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Research Paper

Application of the Methylated Markers (Spectrin Beta and DEAD-Box Protein) for Definitive Differentiation Between Fresh and Aged Semen by evaluating Their Role in Identifying Semen From Mixed Body Fluids

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ABSTRACT

Background: Semen identification is assumed a crucial proof of sexual assault. Moreover, body fluids at the crime scene of a human being, such as blood, semen, and saliva, are often mixed.

Methods: Hence, in our study, we aimed to use methylation analysis targeting DNA epigenetic markers Spectrin beta chain (B_SPTB_03) and DEAD-box protein (DDX4) to differentiate between fresh semen (less than 4 hours) and aged semen (after 24 hours) as well as to differentiate between semen alone and semen mixed with other body fluids (blood and saliva) in the fresh and dried state.

Results: Our findings showed statistically significant differences in the methylation patterns of the SPTB and DDX4 loci to distinguish semen from mixed body fluids in fresh and old samples. We were able to obtain two novel cutoff values to differentiate between fresh and aged semen, which are (52.25) with the SPTB marker and (70.75) with the DDX4 marker.

Conclusion: It is concluded that the methylation approach based on the epigenetic markers of Spectrin beta chain and DEAD-box protein (B_SPTB_03 and DDX4) successfully identified fresh from aged semen and semen-derived alleles from mixed stains, hence it is recommended to be employed in forensic practice.

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1. Introduction

Identification of semen at a crime scene can significantly reflect sexual assault. As a result, its identification methods receive more attention [1]. Various techniques of semen detection have already been studied, such as chemical tests, immunological tests, protein reactant movement tests, microscopy, and spectroscopic strategies. However, these presumptive tests cannot conclusively confirm the presence of sperm [2, 3]. Hence, gigantic interest in research targets advanced molecular approaches that detect semen as bacterial Deoxyribonucleic Acid (DNA), and different DNA methylated regions [4].

Methylation is an epigenetic change associated with gene expression and gene control. The levels of methylation in the control region are correlated with a lack of gene expression and vice versa [5]. Methylated DNA has a magnificent rule in distinguishing various body fluids due to its high tissue specificity. Furthermore, it is less liable to degradation than messenger Ribonucleic Acid (RNA) [6].

DNA methylated regions in various body fluids, such as semen and vaginal secretions have not been broadly scrutinized. Moreover, body fluids at the crime scene of a human, such as blood, semen, and saliva, are often mixed or discolored due to dryness as a result of long exposure, and genotyping from these mixed samples is assumed to be a major drawback in forensic examination [7, 8]. Hence, our study aimed to use DNA methylation markers to differentiate fresh and stored semen, distinguish it from other mixed body fluids, and test its applicability in forensic practice.

2. Materials and Methods

The current study is a clinical prospective study in which the two epigenetic markers (SPTB and DDX4) were employed to differentiate fresh and stored semen and to identify semen from other mixed body fluids. It was carried out at the Forensic Medicine and Clinical Toxicology, the Medical Biochemistry, and Andrology Departments, Faculty of Medicine, Cairo University.

Participants

Twenty male donors participated in this work according to the following:

- Inclusion criteria: healthy Egyptian males aged 20–40 years with normal semen in quality and quantity.

- Individuals with blood diseases, malignant tumors, azoospermia, and genitourinary disorders are excluded.

We obtained written informed consent from all the included participants after being informed about the study's aim and procedure according to the approved ethical guidelines. Semen samples were collected from them, and each sample was divided into eight groups as follows:

Group 1: Fresh semen not mixed with other body fluids (less than 4 h) (n=20).

Group 2: Fresh (mixed semen and saliva) (less than 4 h) (n=20).

Group 3: Fresh (mixed semen and blood) (less than 4 hours) (n=20).

Group 4: Fresh (semen mixed with blood and saliva) (less than 4 hours) (n=20).

Group 5: Aged semen not mixed with other body fluids (after 24 hours) (n=20).

Group 6: Aged (mixed semen and saliva) (after 24 hours) (n=20).

Group 7: Aged (mixed semen and blood) (after 24 hours) (n=20).

Group 8: Aged (semen mixed with blood and saliva) (after 24 hours) (n=20).

Sample collection

Fresh samples were extracted before 4 hours while the other samples were dried in ambient air for 24 hours. Blood samples were collected in Ethylene Diamine Tetraacetic Acid (EDTA) tubes (3 mL), saliva swabs were collected in micro-centrifuge tubes, and freshly ejaculated semen samples (2 mL) were collected in falcon tubes.The sample size analysis was done using GPower software v.3.0.10.

