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Short report

Gender-related difference in bloodstain RNA ratio stored under uncontrolled room conditions for 28 days

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ABSTRACT

Bloodstain age is a parameter that can be used in crime scene investigations. Bloodstain age can be determined by measuring the 18S rRNA:β-actin mRNA ratio by Reverse Transcription-quantitative PCR (RT-qPCR). Since this ratio is a function of time, it can be used as an estimator of bloodstain age. However, it is important to validate the technique in a variety of scenarios before it can be applied. We investigated 18S rRNA:β-actin mRNA ratio in bloodstains from sixteen Chinese subjects in 28 days under uncontrolled room conditions. The ratio changed in a linear fashion. It was also found that the subjects' gender affected the relationship between time and the RNA ratio.

1. Introduction

1.1. Bloodstains as evidence

Bloodstains are frequently used as evidence when investigating different types of crimes. The presence of bloodstains may be evidence of murder or injury, so the examination of stains found at the crime scene is vital. Evaluation of the distribution and appearance of the bloodstains at the scene is usually the province of specially trained and experienced forensic scientists (e.g., bloodstain pattern analysts). From bloodstains many clues can be drawn about the case. The bloodstain pattern can help to reconstruct the crime scene event; e.g., blood flow patterns from an injury can prove that a body has been moved; DNA can be analyzed from bloodstains to identify a possible suspect. Recent progress in RNA techniques may be used to deduce the possible tissue origin[1–6] and even to determine the bloodstain age.[7,8] This is important because bloodstain age can be very useful in criminal investigations to identify when the crime occurred or to include or exclude an individual as a suspect.

1.2. Bloodstain aging

As bloodstains dry and age, they rapidly lose their initial bright-red color within hours and become a dull red; within days they become brownish. Research on bloodstain aging has included chemical assays,[9] enzyme assays,[10] and many methods utilizing deteriorative changes in the visible spectrum of hemoglobin (Hb) over time.[11–14] Several new approaches have recently been introduced: High Performance Liquid Chromatography (HPLC),[15,16] Atomic Force Microscopy (AFM),[17] Electron paramagnetic Resonance Spectroscopy (EPR),[18,19] Diffuse Reflectance Spectroscopy (DRS)[20] and others. All of these methods have limited effectiveness with either weak age correlation or specific sample type or size constraints, and may be adversely influenced by environmental factors.

With the development of gene expression assays, specific mRNA degradation patterns have been proposed as a new tool for forensic investigations. It is now possible to isolate RNA of sufficient quality and quantity from dried blood, semen, and saliva stains to detect particular mRNA species using RT-PCR methods and to determine their tissue origin.[1–6] RNA techniques have considerable sensitivity, and some researchers have succeeded in identifying some mRNA markers in quite old samples of blood (13–16 years) and saliva (2–6 years).[21] Recently the collaborative exercise on RNA/DNA co-

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analysis for body fluid identification and Short Tandem Repeat (STR) profiling organized by the European DNA Profiling Group (EDNAP) supported the potential use of an mRNA-based system for the identification of blood in forensic casework that is compatible with current DNA analysis methodology.[22,23]

Anderson et al. demonstrated that the ratio between different types of RNA (18S rRNA:β-actin mRNA ratio) changed over time in a linear fashion in bloodstains under controlled conditions.[7] This method was sensitive enough to be used on stains containing as little as 1 μl blood. It was also shown that multivariate analysis of different RNA species could be used to differentiate between samples of different ages in the defined population.[8]

1.3. Confounding factors affecting RNA bloodstain aging methods

Based on the belief that mRNA is more vulnerable than rRNA, the RNA technique to deduce bloodstain age is very promising, but before it can be introduced into practice, it needs to be validated across a varied range of conditions that may apply in practice. Thus, we should find out how the technique is affected by different environmental factors (indoors/outdoors; microorganisms, temperature, humidity, and full-spectrum light, etc.). It is also important to evaluate the effects of age, gender, and ethnicity on RNA bloodstain aging. The same technique might be applied to other biological samples such as saliva and hair. Setzer et al. have shown that it is better to store bloodstains dry at room temperature in plastic bags: housekeeping and tissue-specific mRNA recoverability from blood was shown up to 547 days. When samples were outside with no rain, mRNA recoverability from blood could be shown up to 30 days. Rain had detrimental effects on the recoverability of blood, with mRNA recoverable up to 3 days.[3] Preece et al. showed that gender, age at death, and brain pH, all of them have significant effects upon mRNA levels in the post-mortem human brain.[24] When Anderson et al. examined the relative quantity of β-actin and 18S as a function of time using RT-qPCR, it was suggested that the minimum age difference that could be identified was 4 weeks. Therefore, the present study was designed to be carried under room conditions with no strict control of temperature or humidity, and with daylight and shorter time intervals (0, 7, 14, 21, and 28 days), which aimed to observe the progressive degradation of the two species of RNA.

