyper-methylation in FLJ42875 (Table 4 and Fig. 1a). For menstrual blood, the four markers all showed partial methylation, and the methylation percentages were 38.4±6.7% (CD93), 62.9±15.3% (EXD3), 21.9±5.7% (ELOVL5) and 64.7±11.8% (FLJ42875) respectively (Table 4 and Fig. 1b). These two biofluids were observed with different patterns of electropherogram (Fig. 1) and different levels of methylation (Table 4) for these markers. Nevertheless, semen showed the similar methylation level (hypo-methylation) with venous blood at CD93, ranging from 2.9%-9.4% for 3 venous blood samples and 0.0% for 3 semen samples respectively. On the other hand, the other three markers (EXD3, ELOVL5 and FLJ42875) showed an adverse methylation status between venous blood and semen: venous blood was with hyper-methylated CpG (100.0±0.0%) at FLJ42875, while

semen was hypo-methylated (0.0±0.0%); hypo-methylated at EXD3 (5.7±9.9%) and ELOVL5 (0.0±0.0%) for venous blood, while hyper-methylated for semen (100.0±0.0% and 87.1±2.7% respectively) (Table 4, Fig. 1a and 1c). Therefore, venous blood could be discriminated from semen by using the EXD3, ELOVL5 and FLJ42875 markers.

Table 4 The methylation percentage for the selected markers.

	Methylation percentage (%)							
	CD93		EXD3		ELOVL5		FLJ42875	
Biofluid type	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD
Venous Blood (n=3)	2.9-9.4	6.6±3.4	0.0-17.1	5.7±9.9	0.0±0.0	0.0±0.0	100.0±0.0	100.0±0.0
Menstrual Blood (n=3)	34.3-46.1	38.4±6.7	48.9-79.2	62.9±15.3	16.0-27.5	21.9±5.7	53.2-76.8	64.7±11.8
Semen (n=3)	0.0±0.0	0.0±0.0	100.0±0.0	100.0±0.0	84.1-89.3	87.1±2.7	0.0±0.0	0.0±0.0

Conclusions

In this study, a 4-plex MSP system with biofluid-specific methylated CpG markers was developed to discriminate the venous blood and menstrual blood. From the preliminary results, it showed that the methylation patterns and methylation levels of this 4-plex MSP amplification were with the potential to discriminate the venous blood from menstrual blood. When human blood is identified through the presumptive and confirmatory tests, this 4-plex MSP system can be used to clarify whether the blood is from the venous blood or menstrual blood. This study provided an alternative assay to discriminate the venous blood and menstrual blood. However, it needs to test more samples (including the mixtures) to evaluate the feasibility for its applications on forensic practice.

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References

1. Virkler K, Lednev I-K. Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene. Forensic Sci Int 2009; 188(1-3):1-17.
2. Spalding RP. The identification and characterization of blood and bloodstains. In: James SH, Nordby JJ, Bell S, editors. Forensic Science: An Introduction to Scientific and Investigative Techniques. 3th ed. Boca Raton: CRC Press, 2009.
3. Greenfield A, Sloan MA. Identification of biological fluids and stains. In: James SH, Nordby JJ, Bell

S, editors. Forensic Science: An Introduction to Scientific and Investigative Techniques. 3th ed. Boca Raton: CRC Press, 2009.

