**Evaluating the use of hypoxia-sensitive markers for body fluid stain age prediction**

*Abstract:*

*To augment DNA profiling and body fluid identification techniques efforts are being made to increase the amount of information available from a crime scene stain, which includes efforts to identify externally visible characteristics through phenotypic analysis. A key question surrounding crime scene stains is the length of time between deposition of the stain and its subsequent recovery, in that is the stain recovered related to the incident in question or from a previously deposited stain number of weeks earlier? The inability to answer this fundamental question has a detrimental effect upon the successful completion of a criminal investigation. Once a body fluid leaves the body, the oxygen concentration in the environment changes; therefore, it may be that this change could cause a change in the expression of hypoxia-sensitive biomarkers. Here, a range of bloodstains, liquid saliva and liquid semen samples were collected at 0 days, 7 days, 14 days, 21 days and 28 days of degrading at room temperature (19-22oC), before undergoing total RNA extraction and cDNA synthesis. Blood was recovered from filter paper with 3mm2, with saliva and semen being left in their tubes and swabbed at the appropriate times. All samples then underwent quantitative PCR targeting Vascular Endothelial Growth Factor A (VEGFA) and Hypoxia-Inducible Factor 1 Alpha (HIF1A), with B-Actin (ACTB) as a reference gene. A range of linear and quadratic correlation values was obtained from the qPCR data and used to develop a predictive model with a mean absolute deviation (MAD) of 4.2, 2.1, and 5 days for blood, saliva, and semen respectively. Blind testing indicated that a stain age prediction model based upon VEGFA with ACTB as a reference gene could be used on samples up to four weeks old with a margin of error ranging from 2 days through to 5 days. While a sizeable potential time frame exists using this model; this represents a significant step towards the target of having an accurate stain age prediction model.*

**Introduction**

Considerable effort is being made to enhance the amount of genetic information obtained from crime scene stains, thus augmenting the standard DNA profiling processes. For example, the characterisation of mRNA, miRNA, and DNA methylation markers to enhance body fluid identification capability [1-8]. One area of interest that is proving challenging is the ability to predict the age of the body fluid stain, i.e. the time since deposition. The ability to predict the age of any biological sample would be of great benefit to the forensic investigation. It would provide a temporal linkage between the contributor of the stain and the deposition of the stain or it could be used to exclude evidence that does not correspond to the time a crime was committed. For example, was the blood deposited less than 24 hours ago when the alleged incident occurred or did it happen two months ago when the involved party had an accident and cut his finger?

A linkage between ageing of a biological sample or the post-mortem interval (PMI) and the degradation of RNA was investigated by several research groups [12-20]. Most of these studies targeted different markers and different techniques to obtain a correlation between target markers and time or age. Simard *et al.* assessed the possibility of using RNA transcription detection by duplex real-time PCR (RT-qPCR) to determine the age of body fluid stains commonly encountered in forensic biology including blood, saliva, and semen. Over six months of storage, the result showed all targets used including 18S rRNA and β-actin (ACTB), GAPDH and cyclophilin A messenger RNA markers have a similar rate of decay [14] whereas Anderson *et al.* found the ratio between 18S rRNA and β-actin linearly changed over time [13].

However, such studies have focused upon relatively stable markers such as housekeeping genes and rely upon the markers degrading. This means that such degradation may take longer than is operationally relevant, especially if the body fluid stain is dried. Thus, this study explores markers that may change upon exposure to a different environment before the commencement of the degradation process. One obvious factor in changing environment is the oxygen concentration difference from within the body and externally.

