Abstract

The first appearance of ribonucleic acid (RNA) in forensic science research was in 1984 in the study of post-mortem tissues. Since then, many studies have explored the role of gene expression and its potential applications in forensic science. The two main RNA molecules that have been subject to increasing interest in the forensic science community are messenger RNA (mRNA) and microRNA (miRNA). Identification of body fluid type and estimating the time since deposition can be of immense value to criminal investigations. Determining the time since deposition or age of a biological stain can help to indicate either when a crime happened, or whether the biological evidence was deposited before/after a known crime event, in order for samples to be excluded. The research presented here has used reverse transcription quantitative PCR to examine the relative expression ratio (RER) in two types of body fluid-specific markers (saliva and semen), to develop a method to estimate the age of biological stains. mRNA and miRNA markers specific to saliva and semen, along with three reference genes were selected. Biological samples from 20 participants were stored in a dark dry place at room temperature to simulate natural ageing. A series of desired ageing points were set and total RNA was extracted when samples reached each desired point. The degradation behaviour of each RNA marker was analysed, showing that they exhibited unique degradation profiles across a one-year storage interval for saliva and semen samples, where miRNAs and the *U6* reference gene were shown to have high stability. The RERs exhibit a non-linear relationship with body fluid stain age and can be considered as a potential method for body fluid stain age estimation, hence the time since deposition.

Introduction

RNA first appeared in forensic research in 1984, when its synthesis in post-mortem tissues was described [1]. Since then, many more studies have been carried out to try and understand how patterns of gene expression can be useful in forensic science. Some RNA types such as messenger RNA (mRNA) have shown the ability to reveal the activities of genes and their respective cells/tissues, which may be useful in indicating pathological states [2] or any condition that leads to death [3]. In addition, RNA can be used to identify the type of a body fluid and the mechanism of its degradation can give some indication of the time it was deposited [4-8].

A DNA profile obtained from a biological material recovered at a crime scene can be used to identify individuals but does not provide any information regarding the origin of this biological material or the time since deposition. Determining whether a biological stain recovered from a scene of crime originates from blood, saliva, semen, or other body fluids can contribute to criminal investigations significantly. Recently, some RNA types such as mRNAs and micro RNAs (miRNAs) have been shown to be cell type-specific and can provide high specificity for body fluid identification [4, 9].

Time since deposition is a crucial piece of information for criminal investigations. Knowing when a biological material was deposited at a crime scene can provide investigators with information regarding when a crime occurred. Conversely, samples that do not correspond to the time when the crime is proposed to have occurred may be excluded. Previously, variation in solubility, morphological differences in bloodstains and analysis of protein degradation were used as methods to estimate the age of bloodstains, although these methods have some limitations [10]. To date, a method for ageing biological samples using RNA analysis has only been applied to bloodstains [11, 12], saliva [13] and hair [14].

Based on the findings of our previously published work on blood-specific RNA markers [15], which indicated that each RNA marker exhibited a unique degradation profile, the study presented herein is conducted on saliva and semen samples by applying the same method to determine whether their specific RNA markers would show similar patterns. The expression and degradation levels of RNA markers including miRNA and mRNA were analysed in samples stored over a one-year period, with the aim of identifying the

most/least stable RNAs, with the objective of developing a method to estimate the age of body fluid stains.

Materials and Methods:

Sample collection:

Samples were collected from 19 volunteers. The samples were collected from volunteers using procedures approved by the Department of Pure and Applied Chemistry Departmental Ethics Committee. Signed consent sheets were obtained from each donor after they had read a Participant Information Sheet (PIS). Fresh saliva and fresh ejaculated semen samples were directly deposited into sterile containers and then 50µL was transferred onto cotton swabs. All samples were stored at room temperature in a dark dry place to simulate natural ageing. Samples were stored until they reached a number of desired age points. Sample ageing points were 0, 7, 14, 28, 90, 180, 270, and 360 days.

RNA extraction:

Total RNA was extracted using the TRI® Reagent method (Sigma-Aldrich, Gillingham, UK) [12]. Genomic DNA was digested with the TURBO DNA-free™ Kit (Applied Biosystems, Life Technologies, UK) following the manufacturer's protocol. The quantity of extracted total RNA was determined using a NanoDrop-1000 Spectrophotometer (Thermo Scientific).

Reverse transcription quantitative PCR (RT-qPCR):

cDNA was synthesised from extracted RNA using the High-Capacity cDNA Reverse Transcription Kit for mRNA/rRNA markers, and TaqMan® microRNA Reverse Transcription Kit for miRNA markers (Applied Biosystems) following the manufacturer's instructions.