4. Clem D, Maidment J, Ringham J-M. A study into the measurement of α-amylase activity using phadebas, iodine and gel-diffusion procedures. Nutrition & Food Science 2001; 31(3):141-6.
5. Old J-B, Schweers B-A, Boonlayangoor P-W, Reich K-A. Developmental validation of RSID-saliva: a lateral flow immunochromatographic strip test for the forensic detection of saliva. J Forensic Sci 2009; 54(4):866-73.
6. Watson N. Analysis of Body Fluids. In: White PC, editor. Crime Scene to Court: The Essentials of Forensic Science. 4th ed. Cambridge: The Royal Society of Chemistry Press, 2016.
7. Pang B-C, Cheung B-K. Identification of human semenogelin in membrane strip test as an alternative method for the detection of semen, Forensic Sci Int 2007; 169(1):27-31.
8. Vidaki A, Daniel B, Court D-S. Forensic DNA methylation profiling—potential opportunities and challenges. Forensic Sci Int Genet 2013; 7(5):499-507.
9. Bauer M. RNA in forensic science. Forensic Sci Int Genet 2007; 1(1):69-74.
10. Roeder A-D, Haas C. mRNA profiling using a minimum of five mRNA markers per body fluid and a novel scoring method for body fluid identification. Int J Legal Med 2013; 127(4):707-21.
11. Wang Z, Luo H, Pan X, Liao M, Hou Y. A model for data analysis of microRNA expression in forensic body fluid identification. Forensic Sci Int Genet 2012; 6(3):419-23.
12. Sirker M, Fimmers R, Schneider P-M, Gomes I. Evaluating the forensic application of 19 target microRNAs as biomarkers in body fluid and tissue identification. Forensic Sci Int Genet 2017; 27:41-9.
13. Divall GB. Methods for the identification of menstrual blood. Advances in Forensic Science, Biomedical Publications, Foster CA, 1985:1-14.
14. Hausmann R, Pregler C, Schellmann B. The value of the Lugol's iodine staining technique for the identification of vaginal epithelial cells. Int J Legal Med 1994; 106(6):298-301.
15. Asano M, Oya M, Hayakawa M. Identification of menstrual blood stains by the electrophoretic pattern of lactate dehydrogenase isozymes. Forensic Sci 1972; 1(3):327-32.
16. Divall G-B, Ismail M. Lactate dehydrogenase isozymes in vaginal swab extracts: a problem for the identification of menstrual blood. Forensic Sci Int 1983; 21(2):139-47.
17. Baker D-J, Grimes E-A, Hopwood A-J. D-dimer assays for the identification of menstrual blood. Forensic Sci Int 2011; 212 (1-3):210-4.
18. Holtkötter H, Dierig L, Schürenkamp M, Sibbing U, Pfeiffer H, Vennemann M. Validation of an immunochromatographic D-dimer test to presumptively identify menstrual fluid in forensic exhibits. Int J Legal Med. 2015; 129(1):37-41.
19. Hanson E-K, Lubenow H, Ballantyne J. Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. Anal Biochem 2009; 387(2):303-14.
20. Zubakov D, Boersma A-M, Choi Y, Kuijk P, Wiemer E-C, Kayser M. MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. Int J Legal Med 2010; 124(3):217-26.
21. Jakubowska J, Maciejewska A, Pawłowski R, Bielawski K-P. mRNA profiling for vaginal fluid and menstrual blood identification. Forensic Sci Int Genet 2013; 7(2):272-8.
22. Fleming R-I, Harbison S. The use of bacteria for the identification of vaginal secretions. Forensic Sci Int Genet 2010; 4(5):311-5.
23. An J-H, Shin K-J, Yang W-I, Lee H-Y. Body fluid identification in forensics. BMB Rep 2012; 45(10):545-53.
24. Nouzova M, Holtan N, Oshiro M-M, Isett R-B, Munoz-Rodriguez J-L, List A-F, Narro M-L, Miller S-J, Merchant N-C, Futscher B-W. Epigenomic changes during leukemia cell differentiation: analysis of histone acetylation and cytosine methylation using CpG island microarrays. J Pharmacol Exp Ther 2004; 311(3):968-81.
25. Rothenburg S, Koch-Nolte F, Thiele H-G, Haag F. DNA methylation contributes to tissue- and allele-specific expression of the T-cell differentiation marker RT6. Immunogenetics 2001; 52(3-4):231-41.
26. Isagawa T, Nagae G, Shiraki N, Fujita T, Sato N,

Ishikawa S, Kume S, Aburatani H. DNA methylation profiling of embryonic stem cell differentiation into the three germ layers. PLoS ONE 2011; 6(10):e26052.

27. Delaney C, Garg S-K, Yung R. Analysis of DNA methylation by pyrosequencing. Methods Mol Biol 2015; 1343:249-64.
28. Jung S-E, Cho S, Antunes J, Gomes I, Uchimoto M-L, Oh Y-N, Di Giacomo L, Schneider P-M, Park M-S, van der Meer D, Williams G, McCord B, Ahn H-J, Choi D-H, Lee Y-H, Lee S-D, Lee H-Y. A collaborative exercise on DNA methylation based body fluid typing. Electrophoresis 2016; 37(21):2759-66.
29. Lin Y-C, Tsai L-C, Lee J-C, Liu K-L, Tzen J-T, Linacre A, Hsieh H-M. Novel identification of biofluids using a multiplex methylation-specific PCR combined with single-base extension system. Forensic Sci Med Pathol 2016; 12(2):128-38.
30. Lin Y-C, Tsai L-C, Lee J-C, Su C-W, Tzen J-T, Linacre A, Hsieh H-M. Novel identification of biofluids using a multiplex methylation sensitive restriction enzyme-PCR system. Forensic Sci Int Genet 2016; 25:157-65.
31. Lindenbergh A, de Pagter M, Ramdayal G, Visser M, Zubakov D, Kayser M, Sijen T. A multiplex (m) RNA-profiling system for the forensic identification of body fluids and contact traces. Forensic Sci Int Genet 2012; 6(5):565-77.
32. Juusola J, Ballantyne J. Multiplex mRNA profiling for the identification of body fluids. Forensic Sci Int 2005; 152(1):1-12.
33. Madi T, Balamurugan K, Bombardi R, Duncan G, McCord B. The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing. Elecrophoresis 2012; 33(12):1736-45.