Validation of mRNA and microRNA profiling as tools in qPCR for estimation of the age of bloodstains

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Abstract: Background: Bloodstains are the most important source of evidence encountered at crime scenes, due to their regularity and variety of applications. Temporal determination of the age of bloodstains in forensic investigations is essential, as it helps in estimating the time elapsed since occurrence or reporting of the crime. However, little has been explored in this regard forensically. Hence, this study aimed at the temporal estimation of the age of bloodstains using real-time quantitative-PCR for microRNAs (miR-126, miR-150, and miR-451), in addition to Caspase-1 and Caspase-3 mRNA. We also analyzed whether the accuracy and quality of information provided by the parameters help in achieving the purpose of this study. **Method:** A total of 70 bloodstains were randomly divided into two sets, incubated at 25 °C with 50% humidity, and were sorted for profiling of mRNA and microRNA (miRNAs). **Results:** The results indicated that levels of Caspase-1 and Caspase-3 mRNA gradually decreased at days 1, 6, 13, and 27, in comparison to that in fresh bloodstains In addition, the mRNA levels decreased significantly at days 41 and 55, after which they were nearly absent. In contrast, the levels of miR-126, miR-150, and miR-451 had a constant expression level all over the studied time points (days 1, 6, 13, 27, 41, and 55 of incubation of the bloodstains). **Conclusion:** Our findings indicated that profiling of mRNA in the bloodstain is suitable for the accurate aging of blood samples up to 55 days old. On the other hand, since levels of miRNA could be quantified in both fresh and dried bloodstains for long periods, it cannot be a useful tool for aging bloodstains up to 55 days. Another important conclusion of high forensic relevance is that miRNAs were highly stable in aged samples till the studied time points, which could have other applications in forensic casework.
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1. Introduction

Various forms of evidence found at a crime scene are indicators of the time at which the crime was committed, and are considered to be crucial in forensic practice (Bremmer et al., 2012). The biological evidence found in forensic cases is classified into body fluids, hairs, and contact traces (Knock and Davison, 2007) Bloodstains provide valuable information in forensic cases, as they can be used in various applications such as DNA-profiling to prove the suspect's identity, thus solving many crimes (Edelman, 2012)

The bloodstains could be precious evidence in the estimation of the time since death, particularly when there are no witnesses at the crime scene, and bloodstains are the only piece of evidence available (Kind et al., 1972). Moreover, bloodstains could predict the actual time of death more accurately and help in establishing a correlation with other blood drops at a crime scene.

The possibility of using bloodstains to estimate the time at which a crime was committed has not yet been materialized, despite several attempts. Schwarzacher (1930) attempted to correlate the age of a bloodstain with its rate of solubility in water, which begins rapidly and then decreases gradually as the blood stain ages. Recently, Anderson et al. (2005, 2011) and Bauer et al. (2003) demonstrated the possibility of utilizing the nucleus of the white blood cell for age determination of bloodstains, as the white blood cell contains both DNA and RNA. However, DNA appeared to be stable in dried bloodstains as long as there was no bacterial overgrowth. The studies had neither discussed the degradation of the cells themselves, nor its outcome on age estimation of bloodstains (Dissing et al., 2010). RNA, which can be extracted from the nucleus of a white blood cell, is not as stable as DNA. Hence, estimation of the extent of RNA degradation appears to be feasible for the age determination of bloodstains. Several studies have demonstrated that RNA degradation rates are useful in the evaluation of the age of biological specimens (Anderson et al. 2005, 2011, Bauer et al., 2003). RNA exists in different forms (mRNA, tRNA, and rRNA) and it is hypothesized that the diverse types of RNA may also decay at different rates, resulting in a change

in the ratios of RNA species with time. The instability of RNA implies that small changes lead to polymorphs of RNA. Polymorphism occurring between RNA species can be standardized for species-specific tests. Improvements in age estimates were observed when a multivariate analysis of the RNA species was carried out. Using RT-PCR, GAPDH mRNA could be identified from dried blood samples that had been aged up to six months (Bauer, 1999) showing that the RNA in dried blood samples might be sufficiently stable for various forensic analytical methods. However, mRNA of the inducible gene IL-3 was reported to decay more rapidly than the mRNA of the GAPDH housekeeping gene in lung tissue of dead rats (James, 2005).

