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TECHNICAL NOTE

CRIMINALISTICS

Stacey E. Anderson,[1,†] *Ph.D.; Gerald R. Hobbs,*[2] *Ph.D.; and Clifton P. Bishop,*[1] *Ph.D.*

Multivariate Analysis for Estimating the Age of a Bloodstain*

ABSTRACT: Our objective is to provide crime laboratories with a technique for estimating the age of a bloodstain. Toward that goal, we have used multiplexed, real-time RT-PCR (or qPCR) to determine the relative stability of different-sized segments of the same RNA species as well as differences in stability between two different RNAs' change over time in bloodstains. Our results indicate that a multivariate analysis of the changing ratio of the different RNA segments can be used to differentiate between samples of different ages in the defined population. Bloodstains from 29 of 30 donors could be partitioned into different ages using this technique. Although further improvements will be required before this approach can be implemented in crime laboratories, the multivariate analysis holds promise of providing a reliable approach for temporally linking a bloodstain to the commission of a crime or excluding a bloodstain as being irrelevant to the case in question.

KEYWORDS: forensic science, bloodstain, age, RNA, real-time PCR

An accurate estimate of the age of a bloodstain can reveal the time since it was deposited, thereby providing for a temporal link between the bloodstain and the commission of a crime (or conversely, excluding a bloodstain as being irrelevant to the crime). DNA analysis can link an individual to a given scene/location, but in itself provides no information on when the sample was deposited. The majority of previous attempts to estimate the age of a bloodstain have involved changes in hemoglobin characteristics, or measures of residual enzyme activity in the specimen. In 1907, Tomellini (1) developed a chart, which illustrated that changes in the color of blood correlated with the age of the specimen. Schwarz (2) used a guaiacum-based assay to determine catalase and peroxidase activity. This assay showed that the intensity of the reaction's color was inversely related to the age of a bloodstain. More recently, Sakurai et al. (3) and Fujita et al. (4) have used electron paramagnetic resonance (EPR) spectroscopy to monitor denaturation of hemoproteins as bloodstains age. Their technique, under controlled conditions, produced a linear relationship between the age of the blood and the EPR intensity ratios of hemoprotein denaturation products that extended to 432 days with an error range of 25%. They report that their technique is sensitive to temperature, light exposure, and the material onto which the blood absorbed giving potentially inaccurate estimates when any of these variables are not accounted for (no data were presented on humidity or on person/sample variability). This technique is nondestructive but requires a minimum of 10 mg of dried blood and is not useful on other biologic samples such as saliva, hair, or fingerprints. Reverse-phase high performance liquid chromatography (HPLC) (5,6) and atomic force microscopy (7) have also been used to estimate the age of bloodstains but have not gained wide acceptance.

Ribonucleic acid (RNA) analysis may potentially become of increasing importance for forensic investigations (8). Several research groups have examined the different RNAs found in different tissue types as a means to identify tissue types (9–15). Additionally, RNA degradation rates have been used to estimate the age of biologic specimens. Three groups have reported using RNA degradation as a means to estimate the age of a biologic specimen. Inoue et al. (16) examined RNA from dead rats using northern blots and real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. They report that three RNA species isolated from four tissues (brain, heart, liver, and lung) decayed in a linear fashion from zero to 7 days postmortem. Bauer et al. (17) examined bloodstains using semi-quantitative duplex PCR and competitive PCR with an external standard. By examining β-actin amplification products, they were able to generate a timeline that extended to 60 months (n = 6–12 samples per time point assayed). They report that samples stored at 37°C yielded no usable RNA and that specimen exposure to direct sunlight for 2 months had no significant effect on the analysis. The techniques used by Bauer et al. (17) are labor intensive and have been largely replaced by real-time RT-PCR (ref. [18] and see [19–22] for reviews). In our laboratory, real-time RT-PCR was used to compare the relative degradation of β-actin mRNA to that of 18S rRNA in bloodstain samples (23). That proof of concept paper indicated that 18S rRNA was more stable than β-actin mRNA, and that the relative amounts of the two RNA species changed over time in a predictable fashion.