Studies have been conducted using oxygen-regulated factors such as erythropoietin (EPO), vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1 alpha (HIF1A) mRNAs to estimate post-mortem interval (PMI) [21, 22]. Recent advances in molecular biology suggest that hypoxia-inducible factor 1 (HIF1), a transcription factor that functions as a global regulator of hypoxic gene expression, plays a major role in the response of hypoxia together with other factors including erythropoietin (EPO) and vascular endothelial growth factor (VEGF) [23]. The expression of these factors involved in oxygen homeostasis increases the protein and mRNA levels in organisms or cells suffering from hypoxia or ischemia [24]. Therefore, it may be possible to predict the age of biological samples deposited in a crime scene targeting oxygen-sensitive markers, as the exposure to the change in oxygen concentration may cause an over- or under-proliferation of proteins in the short term. Thus, this study aimed to evaluate the degradation patterns of a small range of hypoxia-sensitive mRNA markers to potentially predict the age of blood, saliva, and semen samples. While previous studies have explored this issue over months; this study investigates whether such changes can be reliably characterised over 4 weeks, thus representing a shorter time frame and therefore may be more practical to the criminal investigation.

In addition, current studies have compared the degradation rate with each other, using one stable marker against one relatively unstable marker. Here, the different pairings of different markers are explored, to assess whether a stable marker versus an unstable marker or two unstable markers provides a more accurate prediction model.

Also, this study focuses upon a small number of markers, not only to demonstrate the proof-of-principle that such markers could be utilised for such purposes, but also to factor in the limited number of dye channels available in qPCR based studies. However, for such systems to be robust, additional markers may be required.

**Material and methods**

*Sample collection and RNA extraction*

All samples were collected from healthy individuals with informed consent. Forty samples for each blood and saliva, 8 individuals per each of the 5 time points (From fresh until four weeks) were collected. Whereas in semen, thirty samples were collected (6 individuals per each of the 5 time points). Saliva samples were collected by expectorating into a falcon tube and sampled using buccal swabs (Sarstedt, UK), and blood samples collected using the finger-prick method and depositing on to a sterile filter paper. The semen samples were deposited in sterile tubes as a whole sample and then using buccal swabs to collect them related at each time point. All samples were incubated within their original collection state at room temperature (19-22oC) in the dark (in a laboratory cupboard) to reduce variables such as light and temperature as much as possible for up to four weeks. At relevant times, sections of blood (3mm2 filter paper excised), saliva, and semen (both via dipping a sterile swab into the solution) were removed and immediately underwent total RNA extraction using the RNeasy Mini Kit (Qiagen, U.K.) with on-column DNA digestion as per manufacturer's instruction with minor modifications as indicated by Zubakov *et al.* [25].

*cDNA synthesis and qPCR*

Synthesis of cDNA was carried out using Multiscribe First-Strand Synthesis System for quantitative-PCR (Invitrogen, U.K.) using random hexamers as per manufacturer's instructions. Reactions without the addition of reverse transcriptase (RT-ve) were performed alongside with cDNA synthesis of each sample. cDNA products were characterised using the relative quantitation (R.Q.) method by ABI 7500 Fast Real-Time PCR Machine (Life Technologies, U.K.) using FAST Master Mix mini kit with low Rox with SYBR green (Primer Design, U.K.). The real-time PCR reactions were performed in triplicates with a total volume of 10 µl containing 2 μl of cDNA (or 2 μl of DNA/RNA free H2O (sigma) for negative controls), 5 μl SYBR, 0.5 μl of forward primer (500nM), 0.5 μl reverse primer (250nM) and 2 μl DNA/RNA free water. Following the amplification, a high-resolution melting curve analysis (HRMA) was carried out to verify the product. Unlabelled primers were used (MWG Eurofins) targeting HIF1A, VEGFA, and ACTB. Primers were chosen from RTPrimer database, and the sequences are available in the supplementary data (Supp Table 1). The data were normalised against a chosen reference gene (HIF1A, VEGFA or ACTB) and calibrated against the Day 0/Fresh samples.