MicroRNAs (miRNAs) belong to a class of endogenous, small, 18-24-nucleotide long, noncoding RNA molecules that regulate gene expression at the post-transcriptional level (Kim, 2009). Hanson et al. (2009) reported that the blood-specific miRNAs miR-16 and miR-451 were stable in dried bloodstains. Mature miRNA can cause inhibition of mRNA translation by incorporating into the RNA induced silencing complex (RISC) and hybridizing to the 30UTR of specific mRNA targets, and/or mRNA degradation (Gu et al., 2009). Tavazoie et al. (2008) demonstrated the vital role of miRNAs in physiological functions and pathogenesis, suggesting that miRNAs can express in a tissue-specific manner. Theoretically, the shorter fragments and tissue-specific expression of miRNA make it less susceptible to degradation by chemical and/or physical environmental stress, which makes it a useful biomarker for identification of body fluids. Interestingly, miRNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples were reported to yield feasible and valid profiling results (Hui et al., 2009, Leite et al., 2011). On the lines of the preceding knowledge, we designed this study with the aim of determining the age of bloodstains by profiling miR-126, miR-150, and miR-451 as well as the Caspase-1 and Caspase-3 mRNA, using quantitative real-time polymerase chain reaction (qPCR). Another objective of this study was to compare the accuracy of miRNAs and mRNA in determining the age of bloodstains in rat models.

2. Materials and methods

Experimental animal model

The laboratory animal unit of the faculty of Veterinary Medicine, Zagazig University, Egypt provided 14 healthy, adult male Sprague-Dawley rats weighing 240–250 g. Animals were kept in hygienic conditions at the experimental animal center for five days before collection of blood samples. They were fed *ad-libitum* on a standard rodent chow and filtered water.

Blood sampling

Five milliliters of blood were drawn from the median canthus (orbital vessels) of each rat into dry non-coated BD Vacutainers (Fisher Scientific, Pittsburgh, PA) without anticoagulant. Thus, a total of 70 mL blood sample was obtained and used to prepare 70 bloodstain samples (1 mL each), which were further analyzed in this study.

Preparation of bloodstains

Each blood sample was placed on a piece of white unbleached cotton (dimensions 2 × 2 × 1 cm, weight 160–190 mg). The soaked cotton pieces were then placed in clean, dry plastic Petri dishes, and were left in a chamber (HPAV-48-20; Isuzu Seisakusho, Niigata, Japan) maintained at 25 °C and 50% humidity for 0, 1, 6, 13, 27, 41, or 55 days (10 samples per time point, total = 70). The blood stain samples at each time point were assigned into two sets (n=5); the first set was used for mRNA analysis and the second set for miRNA analysis.

Isolation of white blood cells from bloodstains

The fresh and dried bloodstains on Petri dishes were collected into BD Falcon™ tubes of 50 mL capacity. Five milliliters of Buffer EL (Qiagen, Hilden, Germany) were directly added to the tubes and vortexed for 30 min at 4 °C. The mixtures were transferred to fresh 15 mL tubes using plastic syringes and were incubated on ice for 20 min with brief vortexing twice during the incubation period. The samples were then centrifuged at 800 ×*g* and 4 °C for 10 min (Nakao et al., 2013). The supernatants were then removed completely and added to 2 mL of Buffer EL, followed by brief agitation using vortex. The samples were again centrifuged at 800 ×g and 4 °C for 10 min (Nakao et al., 2013), the supernatants were discarded and the leukocyte pellets were collected.

Transcription levels of genes coding for mRNA and miRNAs

Quantitative RT-PCR (qPCR) was used to measure levels of mRNA (Caspase-1 and Caspase-3), micro RNAs (miR451, miR126, and miR150), and the housekeeping genes (β-actin for mRNA and RNU6b for miRNA), using gene-specific primers (Table 1).