There are advantages in using RNA-based analysis in lieu of DNA or hemoprotein-based approaches. RNA can be co-isolated with DNA so that both a time estimate and the identity of the

[1]Department of Biology, West Virginia University, PO Box 6057, Morgantown, WV 26506-6057.
[2]Department of Statistics, West Virginia University, PO Box 6330, Morgantown, WV 26506-6057.
*Presented in part at the Fourth Annual DNA Grantees' Workshop, June 2003, in Washington, DC.
[†]Present Address: Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road (M/S 4020), Morgantown, WV 26505.

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person depositing the blood can be determined from the same sample. PCR amplification enables estimates from samples as small as 1 μL (~1 mg of "wet" blood) to be made, giving (at least) a 10-fold more sensitive assay than the EPR approach. Unlike hemoprotein-based approaches, RNA primers and probes can be made species specific, and the use of so-called housekeeping RNAs (found in all cell types) makes the PCR approach applicable whenever RNA is present. RNA is more labile than DNA, and any given RNA species is much more abundant than the DNA from which it is transcribed. The DNA encoding for a gene is usually present in only two copies per cell, while the RNA it encodes may be present in hundreds of copies (at least for housekeeping genes). The real-time RT-PCR approach we have developed is less labor intensive than the techniques employed by Bauer et al. (17). In our approach, all RNAs are isolated, converted to cDNA, and the two RNAs being compared are amplified at the same time in the same reaction tube, thus reducing chances of pipetting or other experimental errors.

The Anderson et al. (23) report indicated that 18S rRNA was more stable than *β*-actin mRNA in the same specimens. While both are considered housekeeping genes and are thus present in all cell types, expression of each one is independently regulated. Therefore, they may not always be present in a fixed ratio within cells. Such variables as genetics, health, or medical treatments could alter their relative starting ratio. To avoid this potential problem, we determined that targeting two distinct, yet differently sized regions on the same RNA strand can also be used to estimate the age of a bloodstain. We found that the larger the amplicon (the region amplified in the real-time RT-PCR assay), the more rapidly the signal disappeared. The two different-sized regions on the same RNA molecule will be present in approximately equimolar amounts when blood is first expelled (small deviations from equimolarity may occur because of targeted degradation or incomplete transcription). This relative equivalence is lost over time, and it is this change in ratio that allows for an estimate of the age of a blood sample.

This is the second in an anticipated series of papers on the development of a functional assay for estimating the age of a bloodstain. In addition to demonstrating the use of different-sized amplicons in estimating the age of a specimen, this paper indicates the value of a multivariate analysis of the data and suggests that the development of additional amplicon sets may increase both the precision of the age estimations as well as extending the time frame over which estimates are valid.

Methods

Blood Collection and Sampling

Ten milliliters of blood was collected in plain BD vacutainers by standard venipuncture from 15 men and 15 women of European ancestry on three separate occasions. Men and women of similar ages were recruited to avoid age as a potential variable. Men ranged in age from 21 to 27 (median = 24), and women ranged in age from 21 to 31 (median = 26). Thirty aliquots of blood (10 μL each) were distributed on a nonporous substrate (plastic Petri dishes) and stored at 25°C and 50% humidity in an EC22560 Environmental chamber (Lab-Line, Melrose Park, IL). For each blood draw and individual, a separate Petri dish was used. The blood collection procedure was performed over a 4-week period. Samples were removed from the chamber and processed when they reached the ages indicated on the graphs. Five *ex vivo* ages were assayed for each amplicon set. Two amplicon combinations (18S 501 bp to 171 bp and *β*-actin 169 bp to 18S 171 bp) were assayed at 0, 6, 30, 90, and 120 days *ex vivo*. The values for day zero were derived from fresh aqueous blood. Values for the *β*-actin 301 bp versus 89 bp for the 120-day-old samples were beyond the reliable range of the real-time assays. For this amplicon pair, we added day 15 for the fifth *ex vivo* age measurement. *Ex vivo* ages of 0, 6, 15, 30, and 90 days were assayed for this amplicon pair. In Fig. 3, days are presented on a log scale of 10 where 0 = 0; 6 = 0.77815125; 15 = 1.17609126; 30 = 1.95424251; 90 = 1.95424251; and 120 = 2.07918125.

Our protocols were approved by the West Virginia University Institutional Review Board for the Protection of Human Research Subjects (IRB #15833).