Body fluid-specific markers potentially suitable for saliva and semen identification were selected from the forensic science literature [7-9, 16-18] along with three reference genes (*ACTB*, *18S* and *U6*). Table 1 shows the selected RNA markers for each body fluid. The assays used in this work were designed to amplify only sections of the RNA and not the whole transcripts.

Table 1: **The selected TaqMan® Gene Expression Assays and the TaqMan® MicroRNA Assays for each body fluid.**

Body fluid	RNA marker	RNA type	Applied Biosystems TaqMan® assay ID
Saliva	*STATH*	mRNA	Hs00162389_m1
	miR205	miRNA	000509
Semen	*PRM1*	mRNA	Hs00358158_g1
	PRM2	mRNA	Hs04187294_g1
	miR10b	miRNA	002218
	miR891a	miRNA	002191
Reference genes	*18S*	rRNA	Hs99999901_s1
	ACTB	mRNA	Hs99999903_m1
	U6	snRNA	001973

Real-time PCR was carried out using the TaqMan® Universal PCR Master Mix II Kit, with no AmpErase® UNG (Applied Biosystems) following the manufacturer's protocol with pre-designed primers.

Relative expression ratio (RER):

Similar to what we have applied in our previous work on blood samples [15], the relative expression ratio was obtained by dividing the efficiency-corrected C_q values of the less stable RNA marker by the efficiency-corrected C_q values of the more stable RNA marker, or by dividing the C_q values of the body fluid-specific marker by the C_q values of a reference gene, as shown in the equations (1) and (2) below.

$$RER = \frac{Cq\ of\ less\ stable\ marker}{Cq\ of\ more\ stable\ marker} \qquad \text{(Equation 1)}$$

$$RER = \frac{Cq\ of\ body\ fluid\ marker}{Cq\ of\ reference\ gene} \qquad \text{(Equation 2)}$$

Statistical analysis:

The generated data from RT-qPCR was analysed using MxPro (Agilent Technologies), and GenEx statistical software (version 5.4.4) was used for efficiency correction of the raw C_q data. The line graphs and summary statistics were generated using Microsoft Excel 2016. Minitab® 17 was used for statistical analysis.

Results:

Degradation rate:

The stability and degradation rate of RNA molecules used as saliva and semen-specific markers were assessed along with three reference genes to determine the best marker(s) for estimating the age of body fluid stains or time since deposition.

Figure 1 illustrates that all RNA markers in aged body fluid stains degrade at different rates and have unique patterns of behaviour, with miRNA markers exhibiting the highest stability, as expected due to their small size [19].