DNA extraction

DNA was extracted utilizing a nucleic acid extraction kit (NucleoSpin®) purchased from Macherey- Nagel GmbH and Co. KG- Germany. Sequences of oligonucleotide primers were used for the DNA methylation analysis of all

Table 1. Primers used for the analysis of DNA methylation in all studied samples

Markers Used in this Study	Chromosome Location	PCR and Sequencing Primer	Target Sequence	Number of CpGs for Analysis	Amplicn Size Base Pairs (bp)
Custom_B_SPTB_03	Chr14: 65290186–65290126	F: TGTTGTTTTTGGTTTTT AGGAGAGTT R: ACAAAAACAAAACCCCA TATCATCTACCTA S: TGGTTTTTAGGAGAGTTT	CCGGGGTGCTCCCC-GCGG CGGGGCGGCTG-GTCTGGAG-GAAGCAGCTGGCT-GCGACTGACGT	7	260
Custom DDX4	Chr5: 55033694–55033770	F: AGTTAGTTTTGTATTTA TAGGTTTAATAGG R: ATAACTAACCCCACCAA CCAATCA S: GGTTTAATAGGTTATTT GGTTATG	GCGTCGC-CATAGGGGCCC-GAACGCTAGCGTT-TAGGGAATCCGCAG-GCTAGAAGTGGAG-GCGGGACGCCACTG-GTCGT	9	160

DDX4: DEAD-box protein; SPTB: Spectrin beta; PCR: Polymerase chain reaction.

studied genes. Primers were designed by Life Technologies company (Life Technologies Corporation, Carlsbad, CA). Initially, it was planned to test the following two markers: Spectrin beta (B_SPTB_03) and DEAD-box protein (DDX4) [9]. Primers used for the analysis of DNA methylation in all studied samples are mentioned in Table 1.

Sodium bisulfite was used to determine the methylation status of a DNA sequence. The bisulfite modification of extracted DNA was done using the EpiTect Bisulfite Kit (# K1441 Lot 00134729; Thermo Scientific). Bisulfite-modified DNA was recruited as a template for fluorescence-qPCR (Quantitative real-time PCR) [10]. Primers were designed to specifically amplify two desired genes. Amplification reactions were performed in triplicate in a final volume of 20 L. The qPCR kit was provided by Bioline, a median-sized life science company in the UK (SensiFASTTM SYBR® Hi-ROX): A qPCR master mix was prepared. Samples were measured as triplicates in each PCR run using the universal methylated human DNA standard (Zymo Research) and semen, saliva, and menstrual blood DNA as samples. The relative quantitation (RQ) of methylation is quantified according to the delta-delta Ct calculation (Ct) [11]. The RQ of each gene was calculated by taking $2^{-\Delta\Delta Ct}$ as follows (Equation 1):

1. $\ÄÄCt = [(Ct\ target\ sample) - (Ct\ reference\ sample)] - [(Ct\ target\ control) - (Ct\ reference\ control)]$

Statistical analysis

Coding and data entry were performed by SPSS software v.25 (IBM Corp., Armonk, NY, USA). Data were summarized by the mean and standard deviation for quantitative variables. Comparing between groups was employed using the analysis of variance (ANOVA) test with multiple comparisons, Tukey post-hoc test for comparison between both groups [12]. P values less than 0.05 were considered statistically significant. A Receiver Operating Characteristic (ROC) curve and area under the curve analysis were applied to find the best cut-off value of markers to detect fresh semen from aged semen.

3. Results

Table 2 presents novel cut-off values between fresh and aged semen samples. The first (52.25) with SPTB mark-

Table 2. Cutoff values between fresh and aged SPTB and DDX4 markers

Area Under the Curve		P	95% Confidence Interval				
			Lower Bound	Upper Bound	Cut-off	No	
						Sensitivity	Specificity
SPTB	0.968	<0.001	0.919	1.000	52.25	95	90
DDX4	1.000	<0.001	1.000	1.000	70.75	100	100

P<0.05 is significant.

DDX4: DEAD-box protein; SPTB: Spectrin beta.

