The Effect of Mixing Seminal Fluid and Vaginal Secretion on the Expression of miRNA Markers in a Simulated Forensic Scientific Detection

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ABSTRACT

Forensic investigators usually recover body fluids from crime scenes, and their identification is a necessary part of forensic case study. By establishing a connection between sample donors and actual criminal activities, determining the type and origin of body fluids found at a crime scene will provide crucial information for crime scene reconstruction. A quantitative Real-time PCR technique was used to calculate the expression levels of miR-10b and miR-135b, as well as miR-124-3p and miR-372, in semen, vaginal fluid stains, and their mixture. The identification and stability of the target genes were evaluated using SNORD-47 as a reference gene. The results of this study reported that the MiR-10b and miR-135b were expressed at higher levels in semen stains than in vaginal stains; miR-124-3p and miR-372 were expressed at higher levels in vaginal stains than in semen stains; and four miRNAs were expressed in semen/vaginal mix stains but at lower levels than in primary samples. Additionally, the results revealed that in comparison to the reference gene SNORD-47, the expression levels of miR-10b and miR-135b (semen) and miR-124-3p and miR-372 (vaginal secretion) were higher in this sample. For two weeks, a mixture of semen and vaginal stains has no effect on miRNA expression levels and has good stability.

Keywords: MicroRNAs; Body fluids; Reference genes; RT qPCR.

INTRODCTION

The analysis of mRNA and miRNA expressions particularly in body fluid has been a major focus during the past decade. It offers valuable information on the origin of the body fluid and can provide a DNA profile with evidence strength. MiRNAs are known to control gene expression within the cells in which they are produced, and they may also be secreted into the extracellular space to regulate other cells or communicate between cells [1]. The expression profiles of miRNAs have shown that they are expressed differently in different cell types. These patterns of expression of miRNAs indicate that miRNAs could promise to indicate cell type and tissue identification [2]. One of the most significant issues in using miRNAs as markers for body fluid identification is their stability within the samples. In sexual assault cases, semen can be found in the form of stains on items or clothing, and it can also be retrieved from the victim's body and, vaginal secretion is one of the most significant forensically relevant body fluids [3]. Vaginal fluid, unlike other body fluids, lacks specific proteins that can be used to identify it, making it impossible to prove its existence. Micro-RNAs have recently been explored as an alternative tool for the identification of forensic body fluids [4]. Several studies have been published on the use of microRNA to identify semen and vaginal fluid [3-5]. The first study of miRNA analysis as a possible body fluid recognition tool in forensic casework was conducted by Hanson et al [3], and they reported miRNA markers that could be used to detect semen (miR-10b and miR-135), as well as vaginal secretions (miR-124a and miR-372). Therefore, the present study was carried out to determine the effect of mixing semen and vaginal sexual fluids on the expression of miR-10b; miR-135b; miR-124-3p and miR-372 in simulated dried spots.

Material and Methods

Ethical statement

Every volunteer has informed written consent. The ethics committee of the MOH and MOHSER in Iraq's ethical approval for scientific research has accepted this research.

Collection and preparation of samples

Five samples of seminal fluid, and vaginal secretion were taken from healthy volunteers. Male donors delivered freshly ejaculated semen in sealed Falcon tubes, which were then transferred to sterile stemmed cotton swabs. On a sterile cotton tipped swab, vaginal secretions were collected, and the combination of semen and vaginal were mixed with different mounts. All samples were dried at room temperature, dark and processed for RNA extraction as fresh and after two weeks.

Selection of Micro-RNA

The following Micro-RNAs were selected: for semen: miR-10b and miR-135b, while miR-124-3p and miR-372 for vaginal secretion [3,6] and all markers for mixed semen/ vaginal. For all samples, SNORD-47 was chosen as the reference gene for normalizing Micro-RNA expression.

Total RNA Extraction

Extraction of total RNA by RNAzol RT reagent (Sigma-Aldrich/USA), according to manufacturer's instructions. RNA yield and integrity measurements were performed.

Estimation of Concentration and purity of Total RNA

The concentration and purity of samples were measured at 260 nm and 280 nm by Nanodrop (Biodrop / UK) instrument. A260/280 ratios of pure RNA would usually range from 1.8 to 2.2.

Synthesis of cDNA from microRNA

Total RNA was extracted from two body fluid samples and used for synthesis cDNA synthesis by MiRNA All-In-One cDNA Synthesis Kit from abm / USA.