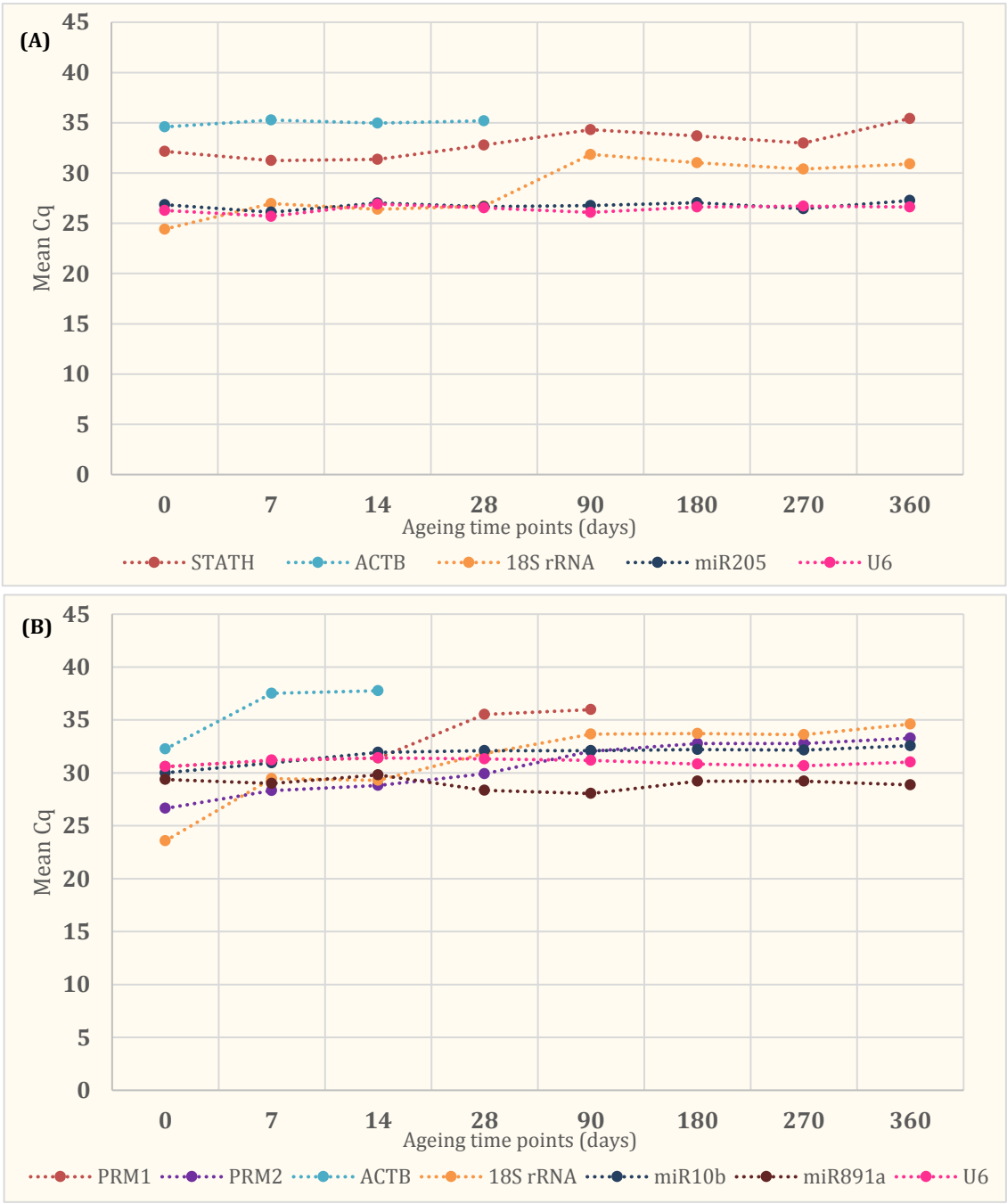

Figure 1: **Mean C_q for RNA specific markers in total RNA extracted from saliva and semen samples stored at room temperature for 360 days.** (A) saliva-specific markers and (B) semen-specific markers. Data represent the mean of n = 10 saliva stains and n = 9 semen stains. Error bars were omitted for clarity.

Saliva:

One mRNA (*STATH*) and one miRNA (*miR205*) saliva-specific marker were examined. *STATH* exhibited very interesting behaviour, showing high stability by remaining relatively at the same level in the first 28 days and starting to degrade slowly after that. After 90 days, the level of *STATH* starts to fluctuate and this marker gave the highest C_q value at 360 days.

The C_q values for *miR205* in saliva showed no notable decrease in their quantity as they remained stable at the same level across all ageing time points.

Semen:

Both *PRM1* and *PRM2* degraded in a linear fashion. The degradation rate of *PRM1* was slow initially but after 14 days of ageing, its level dropped dramatically and fell below the sensitivity level of the assay after 90 days. *PRM2* degraded steadily across the first 90 days of ageing but was still detected across all ageing time points, reaching a plateau after 90 days of ageing.

The *miR891a* marker remained stable across all ageing points, while *miR10b* started to degrade slightly up to 14 days, then showed no pronounced increase in C_q measurements.

Reference genes:

Among the selected reference genes, *U6* was the only marker that showed high stability in both body fluid types, and its C_q values remained at the same level across all time points in both sample types. Both *ACTB* and *18S* were excluded from further analysis due to their relatively low stability.

Relative Expression Ratio (RER):

To determine the RER, the C_q values of the less stable markers were considered relative to those of the most stable markers, or the body-specific marker relative to the reference gene. The relationship between body fluid stain age and RER values was examined. A parametric statistical analysis was applied as an Anderson-Darling normality test showed that data were normally distributed ($p > 0.05$).

Saliva:

Applying a Pearson correlation, only the RER of *STATH/miR205* showed a positive correlation with saliva sample age (r = 0.896, p = 0.039), but no significant relationships with any other RERs were detected. This relationship was only observed up to 90 days, because after this the *STATH* marker started to fluctuate, so the data were not considered at any of the later time points.

Semen:

The relationship between the RERs of semen-specific markers and body fluid stain age was analysed statistically up to 90 days, because at that point the C_q of *PRM1* had fallen below the sensitivity level of the assay and *PRM2* had reached a plateau.