Table 3. Comparison Between all studied groups regarding methylated markers spectrin beta and DEAD-box protein

	Mean±SD								P
	Semen and Saliva Aged	Semen and Blood Fresh	Semen Fresh	Semen Aged	Semen and Saliva Fresh	Semen and Blood Aged	Semen and Blood and Saliva Aged	Semen and Blood and Saliva Fresh	
SPTB	30.72±5.84	40.93±5.30	60.70±5.71	44.44±6.85	47.80±4.24	31.25±5.32	15.82±3.95	23.18±5.32	<0.001
DDX4	47.15±8.61	51.88±5.59	80.62±6.15	61.01±5.48	61.39±7.66	39.39±5.37	17.12±8.69	26.53±6.83	

P<0.05 is significant.

International Journal of Medical Toxicology & Forensic Medicine

DDX4: DEAD-box protein; SPTB: Spectrin beta.

er (<52.25 indicates aged semen and >52.25 indicates fresh semen) where the sensitivity (95%) and specificity (90%) are statistically significant (P<0.05). The second cut-off value obtained to differentiate between fresh and aged semen samples with DDX4 marker is (70.75), (<70.75) indicates aged semen, and (>70.75) indicates fresh semen) with 100% sensitivity and 100% specificity and statistical significance (P<0.05).

Comparisons were made between all studied groups regarding methylated markers (SPTB and DDX4), as demonstrated in Table 3. A highly statistically significant difference was observed between their means (P<0.05).

Table 4 presents a post-hoc pairwise test to compare each pair of the studied groups, fresh (semen not mixed with other body fluids, mixed semen, and saliva, mixed semen and blood, semen mixed with blood and saliva), and aged (semen not mixed with other body fluids, mixed semen and saliva, mixed semen and blood, semen mixed with blood and saliva) regarding methylated SPTB and significant differences were observed between all studied groups (P<0.05) except for the following pairs (aged semen and blood, aged semen and saliva), (aged semen, fresh semen, and blood), (aged semen, fresh semen, and blood) (aged semen, fresh semen, and saliva, (aged semen and blood, aged semen, and saliva) where no statistical significance were observed.

Table 5 presents the comparison of both groups regarding methylated DDX4 using a post-hoc pairwise test and with P value (<0.05) which indicates significant differences between all studied groups. On the other hand, no significant differences were observed between (fresh semen and blood, aged semen and saliva) (aged semen and saliva, fresh semen, and blood) (aged semen and saliva, fresh semen).

Table 4. Post-hoc pairwise comparison between every two groups regarding methylated spectrin beta

Variables	Semen and Saliva Aged	Semen and Blood Fresh	Semen Fresh	Semen Aged	Semen and Saliva Fresh	Semen and Blood Aged	Semen and Blood and Saliva Aged	Semen and Blood and Saliva Fresh
	SPTB							
Semen and saliva aged		<0.001	<0.001	<0.001	<0.001	1.000	<0.001	<0.001
Semen and blood fresh	<0.001		<0.001	0.445	0.002	<0.001	<0.001	<0.001
Semen Fresh	<0.001	<0.001		<0.001	<0.001	<0.001	<0.001	<0.001
Semen aged	<0.001	0.445	<0.001		0.505	<0.001	<0.001	<0.001
Semen and saliva fresh	<0.001	0.002	<0.001	0.505		<0.001	<0.001	<0.001
Semen and blood aged	1.000	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001
Semen and blood and saliva aged	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		0.001
Semen and blood and saliva fresh	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	

P<0.05 is significant.

International Journal of Medical Toxicology & Forensic Medicine

SPTB: Spectrin beta.

Table 5. Comparison between every two groups regarding methylated DEAD-Box Protein (DDX4) using post-hoc pairwise test

Variables	Semen and Saliva Aged	Semen and Blood Fresh	Semen Fresh	Semen Aged	Semen and Saliva Fresh	Semen and Blood Aged	Semen and Blood and Saliva Aged	Semen and Blood and Saliva Fresh
	DDX4							
Semen and Saliva Aged		0.380	<0.001	<0.001	<0.001	0.012	<0.001	<0.001
Semen and blood fresh	0.380		<0.001	0.001	0.001	<0.001	<0.001	<0.001
Semen fresh	<0.001	<0.001		<0.001	<0.001	<0.001	<0.001	<0.001
Semen aged	<0.001	0.001	<0.001		1.000	<0.001	<0.001	<0.001
Semen and saliva fresh	<0.001	0.001	<0.001	1.000		<0.001	<0.001	<0.001
Semen and blood aged	0.012	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001
Semen and blood and saliva aged	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		0.001
Semen and blood and saliva fresh	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	

P<0.05 is significant.

International Journal of
Medical Toxicology & Forensic Medicine

DDX4: DEAD-box protein.