Data analysis

The Livak or ΔΔCq method was used to evaluate RNA degradation as an indicator of stain age prediction [26]. Fresh samples of blood, saliva, and semen were selected as calibrator to look at how chosen markers were degraded. Delta cycle quantification (ΔCq) was also calculated (Cq Max –Cq Target), to show the abundant of selection markers. The models for age prediction were obtained based on a regression line between selected markers and actual age. The models were achieved by using single and multiple regression analysis with confidence and prediction intervals at 95%. The correlation between dependent (y) and independent (x) variables were used to generate a model. Therefore, the relation between the dependent variable (actual age) and independent variable (R.Q.) was investigated using SPSS statistical software v22. The level of significance was set for p<0.05, and the interpretation of Pearson's correlation values was done as the same as described by Mukaka *et al*. as the data satisfied the criteria for parametric distribution [27].

Blind testing set up

Aliquots of the all the samples collected were taken and provided to an independent researcher unrelated to the study, who selected 15 samples at random (ensuring that all body fluids and a representative sample of the full-time range are selected) and returned them labelled anonymously. A note was made as to the identity of the blind samples and retained by the independent researcher until after the study was completed. All selected aliquots then underwent analysis using the above protocols.

**Results**

*Verification of selected markers*

Verification of the three markers was conducted to ensure that their amplification efficiency was suitable for the study. The amplification efficiency of each target was approximately equal to that of the reference gene (ACTB), which demonstrated the validity of using the ∆∆Cq method for relative quantification [22, 28, 29]. This data is available in the supplementary material (Supp Figure 3 and 4).

The ∆Cq and the mean R.Q. values of assays were calculated, and the results showed these markers are more abundant in fresh saliva rather than fresh blood and semen. ACTB as the reference gene was the most abundant marker in all samples under investigation, as expected. All markers showed clear linearity in saliva as a function of time. Also, VEGFA and HIF1A in blood, saliva, and semen have a similar amplification value, and this is advantageous in normalising both markers. This data is available in the supplementary material (Supp Figure 5 and 6).

In all samples, two curves were tested, and the result indicated that the HIF1A/VEGFA has the highest quadratic and linear curves in saliva with an R2 value of 0.986 and 0.995, respectively. In semen, the HIF1A/VEGFA has a highest quadratic curve with an R2 value of 0.96, whereas in blood HIF1A/ACTB showed the highest quadratic curve and VEGFA/ACTB has the highest linear curve.

*Degradation profiling*

Relative quantification was performed on the samples to look at the pattern of degradation over a given period as shown in Figures 1, 2 and 3. Therefore, the relationship between the dependent (age) and independent variables (R.Q. values) was investigated. The Pearson's Correlation was calculated, and the results showed there were strong negative correlations between the assays and actual age with highly significant differences. Simple and multiple regression analyses with 95% confidence and prediction intervals were performed to assess the ability of the R.Q. values for the selected markers in predicting the age of unknown samples. Linear, quadratic and cubic curves were tested to find an optimal curve.

A simple regression analysis was conducted using the R.Q. values from hypoxia markers with actual age in blood, saliva, and semen. The VEGFA marker was normalised against ACTB (VEGFA/ACTB). It showed two different curves, cubic with blood, and saliva, and linear with semen with an R2 value of 0.70, 0.75, and 0.55 respectively, whereas HIF1A (HIF1A/ACTB) has optimal quadratic curves with an R2 value of, and 0.52 for, saliva, and semen respectively. Whereas in the blood, it has a cubic curve with an R2 value of 0.65. For HIF1A with VEGFA as the reference gene (HIF1A/VEGFA) the result showed this model has an optimal linear curve in both saliva and blood with R2 values with 0.92 and 0.70 respectively and quadratic curve in semen with an R2 value of 0.54. Three equations were obtained per marker for blood, saliva, and semen from the regression lines (Table 1) and the age predictions were calculated using these formulas (Figures 1, 2 and 3) for blood, saliva, and semen respectively.