The RERs of *PRM2* to both *miR10b* and *miR891a*, as well as the *U6* reference gene, showed a positive correlation with ageing time points (Table 2). In addition, the RERs of *PRM1* to the two miRNA markers and *U6* also showed a slight increase with increasing time ageing points (data not shown), however, when applying a correlation analysis, no significant relationship was found.

Table 2: **Spearman's correlation between ageing time points and the RERs of semen-specific markers.**

RER	r	p-value
PRM2/miR10b	0.97	0.0062
PRM2/miR891a	0.94	0.0161
PRM2/U6	0.97	0.0061

Regression analysis:

Regression analysis was used to estimate the relationship between the RERs of body fluid-specific markers and the age of the stains. Figure 2 illustrates the relationship between the RERs of *STATH/miR205* (Figure 2A) in saliva, *PRM2/miR10b* (Figure 2B), *PRM2/miR891a* (Figure 2C) and *PRM2/U6* (Figure 2D) in semen samples, all of which give very high R^2 values with narrow confidence intervals, when applying a second-order polynomial. All data were analysed up to 90 days before the level of

PRM1 dropped below the detection sensitivity of the assay, *PRM2* reached plateau, and *STATH* began to fluctuate.

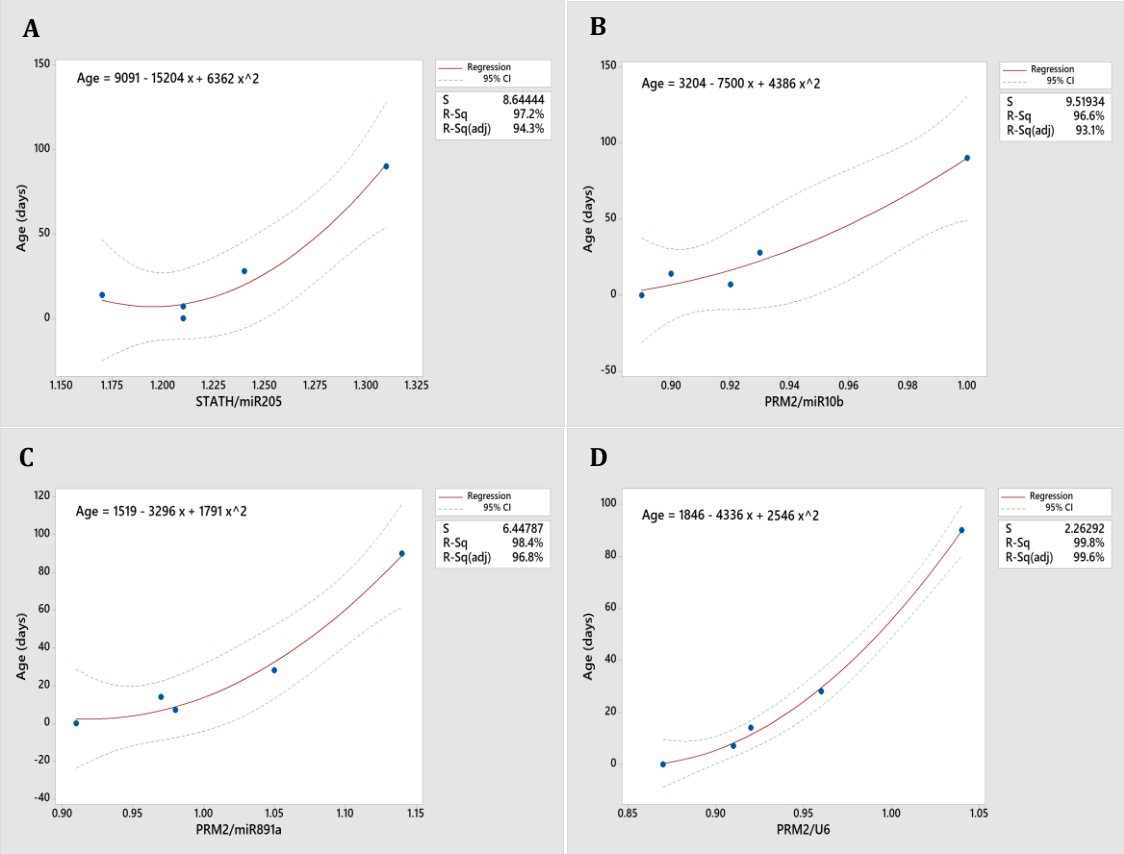

Figure 2: **Regression analysis of the RERs of body fluid samples over ageing time points using a second-order polynomial curve with 95% confidence intervals** (A) RER of *STATH* to *miR205* (saliva), (B) RER of *PRM2* to *miR10b* (semen), (C) RER of *PRM2* to *miR891a* (semen) and (D) RER of *PRM2* to *U6*. Data represent mean of n = 10 saliva stains and n = 9 semen stains.