301bp: Forward Primer = 5' CTT CAA CAC CCC AGC CAT GT 3'

Reverse Primer = 5' CTC TTG CTC GAA GTC CAG GG 3'

TAMRA Probe (FAM)= 5' CTG TGC TAT CCC TGT ACG CCT CTG GC 3'

89bp: Forward Primer = 5' TCA TTC CAA ATA TGA GAT GCA TTG T 3'

Reverse Primer = 5' GGA CTG GGC CAT TCT CCT TAG 3'

TAMRA Probe (VIC) = 5' AAG TCC CTT GCC ATC CTA AAA GCC ACC 3'

Note: **hsBA** is a proprietary primer and probe combination from Applied Biosystems for which the primer/probe sequences are not available.

FIG. 1—*β-actin amplicons. Relative location of amplified regions and primer and probe sequences.*

RNA Isolation

RNA was isolated from 10 μL dried bloodstains using a monophase solution containing both phenol and guanidine thiocyanate (TRI Reagent BD; Molecular Research Center, Cincinnati, OH). For each time point (*ex vivo* age) and for each blood draw, RNA from three separate 10 μL bloodstains was isolated for each subject. For each subject, therefore, a total of nine RNA samples were isolated for each time point. A volume of 200 μL of water and 3 μL of a polyacryl carrier (Molecular Research Center) were added to 750 μL of TRI Reagent BD. The dried bloodstains on Petri dishes were collected with a moistened pair of tweezers and added directly to this solution, which was then mixed briefly using a vortex and incubated at 50°C for 10 min. One hundred microliters of 1-bromo-3-chloropropane (Molecular Research Center) was added to each sample and then mixed using a vortex for 15 sec followed by incubation at room temperature (23°C) for 3 min. Samples were then centrifuged for 15 min at 4°C. All centrifugations were performed at 16,099×*g* at 4°C. The upper aqueous layer (~500 μL containing the RNA) was transferred to a new 1.5- mL tube and 500 μL of cold (4°C) isopropanal was added. The samples were inverted two times, incubated at room temperature for 7 min, and they were centrifuged for 8 min. The liquid supernatant was discarded, and 1 mL of 75% ethanol was added to wash the RNA pellet. The samples were briefly mixed with a vortex 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 nuclease-free water (Fisher Scientific, Pittsburgh, PA) was added and incubated at 55°C for 10 min. A sham RNA isolation of just TRI Reagent BD containing no bloodstain was performed with every assay as a negative control.

Reverse Transcription

Applied Biosystem's Taqman Gold RT-PCR kit was used for all reverse transcription reactions mentioned in this manuscript. A reverse transcription master mix (final concentration: 1X TaqMan buffer A; 5.5 mM magnesium chloride; 500 μM each dATP, dCTP, dGTP, and dTTP; and 2.5 μM random hexamers) was made, aliquoted into individual PCR tubes, and stored at −20°C until time of use. Forty microliters of the RNA sample, 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 centrifuged briefly (1–2 sec) and placed in a Techne Touchgene Gradient thermocycler (Burlington, NJ) under the following conditions: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min.

Genomic DNA

To ensure the isolation was free of any contaminating DNA, a portion of each RNA sample was taken through the same steps as the actual assay except that the enzyme reverse transcriptase was omitted. Any signal from this control would have to be from contaminating genomic DNA. Samples contaminated with DNA were treated with DNase and re-assayed.

Primer Optimization

Primer Express software from Applied Biosystems was used to generate potential primers and probes. The specific primers and probes and their location on the targeted RNAs are shown in Figs. 1 and 2 (*β*-actin and 18S, respectively). All custom designed primers and probes were synthesized by and ordered through Applied Biosystems. Optimal primer and probe concentrations for use in real-time PCR range from 25 to 900 nM. An optimization matrix was performed on each primer/probe combination (singleplex) used in the experiments described herein to determine which primer concentrations produced maximum PCR product. Optimal concentrations were identified based on the lowest Ct value and the highest ΔRn for the less abundant/stable and the lowest ΔRn for the more abundant/stable identified based on all reactions in the matrix. The Ct value is the number of PCR cycles when the fluorescent signal is significantly above that of background noise and still in the exponential growth region of the curve. The Rn or normalized reporter value is the ratio of the fluorescence emission intensity of the reporter dye relative to that of the passive internal reference dye. The passive reference dye is present in all assays to correct for any variance in concentration or volume. The ΔRn is the magnitude of the Rn above background. This ensures that there are always sufficient PCR reagents for the amplification of both amplicon species.