Extraction and purification of total RNA

Total RNA was extracted from the leukocytes obtained from fresh and dried bloodstains using E.Z.N.A.™ spin column RNA extraction kit (Omega Biotech, Cat No. R6834-01, Canada), according to the manufacturer's instructions. The miRNAs were extracted using RNeasy Mini Spin Column kit (Qiagen, Heidelberg, Germany). The samples were centrifuged at 10,000 ×*g* for 15 s at room temperature. The flow-through containing the miRNA was collected in a fresh collection tube. The miRNA-

enriched fraction of the flow-through was purified with the RNeasy MiniElute Cleanup Kit (Qiagen, Heidelberg, Germany) according to the manufacturer's instructions. Finally, the purified miRNA-enriched fraction was eluted with 14 µL of RNase-free water.

Synthesis of complementary DNA (cDNA) and analysis by qPCR

Complementary DNA for the qPCR was synthesized using the miScript II RT Kit (Qiagen, Heidelberg, Germany), following the manufacturer's instructions. The reverse transcriptase reactions were performed on reaction mixtures of volume 20 µL, using the GeneAmp® PCR System 9700 (Applied Biosystems, USA). The reaction mixtures were incubated at 37 °C for 60 min and then at 95 °C for 5 min to inactivate the reverse transcriptase. Electrophoresis and melting curve analysis were performed after qPCR to check whether the constructed primer pair had produced any by-products.

Table 1. Primer sequences used for the qPCR analysis

Genes	Sequence	References/ Accession No.
miRNA		
miR-126	UCUCCCAACCCUUGUACCAGUG	(Courts and Madea, 2011)
miR-451	UCGUACCGUGAGUAAUAAUGCG	(Courts and Madea, 2011)
miR-150	AAACCGUUACCAUUACUGAGUU	(Courts and Madea, 2011)
RNU6b	CTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT	(Courts and Madea, 2011)
mRNA		
Caspase-1	F: 5'CACGTCTTGCCCTCATTATC3' R: 5′ CTGTCAGAAGTCTTGTC3′	(Eriksson et al., 2010)
Caspase-3	F: 5'AATTCAAGGGACGGGTCATG3' R: 5'GCTTGTGCGCGTACAGTTTC3'	NM 012922.2
β-actin	F: 5' TTGCTGATCCACATCTGCT 3' R: 5'GACAGGATGCAGAAGGAGAT3'	NM 031144.3

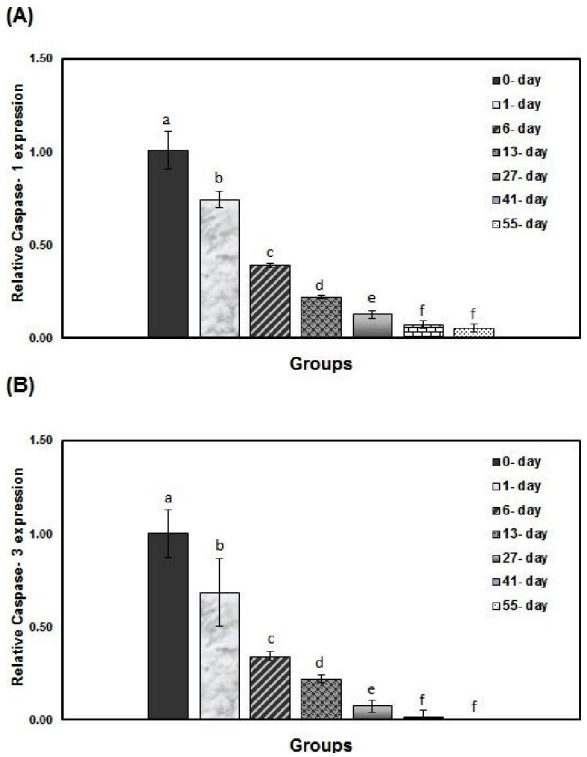

Fig. 1 Caspase-1 (A) and Caspase- 3 (B) mRNA expression profiling in bloodstains from rat blood at different experimental time points (days 0, 1, 6, 13, 27, 41, and 55) using qPCR analysis.