The mean age predictions were calculated per model and plotted against actual age. Two parameters were used to evaluate which model was the best, R2 value and the standard deviation. The data demonstrate that HIF1A/VEGFA was the best model for age prediction in saliva with highest an R2 value and the lowest standard deviation, whereas, in semen was the lowest with an R2 value of 0.63. In an attempt to improve the models obtained, multiple regression analysis was also performed. Three models were tested together, and the data generated from this analysis showed that only two models have a significant difference with actual age. New equations were obtained (Table 2), and the predicted ages were calculated for each sample under investigation. New graphs were generated, and the results showed an improved R2 value especially in blood and semen (Figure 4). In the same way, the mean age predictions were calculated, and the result showed all models were very close to the actual time points, especially at 0 days.

FIGURE 1

***Figure 1. The degradation patterns of Hypoxia markers using SYBR Green in blood samples. (A)The mean delta cycle quantification (∆Cq) of assays among four weeks. (B, C) The R.Q. patterns of VEGFA and HIF1A with ACTB as the reference gene (R.G.), respectively. (D) The R.Q. patterns of HIF1A with VEGFA as the reference gene (HIF1A/VEGFA). VEGFA/ACTB (VEGFA marker normalised with ACTB) has the highest linear curve with R2 values of 0.93 (as indicated by the dotted line), whereas, HIF1A/ACTB has the highest quadratic curve with an R2 of 0.997.***  ***Columns denoted with one asterisk (\*) indicates a p < 0.05, the columns with two asterisks (\*\*) indicates p <0.01 and columns with N.S. indicates no significant difference. Error bars represent one standard deviation.***

FIGURE 2

***Figure 2. The degradation patterns of Hypoxia markers using SYBR Green in saliva samples. (A)The mean delta cycle quantification (∆Cq) of assays among four weeks. (B, C) The R.Q. patterns of VEGFA and HIF1A with ACTB as the reference gene (R.G.), respectively. (D) The R.Q. patterns of HIF1A with VEGFA as the reference gene (HIF1A/VEGFA). HIF1A/VEGFA model (HIF1A normalised with VEGFA) has the highest linear and quadratic curves with R2 values of 0.99 (as indicated by the dotted line). Columns denoted with one asterisk (\*) indicates p < 0.05, columns with two asterisks (\*\*) indicates p <0.01 and columns with N.S. indicates no significant difference. Error bars represent one standard deviation.***

FIGURE 3

***Figure 3. The degradation patterns of Hypoxia markers using SYBR Green in semen samples. (A)The mean delta cycle quantification (∆Cq) of assays among four weeks. (B, C) The R.Q. patterns of VEGFA and HIF1A with ACTB as the reference gene (R.G.) respectively. (D) The R.Q. patterns of HIF1A with VEGFA as the reference gene (HIF1A/VEGFA). HIF1A/ACTB has highest linear and quadratic curves with R2 values of 0.77*** ***and 0.99 respectively(as indicated by the dotted line). ACTB as the reference gene was the most abundant marker in all samples under investigation and also ACTB was generally stable, especially in blood and semen. Also, the result showed VEGFA and HIF1A in blood, saliva, and semen have a similar amplification value, suggesting the advantage in normalising both markers with each other. Columns denoted with one asterisk (\*) indicates p < 0.05, columns with two asterisks (\*\*) indicates p <0.01, columns with three asterisks (\*\*\*) indicates p < 0.001, and column with N.S. indicates no significant difference. Error bars represent one standard deviation.***

FIGURE 4

***Figure 4. Mean age predictions were calculated and plotted against actual age. (A, B, and C) VEGFA/ACTB model in blood, saliva, and semen, respectively. (D, E, and F) HIF1A/ACTB model in blood, saliva, and semen, respectively. (G, H, and I) HIF1A/VEGFA model in blood, saliva, and semen, respectively. Two parameters were used to evaluate which model was the best, R2 value and the standard division. The result indicated that HIF1A/VEGFA model was the best model for age prediction in saliva with the highest R2 value (as indicated by the dotted line) and the lowest standard division. Overall, all models were able to accurately predict some time points, namely samples at time 0 and 21 days.*** ***Error bars represent one standard deviation.***

*Table 1. Summary of all models obtained using simple regression analysis.*

TABLE 1

*Table 2. Summary of all models obtained using multiple regression analysis.*

TABLE 2

**Blind samples:** -

To evaluate the models obtained above a total of 15 unknown age samples of blood, saliva, and semen were tested, with a range of ages from day 0 through day 30. The result demonstrated that all prediction values were around the actual age with mean absolute deviations (MAD) listed in tables (3, 4, and 5).