4. Discussion

Two epigenetic markers (SPTB and DDX4) were presented in this work to test their potential in distinguishing between fresh and aged semen, as well as semen and semen mixed with blood and saliva in both fresh and dried forms aiming to find out the probability of their application in forensic practice. Bisulfide treatment was used to determine the DNA methylation profiles of the two markers using pooled DNA (semen, blood, and saliva). The RQ of methylation was quantified according to delta-delta Ct calculation, and the two markers demonstrated hypermethylation in semen samples. We used a post-hoc pairwise test to compare methylated DDX4 and SPTB patterns in semen alone and semen mixed with saliva and blood in fresh and dried states. Our findings showed statistically significant differences in their methylation patterns to distinguish semen from mixed body fluids.

Following our results regarding SPTB, six markers, DACT1, USP49, DDX4, Hs_INSL6_03, Hs_ZC3H12D_05, and B_SPTB_03, had tissue-specific methylation in the study of Balamurugan et al. [5]. All six markers successfully differentiated semen samples from the other four tissue types. The SPTB marker is the only marker that shows hypermethylation in semen. However, other markers showed hypomethylation. Contrary to our results regarding DDX4 hypermethylation in semen, the same study showed hypomethylation of the marker in semen DNA, probably due to a different methodology.

Ledgerwood [13] also showed the effectiveness of SPTB and DDX4 epigenetic markers using high-resolution melting curve analysis in the identification of sperm from other tissues, where SPTB had a melting temperature higher than the other tissues and DDX4 had a melting temperature lower than the compared tissues. Park and other researchers [6] applied the Illumina human methylation 450K bead array to differentiate body fluid targeting the DNA methylation markers, including cg06379435 and cg08792630 for blood, cg26107890 and cg20691722 for saliva, cg23521140 and cg17610929 for semen, and cg01774894 and cg14991487 for vaginal secretions, all of which express the probability of distinguishing various fluids.

Blackman and his colleagues [14] designed the ParaDNA® body fluid identifier system to identify mixed body fluids. This system can identify messenger RNA (mRNA) targets for various body fluids, such as semen, vaginal secretions, blood, and saliva. A study by Stravers and other researchers [15] highlights the use of surface plasmon resonance imaging with antibody-based detection to identify mixed biostains, such as semen, blood, and vaginal fluids and shows the usefulness of the feasibility of SPRi in discriminating between these mixed stains. Abbas and his colleagues [16] applied a multiplex detection platform for the detection of semen

and vaginal secretions on a silver (Ag) vertical nanorod metal enhanced fluorescence substrate. Discrimination between the two stains by the developed sensor is successful without any cross-reaction.

In the present study, SPTB and DDX4 showed a significant hypermethylation pattern in fresh semen and fresh mixed body fluids compared to aged semen and aged mixed body fluids, respectively. The cut-off value was measured by receiver Operating Characteristic (ROC) curve to differentiate fresh from aged semen regarding SPTB and DDX4 markers, where significant differences were detected ($P<0.05$). Therefore, we were able to gain two novel cutoff values to discriminate between fresh and aged semen. The first one (52.25) is with an SPTB marker (52.25 indicates aged sperm and >52.25 indicates fresh sperm) with 95% sensitivity and 90% specificity. The second value is 70.75 with the DDX4 marker (70.75 indicates aged sperm and >70.75 indicates fresh sperm), and the sensitivity and specificity are both 100%.

In agreement with our results, Zapata and his colleagues [17] applied FTIR spectroscopy with chemometrics to differentiate between semen-vaginal fluid mixture stains which were allowed to dry overnight. They assumed that IR spectra did not show any bands due to the color of the fabric.

ConclusionFrom the present study, it can be confirmed that methylation analysis targeting DNA epigenetic markers of B_SPTB_03 and DDX4 can be applicable to differentiate between fresh semen and aged semen and also differentiate between semen alone and semen mixed with other body fluids, hence it is recommended to be employed in forensic practice.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article. The participants were informed of the purpose of the research and its implementation stages. They were also assured about the confidentiality of their information and were free to leave the study whenever they wished, and if desired, the research results would be available to them. A written consent has been obtained from the subjects. Principles of the Helsinki Convention was also observed.