Analysis of PCR data

Expression levels of the target genes were standardized with respect to the reference genes. To determine the efficiency of PCR amplification for reference and target genes, a 10 fold dilution series of cDNA was prepared. Each dilution was amplified using qPCR, and the obtained threshold cycle (C_t) values were plotted against $\log_{10}$ of the cDNA sample dilution on a graph. The slope of the graph indicated the reaction efficiency according to the formula: efficiency = $[10^{(-1/\text{slope})}]-1$. Relative gene expression was then calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001), where the statistical calculation was based on $2^{-\Delta Ct}$ values.

Statistical analysis

All values were expressed as a mean ± standard error (SE). Differences between the values at day 0 and at other time points were determined by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. The level of significance was set at $p< 0.05$.

3. Results

Transcriptional mRNA concentration in bloodstains

After incubation of the bloodstains till the experimental time points, the relative levels of Caspase-1 and Caspase-3 mRNAs showed a tendency to decrease with time, at days 1, 6, 13, and 27 of bloodstain aging, when compared with the fresh

bloodstains ($p < 0.05$) (Fig. 1). On the other hand, there was a high significant decrease in the expression levels of Caspase-1 and Caspase-3 on days 41 and 55 when compared with the samples on day 0 ($p < 0.05$). The levels of Caspase-3 mRNA were nearly absent on day 55.

Transcriptional miRNA concentration in bloodstains

The relative levels of miR-126, miR-150 and miR-451 are shown in Fig. 2. The levels of the three miRNAs examined were observed to be constant and not declined throughout the time points of bloodstain incubation (days 0, 1, 6, 13, 27, 41 and 55).

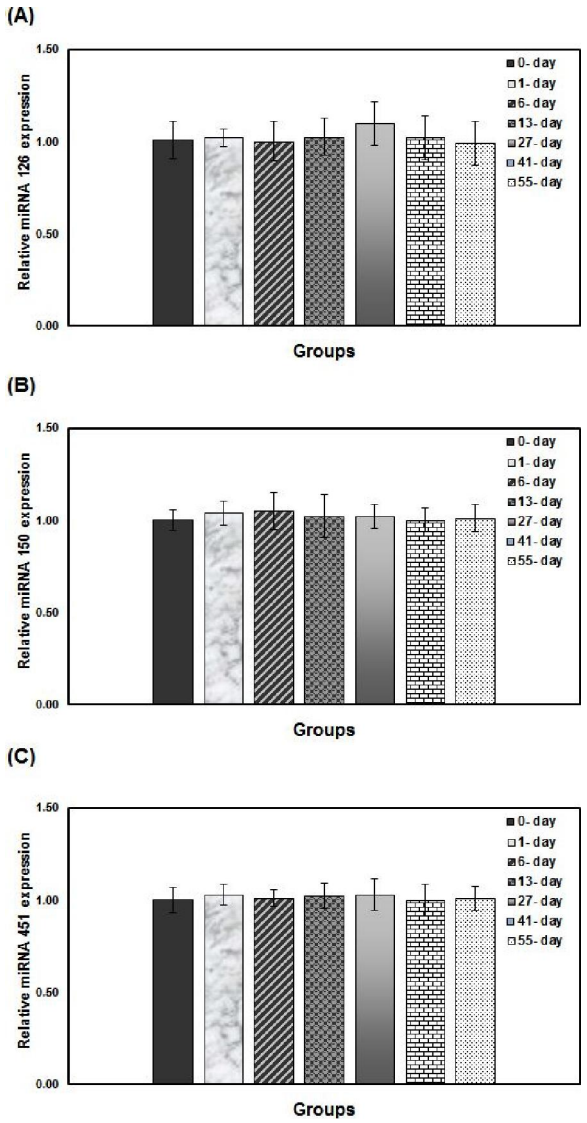

Fig. 2 miR-126 (A), miR-150 (B), and miR-451 (C) microRNA expression profiling in bloodstains from rat blood at different experimental time points (days 0, 1, 6, 13, 27, 41, and 55) using qPCR analysis.