*Table 3. Blind blood samples using the selected markers*

TABLE 3

*Table. 4. Blind saliva samples using the selected markers*

TABLE 4

*Table 5. Blind semen samples using the selected markers*

TABLE 5

**Discussion: -**

This study aimed to evaluate relatively unstable markers by utilising hypoxia sensitive markers, the rationale being that earlier changes in expression might be observed, rather than waiting for degradation to occur. In this study, the amplification of all markers was successfully detected over 30 days, which contradicted general expectation about RNA stability but is supported by recent studies into RNA stability. Messenger RNA has been detected on semen stains aged 33 and 56 years old [32], whereas Lindenbergh *et al.* detected mRNA in 28-years old samples [33]. Bauer *et al*. demonstrated that although RNA gradually degrades in dried bloodstains, mRNA is still suitable for reverse transcription-polymerase chain reaction(RT-qPCR) and can be extracted from blood samples stored for at least 15 years [17]. Another study demonstrated that the blood and saliva –specific mRNA markers can be amplified successfully and reliably in old samples up to 16 years old and 6 years old, respectively, suggesting their suitability for tissue identification in forensic studies [34].

Such stability means that simply looking at degradation rates of mRNA markers may be more of a long term strategy and could therefore only be used in cases where the stain is months or even years old. Thus for stain age prediction to be more operationally effective, shorter time frames were investigated targeting markers that may be expected to degrade more rapidly than those markers previously studied.

Therefore, this study aimed to explore whether hypoxia-sensitive biomarkers could be viable candidates for stain age prediction, based upon the change in oxygen concentrations in the surrounding environment (i.e. in the blood compared to atmosphere). Hypoxic conditions in blood, saliva, and semen were investigated, and the result indicates that hypoxia-responsive genes could be used as a good indicator for stain ageing because this rate showed an increase in *in-vitro* storage [35]. This was achieved by allowing blood, saliva, and semen samples to decompose at room temperature for up to a month (30 days). Once all samples were extracted, they underwent cDNA synthesis and qPCR targeting ACTB, HIF1A, and VEGFA. HIF1A and VEGFA underwent a verification study to assess amplification efficiency. A correlation value of 0.99 was achieved, indicating the validity of the chosen markers. However, it is not clear whether the changes were down to a more rapid degradation or if it is linked to changes in oxygen concentration. Given the marked linearity observed, it is more likely to be linked to the use of an unstable marker rather than specifically environmental changes. If changes in oxygen concentrations had an influence, a two-stage change in expression would be expected – with the first stage being associated with changes in oxygen concentration and the second stage being associated with degradation. Since only one stage could be observed, then this implies that degradation is responsible for the change in expression rather than oxygen concentration.

The comparative Cq method has been widely used as a relative quantification strategy for RT-qPCR method (Rao et al. 2013). This method is a convenient way of calculating relative target gene levels using the threshold cycles (Cq) of different samples directly. However, this approach is mostly dependent on an invalid assumption of 100% amplification efficiency across all samples under the investigation. Relative quantification method is widely used to quantify degraded RNA to determine PMI and age estimation of bloodstains [22, 36, 37]. In the present study, R.Q. was performed on the samples to look at the degradation pattern over a given period. Therefore, the relationship between the actual age and R.Q. values was investigated using single and multiple regression analysis.