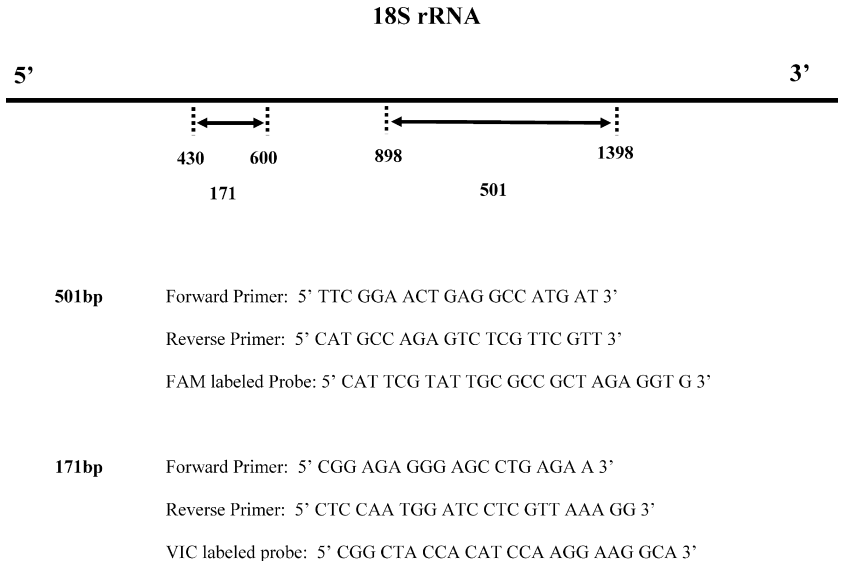

FIG. 2—*18S amplicons. Relative location of amplified regions and primer and probe sequences.*

RNA Detection

Solutions consisting of Applied Biosystems' universal PCR master mix plus the primers and probes for each pair of amplicons to be tested were prepared and used in the assays. The concentration of the probes used in all experiments was 250 nM. The forward primers (FP) and reverse primers (RP) for each combination are as follows: *β*-actin 89 bp FP = 100 nM and RP = 25 nM; *β*-actin 301 bp FP = 500 nM and RP = 500 nM; 18S 171 bp FP = 75 nM and RP = 50 nM; and 18S 501 bp FP = 900 nM and RP = 900 nM. The concentrations of the primers used in the Applied Biosystems' kit for the *β*-actin 169- bp amplicon are proprietary information. Ten microliters of cDNA (used as the template for the real-time PCR) was added to each reaction, and the total volume was brought up to 50 μL with water. Figures 1 and 2 indicate the primer sets, amplicon sizes, the relative location of each amplicon, and the dye labels used in these experiments.

Relative RNA Quantification

All samples were briefly centrifuged before real-time analysis, placed in Applied Biosystem's 7700 Sequence Detection System, and run on default conditions for 40 PCR cycles. Results were analyzed and threshold values adjusted as described by Applied Biosystems. In brief, this was achieved by increasing the threshold bar to ensure it was above any background noise (nonexponential amplification) of the real-time PCR run and at a point where there is little to no variation between duplicate assays. A set threshold was identified for both components of the reaction (*β*-actin mRNA and/or 18S rRNA) and kept constant between all assays. The baseline is identified as a defined range of cycles before amplification of the PCR product occurs. This was set to be specific for each RNA region amplified. Control cDNA was used to adjust for experimental variation caused by the 7700 machine. All runs were normalized to the same control cDNA before statistical analysis. Results were exported into Microsoft Excel for analysis.