4. Discussion

Estimating the age of bloodstains at crime and accident scenes provides a temporal connection between the evidence and the incident. Bloodstains are formed from droplets of whole blood which dry out at the scene of the crime where many traces can be picked up and investigated forensically. Whole blood contains blood cells, proteins, and amino-acids suspended in a fluid called blood plasma. Ribonucleic acid (RNA), one of the main components of leukocytes, is relatively new to the field of forensic science but has immense potential in routine forensic investigations (Bauer, 2007). Measurements of RNA degradation appear to be a viable possibility for age estimation of bloodstains. However, the susceptibility of mRNA stability and susceptibility to degradation has always been an issue for mRNA-based gene expression analysis, and it was demonstrated that altered integrity of mRNA has an effect on the reproducibility of results by presenting a variable degree of bias (Schroeder et al., 2006, Auer et al., 2003). This is particularly true for routine forensic applications using mRNA, as the biological stains from forensic cases are usually affected by ambient moisture and temperature, UV light, suboptimal environmental pH, all of which degrade mRNA beyond use (Courts and Madea, 2011). Interestingly, and as previously discussed (Zubakov et al., 2008, 2009), Caspase-1 is one of the stable-mRNA tissue-specific genes, which made it a candidate for our study. The selected genes were identified to be highly specific markers, by virtue of high blood-specificity on a microarray-based genome analysis of blood samples degraded over time. In addition to this is the vital role played by Caspase-3 in apoptosis, wherein it is considered to be an executioner of apoptosis in all nucleated cells of the body including the leukocytes (McIlwain et al., 2013). This process may be quickened slightly post-mortem, as proved by Kemp et al. (2014) in longissimus muscle. Moreover, Caspase-3 was also isolated from non-nucleated erythrocytes in whole blood samples (Berg et al., 2009), wherein it can perform the same role as the executioner of apoptosis, in exactly the same manner as in the nucleated cell, in the absence of the standard regulators of apoptosis.

The results of the present study indicated that incubation of the examined bloodstains tends to decrease the degradation process with time. The degradation of mRNA was observed to be time-dependent, wherein relative levels of mRNA declined gradually on days 1, 6, 13, and 27, while they declined rapidly on days 41 and 55 to near absence on day 55 of incubation. These findings were consistent with those of Zubakov et al. (2008), who identified blood and saliva-specific mRNA markers that showed stable

expression patterns in stains after up to 180 days of storage. These findings are encouraging as even the stains discovered and analyzed after a long period of time may facilitate accurate identification of the type of body fluid. Furthermore, Setzer et al. (2008) studied the influence of several storage conditions on the analysis of different transcripts and reported that detection of mRNA from samples stored indoors (at room temperature) was possible even after 547 days. They also explained that if samples were kept outdoors but protected from precipitation, tissue-specific mRNAs could still be analyzed in saliva (up to seven days), blood (up to 30 days), and vaginal secretions (up to 180 days). Another study by Bauer et al. (2003) analyzed whole blood samples from living individuals and post-mortem blood and brain samples which were stored at 4 °C for up to five days. The researchers observed that RNA degradation was significantly correlated with the storage interval of blood samples taken from living individuals, and with the post-mortem interval in autopsy cases**.** However, some studies have also concluded that certain mRNAs are expressed in a tissue-specific manner, and could be identified in stains even after a considerable period of time (Juusola and Ballantyne, 2003). A study by Zubakov et al. (2009) reported that humidity, heat, UV light, and physical fragmentation by ubiquitous ribonucleases were detrimental to the stability of mRNA as a specific and sensitive biomarker for forensic applications. The mechanism of *ex vivo* RNA degradation is not as well understood as *in vivo* degradation. RNA degradation in fresh post-mortem samples which are not preserved or dried is driven by cellular RNases that remain active in the moist cellular material. However, in samples that are dried, RNases are largely inactivated, resulting in RNA degradation which is driven mostly by physical and chemical factors such as sunlight or pH (Fordyce et al., 2013). Although RNA is certainly more prone to degradation than DNA in post-mortem or deposited samples, it is often much more stable *ex vivo* than *in vivo.* This is due to the inactivation of RNases in many *ex vivo* samples (such as those that have been dried, frozen, or preserved) (Fordyce et al., 2013). Thus, RNA is considerably more stable in biological samples than assumed earlier. Moreover, the rate of RNA degradation *ex vivo* is measurable over days and weeks and can be utilized to provide information about sample deposition time **(**Anderson et al., 2011).