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Authors' contributions

All authors equally contributed to preparing this article

Conflict of interest

The authors declared no conflict of interest or financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

References

[1] Wasserstrom A, Frumkin D, Davidson A, Shpitzen M, Herman Y, Gafny R. Demonstration of dsi-semen--a novel DNA methylation-based forensic semen identification assay. Forensic Science International: Genetics. 2013; 7(1):136-42. [DOI:10.1016/j.fsigen.2012.08.009] [PMID]

[2] An JH, Shin KJ, Yang WI, Lee HY. Body fluid identification in forensics. BMB Reports. 2012; 45(10):545-53.[DOI:10.5483/BMBRep.2012.45.10.206] [PMID]

[3] Pood E. Methylation-specific differentiation of vaginal epithelial cells for forensic tissue typing by bisulfite conversion and pyrosequencing [MSc Thesis]. Hattiesburg: University of Southern Mississippi; 2019. [Link]

[4] Sijen T. Molecular approaches for forensic cell type identification: On mRNA, miRNA, DNA methylation and microbial markers. Forensic Science International: Genetics. 2015; 18:21-32. [DOI:10.1016/j.fsigen.2014.11.015] [PMID]

[5] Balamurugan K, Bombardi R, Duncan G, McCord B. Identification of spermatozoa by tissue-specific differential DNA methylation using bisulfite modification and pyrosequencing. Electrophoresis. 2014; 35(21-22):3079-86. [DOI:10.1002/elps.201400175] [PMID]

[6] Park JL, Kwon OH, Kim JH, Yoo HS, Lee HC, Woo KM, et al. Identification of body fluid-specific DNA methylation markers for use in forensic science. Forensic Science International: Genetics. 2014; 13:147-53. [DOI:10.1016/j.fsigen.2014.07.011] [PMID]

[7] Haas C, Klesser B, Maake C, Bär W, Kratzer A. mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR. Forensic Science International: Genetics. 2009; 3(2):80-8. [DOI:10.1016/j.fsigen.2008.11.003] [PMID]

[8] Watanabe K, Akutsu T, Takamura A, Sakurada K. Evaluation of a blood-specific DNA methylated region and trial for allele-specific blood identification from mixed body fluid DNA. Legal Medicine. 2016; 22:49-53. [DOI:10.1016/j.legalmed.2016.08.004] [PMID]

[9] Lee HY, Park MJ, Choi A, An JH, Yang WI, Shin KJ. Potential forensic application of DNA methylation profiling to body fluid identification. International Journal of Legal Medicine. 2012; 126(1):55-62. [DOI:10.1007/s00414-011-0569-2] [PMID]

[10] Brait M, Maldonado L, Begum S, Loyo M, Wehle D, Tavora FF, et al. DNA methylation profiles delineate epigenetic heterogeneity in seminoma and non-seminoma. British Journal of Cancer. 2012; 106(2):414-23. [DOI:10.1038/bjc.2011.468] [PMID] [PMCID]

[11] Pfaffl M. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research. 2001; 29(9):e45. [DOI:10.1093/nar/29.9.e45] [PMID] [PMCID]

[12] Chan YH. Biostatistics 102: Quantitative data--parametric & non-parametric tests. Singapore Medical Journal. 2003; 44(8):391-6. [PMID]

[13] Ledgerwood SM. High resolution melt analysis of samples with differential DNA methylation to identify tissue source of origin [MSc Thesis]. Hattiesburg: University of Southern Mississippi; 2015. [Link]

[14] Blackman S, Stafford-Allen B, Hanson EK, Panasiuk M, Brooker AL, Rendell P, et al. Developmental validation of the paraDNA® body fluid id system-a rapid multiplex mRNA-profiling system for the forensic identification of body fluids. Forensic Science International: Genetics. 2018; 37:151-61. [DOI:10.1016/j.fsigen.2018.08.012] [PMID]

[15] Stravers CS, Gool EL, van Leeuwen TG, Aalders MC, van Dam A. Multiplex body fluid identification using surface plasmon resonance imaging with principal component analysis. Sensors and Actuators B: Chemical. 2019; 283:355-62. [DOI:10.1016/j.snb.2018.12.050]

[16] Abbas N, Lu X, Badshah M, il Heo W, Seo SJ, Kim SM. Identification of body fluids using metal enhanced fluorescence substrate with glancing angle deposited Ag nanorods. Paper presented at: Frontiers in Biological Detection: From Nanosensors to Systems XI. 4 March 2019; San Francisco: United States. [DOI:10.1117/12.2511045] [PMCID]

[17] Zapata F, de la Ossa MÁ, García-Ruiz C. Differentiation of body fluid stains on fabrics using external reflection fourier transform infrared spectroscopy (FT-IR) and chemometrics. Applied Spectroscopy. 2016; 70(4):654-65. [DOI:10.1177/0003702816631303] [PMID]