Real-time Analysis of Dried Blood

Real-time PCR was performed on cDNA from the selected time points for all individuals using three multiplexed sets (*β*-actin 301 vs. 89 bp, 18S 501 vs. 171 bp, and 18S 171 bp vs. *β*-actin 169- bp amplicon combinations). Duplicates of each cDNA from each time point for each individual and blood draw were assayed. This totaled 18 measurements for each time point per individual that resulted in a total of 540 measurements for each *ex vivo* age of blood (three blood draws × 3 RNA samples × 2 assays × 30 individuals = 540). Assays were performed on all samples from the same individual in

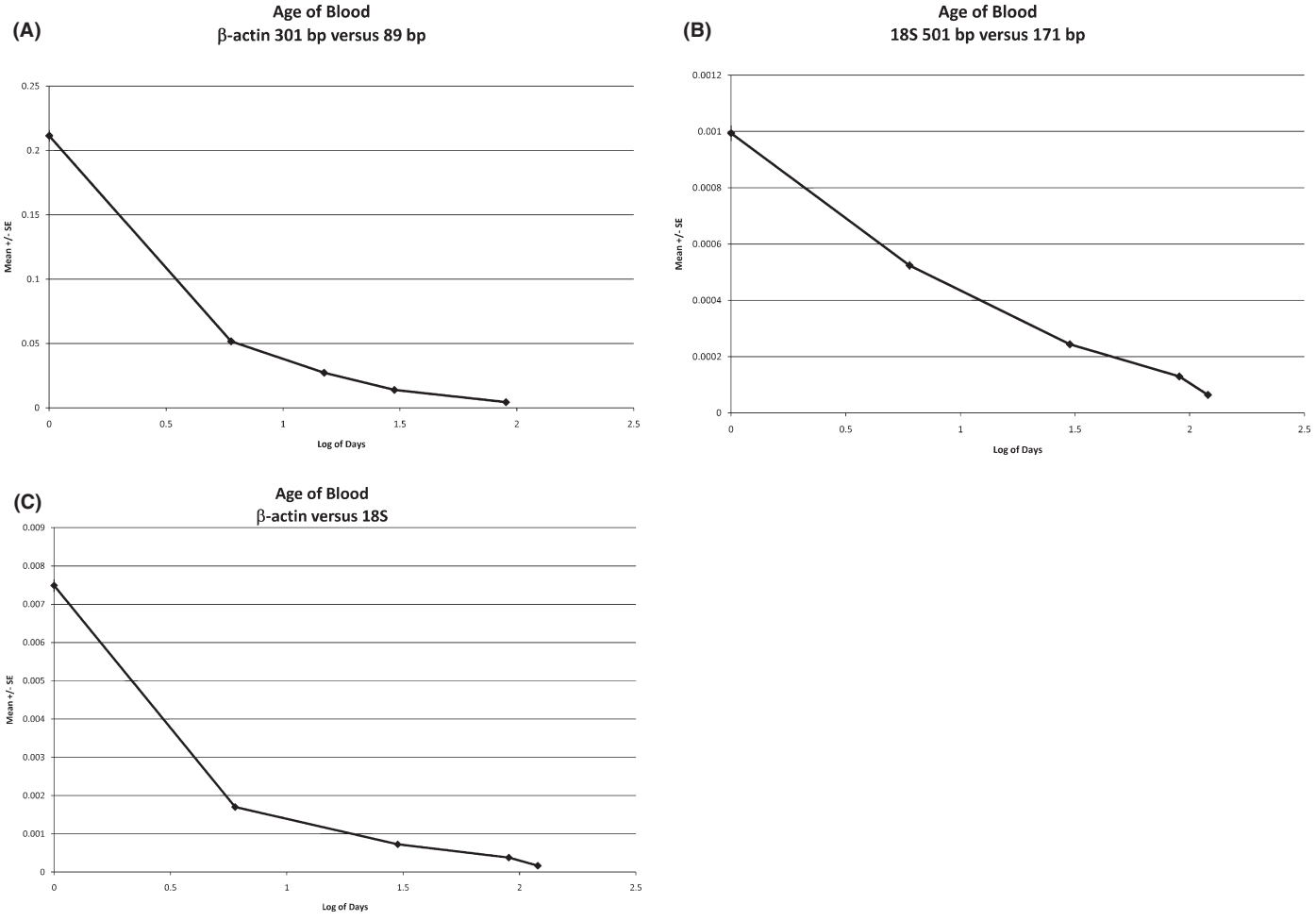

FIG. 3—*Data from real-time RT-PCR analysis for all three pairs of amplicons on* ex vivo *bloodstains. Ct values were converted to* $2^{-\Delta Ct}$ *as discussed in the Methods and the mean ± standard error calculated. The ages of the bloodstain in days were converted to log of 10 scale. (A) β-actin 301 bp versus 89 bp; (B) 18S 501 bp versus 171 bp; and (C) β-actin 169 bp versus 18S 171 bp.*