Blood, saliva, and semen samples were taken at 0 days, 7 days, 14 days, 21 days, and 28 days and characterised, to assess their decomposition profile over a month. While no trend could be observed when considering each marker on its own, it was clear that a linear trend with a correlation value of 0.94 and 0.92 could be observed in VEGFA and HIF1A respectively when ACTB is used as a reference gene and a correlation value of 0.99 when HIF1A with VEGFA as the reference gene, thus indicating a linear decrease in marker expression over a month (Figure 3). The reference gene was selected due to its housekeeping function and high theoretical stability. However, when two hypoxia-sensitive genes were calibrated against each other, a stronger linear correlation was observed (0.99). This was unexpected, but it was hypothesised that using a decay rate ratio from two relatively unstable markers could potentially eliminate any external factors affecting the decomposition, thus smoothing out the degradation rate and making it more linear than using one unstable marker and one stable marker as previous studies have done.

Linear correlations could be observed in the data from the blood samples taken at the same intervals; however, the correlation was higher (0.93, 0.87and 0.84, respectively) and also linear correlation was observed in semen ( 0.66, 0.77, and 0.73, respectively) as seen in figures 2 and 4. It is thought that saliva shows a higher linear correlation as it is a less dynamic fluid than blood and saliva with the latter having more intracellular processes in progress and thus introducing a more extensive range of variables, thereby making any degradation processes less linear.

The data then underwent simple and multiple regression analysis, using standard confidence and prediction intervals of 95%, to develop the basis for predictive modelling. All prediction models were developed for blood, saliva, and semen, with the lowest mean absolute deviation (MAD) of 2.1, 4.2, and 5.00 days which detected with HIF1A/VEGFA model in saliva, blood, and semen respectively. Given the lower linear correlation value for the blood samples, it was expected that the MAD for blood and semen would be greater than that for saliva.

The pairing of the two hypoxia markers to use as a prediction model gave greater linearity than any other pairing, thus indicating potentially greater accuracy. This was unexpected as the intention was to use a reference gene to normalise the data. However, it was hypothesised that by using a decay rate ratio between two relatively unstable markers may well eliminate the influence of any external factors affecting the degradation – in that what affects one marker will also affect the other. Whilst this is true for the use of one stable marker vs one unstable marker, it appears to be even more effective when using two unstable markers, at least for more short term degradation profiles. However, one of these issues that this raise is that it is unknown whether the change in expression is down to hypoxia-related elements or down to a more unstable marker degrading at a faster rate.

Once the predictive models for blood, saliva, and semen were developed, blind testing was carried out. The data were analysed in three ways, targeting VEGFA and HIF1A with ACTB as the reference gene, and then targeting HIF1A with VEGFA as the reference gene. Also, multiple regression models were tested in blood, saliva, and semen.

The predicted stain age was calculated using the models developed and, compared with the actual age, deviation, R2 value, and MAD recorded. In blind blood and saliva samples, the strongest R2 value was observed with multiple regression model targeting VEGFA with ACTB as the reference gene, and HIF1A with ACTB. This was an expected result because this model has the strongest R2 for all models obtained in blood. In the blind semen sample, the VEGFA/ACTB showed the highest R2, despite a stronger linear correlation being observed with multiple regression models.

Consequently, it has been demonstrated that hypoxia-sensitive biomarkers could be used for predicting in stain age up to 1 month, at least in saliva samples and, to a lesser extent, blood and semen samples. There is a substantial margin of error in that any calculated day could be within a time frame ranging from ~2 days through to ~5 days. However, while this time frame is rather substantial, it is an improvement upon existing capability, whereby the difference between fresh samples and 1-month-old samples cannot be determined.

In addition, variables surrounding sample recovery and storage needs to be explored. For example, the blood stains were dried on to filter paper and the saliva and semen samples were retained in liquid format, thus it may be possible that degradation affected them differently. Whilst the viability of these markers for stain age prediction has been shown, there is still a considerable amount of work to carried out before this could be used in forensic casework.

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