2. Material and methods

2.1. Blood collection and sampling

Blood was drawn from 16 healthy individuals (eight males and eight females) from the Chinese Han population. Written informed consent was taken from all donors. All of the donors were aged between 20 and 30 years old. 5 ml of blood was collected by venipuncture on three separate occasions from each donor. Ten microlitres aliquots were immediately spotted onto one piece of 100% cotton fabric and dried at room temperature. A separate piece of fabric was used for each individual, but all pieces were taken from one large piece of cloth. The blood collection procedure was performed over a 1-week period. The cloth which was used to collect bloodstains was suspended on a frame near the ceiling of a room maintained at a temperature of 18–22 °C, with relative humidity maintained around 50%. The cloth was not subjected to direct sunlight, but was subject to normal room light. Since bloodstains are often retrieved from rooms in which ambient temperature and humidity vary, it was considered to be a reasonable simulation of conditions that might affect "real" forensic blood samples. Samples were processed when they reached the desired ages of 0 (4 h after blood draw), 7, 14, 21, and 28 days.

2.2. RNA extraction

TRIzol Reagent (Invitrogen, Carlsbad, CA) was used to isolate RNA from the dried blood samples at the various *ex vivo* ages. For each time point and for each blood drawn, RNA from three separate 10 μl bloodstains was isolated. For each subject, therefore, a total of 9 RNA samples were isolated for each time point. Similar-sized samples of dried bloodstains were cut from the fabric, and directly added to 1 ml of Trizol, which was then vortexed briefly and incubated at room temperature for 10 min. Two-hundred microlitres of chloroform was added to the Trizol solution, vortexed for 15 s followed by a room temperature incubation for 3 min. Samples were then centrifuged for 15 min. All centrifugations were performed at 11,000 g at 4 °C. The upper aqueous layer (approx. 500 μl containing the RNA) was transferred to a new tube, and 500 μl of isopropanol was added. The samples were inverted twice, and incubated at room temperature for 10 min. The liquid supernatant was discarded, and 1 ml of 75% ethanol was added to wash the RNA. The samples were briefly vortexed and centrifuged for 5 min. The liquid supernatant was removed and the RNA pellets were allowed to air-dry for 5 min at room temperature. To resuspend the RNA pellet, 40 μl of RNase-free water was added. A sham RNA isolation of cotton containing no bloodstain was preformed with every assay as a negative control.

2.3. Reverse transcription

TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA) was used for the reverse transcription reaction. A reverse transcription master mix was produced (final concentration: 1× TaqMan buffer A; 5.5 mM magnesium chloride; 500 μM each dATP, dCTP, dGTP, and dUTP; 2.5 μM random hexamers), aliquoted into individual PCR tubes, and stored at −20 °C until time of use. Forty microlitres of the RNA suspension, 2.0 μl of RNase inhibitor (0.8 U), and 2.5 μl of multiscribe reverse transcriptase (3.25 U) were added to each reaction. Samples were pulse-centrifuged and placed in a thermocycler Biometro T gradient (Göttingen, Germany) under the following conditions (25 °C for 10 min, 42 °C for 45 min, and 95 °C for 5 min). A no-enzyme control was run with every assay.

2.4. Real-time PCR

A real-time PCR master mix comprising β-actin control reagents (Applied Biosystems), 18S rRNA control reagents (Applied Biosystems), and TaqMan Universal PCR Master Mix was generated to be used for RNA detection. The following list represents the final concentrations of each component of the reaction: 50 nM 18S rRNA forward primer, reverse primer, and 200 nM probe (VIC dye layer); 300 nM β-actin forward primer, reverse primer, and 200 nM probe (FAM dye layer); and 1× TaqMan Universal PCR Master Mix. Real-time PCR master mix (34.75 μl), was added to real-time optical tubes (Applied Biosystems). Each cDNA sample (15.25 μl) was added to the tubes for a total volume of 50 μl. The samples were briefly centrifuged before real-time analysis. Duplicate samples were run for each RNA sample. Samples were placed in a 7300 Sequence Detection System (Applied Biosystems) and run on default conditions. Positive (control cDNA) and negative (water) controls for real-time analysis were included. The sham RNA isolations (cotton cloth lacking a bloodstain) were analyzed at this point for detection of false positives. Analysis of the data was performed using Sequence Detection Software Version 1.4 (Applied Biosystems). Results were analyzed and threshold values adjusted as described by the manufacturer. Control cDNA was used to adjust for experimental variation caused by 7300 machines. All runs were normalized to the same control cDNA before statistical analysis.