Discussion:

In this work, the expression level of individual RNA transcripts was quantified by RT-qPCR in saliva and semen samples stored in a dark dry place at room temperature for up to one year. The findings demonstrate that each RNA transcript showed a unique pattern of degradation behaviour and that the gradual increase of C_q values across storage time can be used to characterise the degradation of RNA. These results are similar to previous observations in blood-specific markers and suggest that RNA degradation in these three body fluid types follows a broadly similar pattern [15].

In saliva samples, the degradation rate of *STATH* stayed relatively stable up to the 28 days ageing time point, indicating that some mRNA markers can stay stable in saliva for a few weeks. The presented results are in agreement with a more recent study by Watanabe et.al (2017) [20], who stored saliva samples for up to one year under dry conditions, and showed that the mean C_q of *STATH* remained at the same level for one

month, started to show gradual degradation after that, and then was almost undetectable after one year of ageing. Therefore, the high stability of *STATH* makes it a very good candidate as a saliva-specific marker

The degradation rate of the semen-specific markers (*PRM1* and *PRM2*) in this study supports the data presented in Weinbrecht (2014) and Nakanishi et al. (2014), where *PRM1* was shown to degrade over a 6 month period [21], and *PRM2* was still detected in very old semen samples (33 and 56 years old) [22].

The miRNA markers in both body fluids have shown high stability in comparison with mRNA markers. These findings are in concordance with our previous study [15], where the C_q values for *miR16* and *miR451* in blood samples were stable across ageing points indicating high stability, whilst the level of *HBA* dropped dramatically across the same time period.

In gene expression studies, it is crucial to use appropriate reference genes for normalization in order to obtain a reliable assessment and proper interpretation of the data. The main specification of reference genes is that their expression level should be stable under different experimental or environmental conditions. The reference genes must be selected very carefully depending on the conditions of the experiment and the type of samples, in order to generate accurate data. *ACTB*, *18S* and *U6* are commonly used as reference genes and were analysed in this work. However, only *U6* remained stable across all ageing time points while *ACTB* and *18S* exhibited high levels of instability.

The RERs of less stable RNA markers to more stable markers were calculated, and it was demonstrated that these values were related to the age of biological stains, since they were shown to be significantly correlated with the ageing time period in both body fluids stored under controlled conditions. The miRNA markers showed high stability, likely due to their small size, while the C_q values for mRNA markers increased over time, indicating a reduction in their levels as a result of degradation. Additionally, the RERs of semen-specific markers to *U6* (the most stable reference gene detected in this project) were also significantly related to age (Table 2). Supporting these findings, in

blood-specific markers, the RERs of *HBA/U6* and *miR16/U6* exhibited a positive correlation with ageing time points [15].

Figure 2 illustrates regression analysis using a second-order polynomial (non-linear) of the RERs of different body fluid-specific markers and stain age. The equation displayed in each figure can be used to estimate the age of the biological stains from the RER value for a stain of unknown age. However, although these equations have been proposed to be useful in estimating the age of biological stains for each of these body fluid types, it should be acknowledged that there are some limitations associated with this study and its application. For example, the relatively small sample sizes (n=10 for each body fluid type), and the fact that the study did not cover a more extensive selection of body fluid-specific markers. Additionally, the samples were stored under controlled conditions (room temperature and in dark dry conditions) and other environmental effects such as sun exposure (UV) and rain, high temperature, and high humidity were not examined. It will be important to examine these effects in the future, as forensic samples are not always stored in similar conditions. Nevertheless, we have identified RNA markers whose degradation patterns are significantly related to biological stain age for blood [15], semen and saliva. The relative levels of these markers can therefore, in principle, be used to estimate the age of stains of all three body fluid types.

Conclusion:

The findings presented here demonstrate that different RNA molecules degrade at different rates in body fluid stains, with miRNA markers exhibiting the highest stability over time across different body fluids, likely due to their small size. The Relative Expression Ratios of body fluid-specific markers represent a potentially powerful method for estimating the age of biological stains, and this has a wide range of applications in forensic science.

Conflict of interest

None.

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