RT-qPCR

The reverse transcription and RT-qPCR reactions were carried out according to the manufacturer's instructions for Bright Green miRNA qPCR Master Mix. In all samples and the negative control, the expression levels of ten reference genes were determined in duplicate. RT-PCR mixture was in 20 µl including 10 µl (1x) Bright Green miRNA qPCR Master Mix, 300 nM of each primer, 3 µl cDNA product. The qPCR reactions were performed, the samples were subjected to 95 ℃ for 10 min and then 40 cycles of 95 ℃ for 10 second, followed by 63 ℃ for 15 seconds, and 30 seconds extension at 72 ˚C.

Biostatistical Analysis

The SPSS statistical package for the Social Sciences was used to analyze the results (version 20.0 for windows, SPSS, Chicago, IL, USA), [7].

Results and Discussion

Expression of microRNA

The miRNA targets that were studied were compared to SNORD-47. Each sample's triplicate was used to calculate an average CT value. The CT value was calculated by subtracting the average CT for the endogenous control from the target miRNA of interest (CT = CT (Target

miRNA) – CT (Endogenous Control)). The vaginal secretion and seminal fluid were mixed at different concentrations of total RNA (100/0;80/20;60/40;40/60;20/80;0/100) ng/µl. The present results mentioned in table 1.

The results from table 1 reported that the MiR-10b and miR-135b were expressed at higher levels in semen stains than in vaginal stains; miR-124-3p and miR-372 were expressed at higher levels in vaginal stains than in semen stains; and four miRNAs were expressed in semen/vaginal mix stains but at lower levels than in primary samples. A housekeeping gene was used in a normalization strategy to calculate the sample's Relative Quantification [8]. Previously, SNORD47 was described as a housekeeping transcript for miRNA normalization for expression quantification using various types of biological samples [9]. Other housekeeping genes for forensically related body fluids have been proposed and should be considered in the future [10].

Stability of miRNAs

To test the stability of miRNAs, stain samples and a mixture of body fluids were kept at room temperature for two weeks in a dark, dry conditions. The current results in (fig 1) show gene expression of miRNAs with fresh samples without storage (untreated). The results of present study revealed that in comparison to the reference gene SNORD-47, the expression levels of miR-10b and miR-135b (semen) and miR-124-3p and miR-372 (vaginal secretion) were higher in this sample. Whereas (fig 2) shown gene expression values of mixed semen/vaginal Secretion stain for two weeks in room temperature and dark (treated sample). Detection of old or deteriorated forensic samples is a challenge (Hanson et al., 2009). Micro-RNAs (miRNA) are less affected by degradation than mRNA because of their short length of 18–23 bp. Since 2009, several groups have been testing the feasibility and practicability of forensic miRNA analysis and miRNA expression analysis based on quantitative PCR (qPCR) in forensic settings [3,11].

It's also crucial to be able to differentiate between different species of body fluids at a crime scene. Using Raman spectroscopy, a previous study was unable to differentiate between cat, dog, and human blood samples [12]. Raman microspectroscopy, which uses non-actinic (non-destructive) near-infrared light to excite, had been used to analyze dry traces of body fluids such as blood, semen, saliva, vaginal fluid, and sweat [13]. MiRNAs tend to degrade in samples under environmental challenges and harsh chemical exposure, according to studies of miRNA stability in different situations [14]. [5] They found that the stability of miRNAs in old samples did not seem to have deteriorated.

Table 1. The ct and ^ct values for mixed seminal fluid and vaginal secretion samples

Sample	Untreated S/V.S. (ng)(µl)	miR-10b ct	miR-135b ct	miR-124-3p ct	miR-372 ct	HKG SNORD-47 ct	miR-10b Δct	miR-135b Δct	miR-124-3p Δct	miR-372 Δct
M1	100/0 5µl/0	20.20 5	21.43 5	0	0	19.28 5	0.92	2.15	0	0