The agents detrimental to RNA have led many forensics and genetic scientists to validate and employ very short amplicons which can resist the adverse environmental conditions and survive many years for successful PCR detection. These intrinsically short miRNAs have the potential for being ideal markers in forensic applications and in the determination of the age of bloodstains, especially when samples are not suitable for extraction and analysis of DNA (Weber et al., 2010).

The hypothesis that miRNA originates from both endothelial and blood cells is being explored increasingly. For instance, miR-126 was reported to have originated from endothelial and blood cells **(**Kozomara and Griffith- Jones, 2010, Pritchard et al., 2012), while miR-150 and miR-451 were found to originate from blood cells only (Courts and Madea, 2010). Therefore, these candidate miRNAs were selected for the present study.

The present study confirmed that the relative levels of miR-126, miR-150, and miR-451 steadily and significantly increased on days 1, 6, 13, 27, 41, and 55 of incubation of the bloodstains. These results were in accordance with a previous study by Nakao et al. (2013), who summarized that the miR-16 and miR-451 levels in bloodstains prepared using either ethanol or mixture of amphetamine sulfate and methamphetamine hydrochloride, and incubated at 25°C and 50% humidity, decreased significantly from 5–28 days of incubation in the chamber.

Hansen et al. (2009) determined the age of bloodstains using miRNAs. Although miRNAs are among the most stable components in blood, they tend to degrade when bloodstains dry incompletely and in high humidity **(**Patnaik et al., 2010). Bremmer *et al.* (2012) recommended humidity levels of less than 40% for experiments on the estimation of the age of bloodstains. However, in the present study, we placed bloodstains in a chamber at 25 °C with 50% humidity. The significantly elevated of miR-126, miR-150, and miR-451 in the bloodstains can be explained by the fact that the bloodstains were dried before being placed in the chamber, which was maintained at a humidity of 50%. Wet bloodstains are more suitable for use in medico-legal investigations than dried ones. Our results indicated that the relative levels of miR-126, miR150, and miR-451 may be useful for estimating the age of bloodstains.

The miRNAs have many characteristic features which are an improvement over mRNA. Firstly, a small size of about 22 nucleotides renders mature miRNAs much more stable than mRNAs, which is of fundamental importance. In a forensic setting, the small size makes mature miRNAs less susceptible against fractionation by chemical or physical strain. This also applies to formalin-fixed paraffin embedded (FFPE) tissues where intense nucleic acid fractionation occurs, which can be of significant importance to forensic casework. Not only is the recovery of miRNA from FFPE tissues possible and capable of producing valid results **(**Andreasen et al., 2010, Leite et al., 2011, Hui et al., 2009), but it also outperforms FFPE tissue mRNA expression profiling,

by maintaining higher similarity to fresh tissue profiles, which consequently provides significantly better correlation (Liu et al., 2009). Secondly, as described above, miRNA profiling has higher discriminatory potential than mRNA profiling, which makes it possible for miRNA profiling to outperform mRNA profiling in the identification of mixed as well as heavily environmentally challenged stains.

Conclusion

On the basis of our study results, we can conclude that mRNA profiling of bloodstains is capable of accurately predicting the age of blood samples from dead and living individuals for up to 55 days. On the other hand, miRNA concentration can be estimated in both fresh and dried bloodstains for long periods, due to which it is not a promising tool for predicting the age of bloodstains up to the examined point times. Another important finding of a high forensic relevance is the considerable stability of miRNAs in aged samples and the ability of high sensitivity PCR assays to detect miRNAs from minute bloodstains, which makes miRNA profiling more sensitive than the forensic mRNA quantification methods presently in use. Further research is required for the analysis of miRNA in bloodstains aged for a prolonged duration beyond the presently studied time points.

Abbreviations

MicroRNAs (miRNAs), RNA induced silencing complex (RISC), quantitative real-time polymerase chain reaction (qPCR), standard error (SE), one-way analysis of variance (ANOVA), formalin-fixed paraffin embedded (FFPE)

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