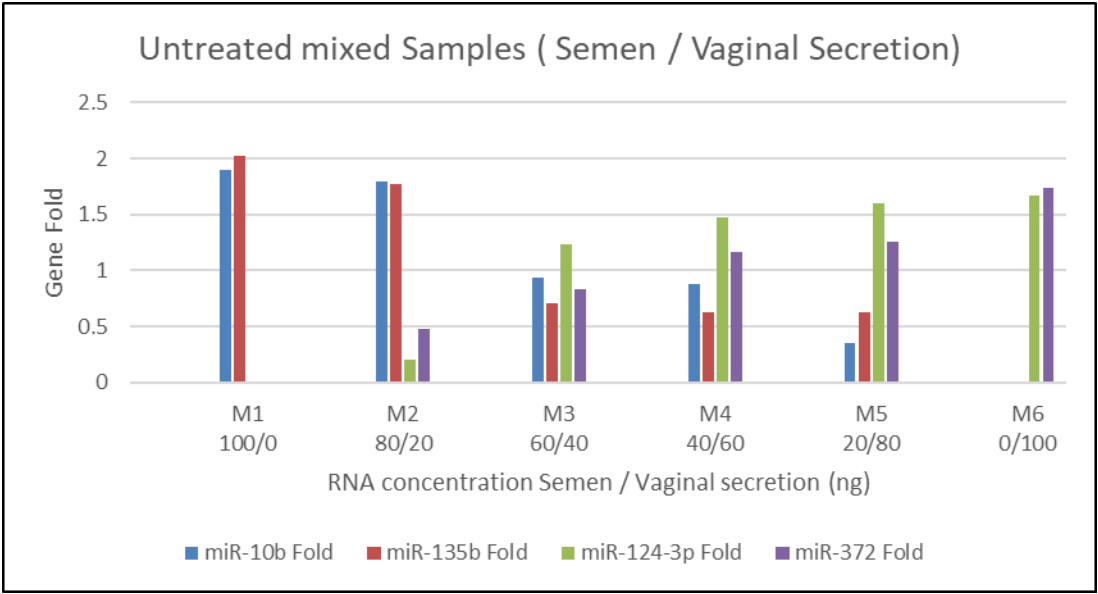|	µl								
M2	80/20 4µl/1 µl	20.88	22.22	27.48	25.075	19.885	0.995	2.335	7.595	5.19
M3	60/40 3µl/2 µl	21.545	23.28	24.64	23.985	19.615	1.93	3.665	5.025	4.37
M4	40/60 2µl/3 µl	21.48	23.285	24.22	23.345	19.455	2.025	3.83	4.765	3.89
M5	20/80 1µl/4 µl	22.775	23.28	24.1	23.225	19.45	3.325	3.83	4.65	3.775
M6	0/100 0µl/5 µl	0	0	24.17	22.895	19.585	0	0	4.585	3.31

Figure 1. Gene Expression Values of Mixed Sample (Semen/Vaginal Secretion) for fresh Sample
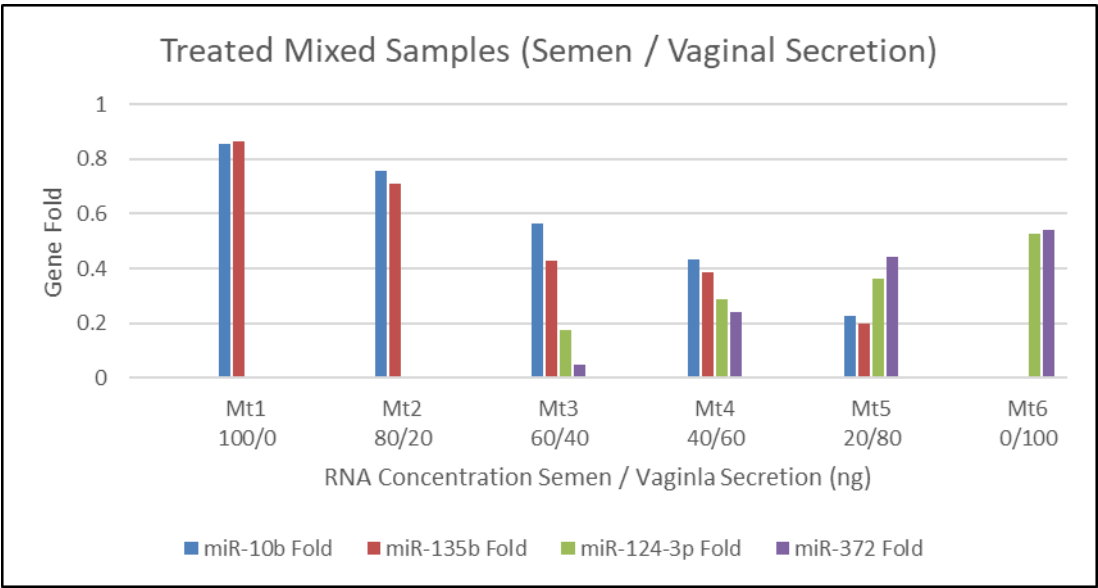

Figure 2. Gene Expression Value of Mixed Sample (Semen/Vaginal Secretion) for Treated Sample (two week in room Temperature and dark)

Conclusion

MiR-10b, miR-135b, miR-124-3p, and miR-372 were found to be stable in seminal fluid, vaginal secretion, and their mixtures in this study. As a result, these miRNAs can be used to identify criminal samples at crime scenes when they are linked together.

Conflict of Interests
The authors have declared no conflict of interests.

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