the same real-time run. Three primer/probe sets were used to analyze every cDNA sample from every individual. A control cDNA (same cDNA aliquot used in all 90 real-time runs) was assayed in each real-time reaction to control for machine, primer and probe, and master mix variability. All results were adjusted based on this value. Thresholds were adjusted for all amplicon combinations before the Ct values were exported into Excel.

Statistical Analysis

The ΔCt, the difference in Ct values between the two amplicons being assayed, was determined for each pair. The Ct values obtained for the more stable amplicon were subtracted from that of the less stable amplicon (*β*-actin 301 bp—89 bp, 18S 501 bp—171 bp, and *β*-actin 169 bp—18S 171 bp). The ΔCt was then converted into $2^{-\Delta Ct}$ for statistical analysis. This conversion is necessitated by the fact that the Ct values are measured on a logarithmic scale (24).

The results of the population study were analyzed using a general linear mixed model appropriate to the repeated measures design from which the data were collected. The factors included sex; donor, which was nested within sex: donor (sex); sample, nested within donor and sex: sample (donor [sex]); duplicate number, nested within sample: duplicate (sample [donor (sex)]); and age of blood, crossed with sex: (age*sex). All factors except age of blood and sex were taken to be random. Significance tests were based on restricted maximum likelihood methods using Kenward-Roger degree-of-freedom adjustments. Because the variation associated with samples and duplicates was small, we averaged those values when we looked at the age of blood profiles.

The results from the population study were analyzed using a nested analysis of variance (method of restricted maximum likelihood) to determine both between donor and within donor variability. This analysis also examined the significance of any experimental errors by comparing samples from the same person collected on the same blood draw date. These samples are replicates differing only in variability introduced through experimental error. For the analysis, date of blood draw was nested within donor: date (donor). *Ex vivo* age of blood was nested within date: age (date [donor]). Samples (RNA isolates) were nested within *ex vivo* age, and duplicate (real-time RT-PCR) assays were nested within samples: duplicate (samples [age (date [donor])]). All factors except sex and age of blood were taken to be random. These included intraperson (blood draw/ID), interperson (ID), experimental error (RNA isolation, conversion to cDNA, and/or machine error), and residual variability.

If all three components (amplicon pairs) of RNA degradation are viewed three-dimensionally, such as using dynamic graphics on a computer, there is a visible separation of the values by age of blood. That separation is less clear in two-dimensional projections. We used cluster analysis, specifically a hierarchical clustering algorithm known as Ward's method to identify clusters of points (RNA triples) to demonstrate that the clusters thus formed correlated with age of blood.

As an alternative to the three-dimensional graphics, an agglomerative hierarchical clustering analysis was used to generate the dendrogram presented in Fig. 4. The dendrogram is a tree diagram that illustrates how the results from bloodstain samples of the same *ex vivo* age cluster together and are separate from samples of a different age, allowing for a more precise estimate of the age of a bloodstain. The dendrogram was developed using those *ex vivo* ages that all three sets of primers and probes had in common (0, 6, 30, and 90 days).

Results

The variability between population samples, with each population consisting of bloodstain samples of the same *ex vivo* age, was analyzed with a nested analysis of variance. These results indicated that the only significant factor contributing to the differences in the population samples was the age of the bloodstain itself. The analysis of variance for the age of the bloodstain produced R^2 values of 0.95 (95%) for the *β*-actin 301 versus 89 bp, 0.86 (86%) for the 18S 501 versus 171 bp, and 0.93 (93%) for the 18S 171 bp versus *β*-actin 169 bp amplicon combinations. The remainder of the differences between populations was accounted for by interperson, intraperson, and residual effects. The residual effects are those not ascribed to the other potential sources of variability and for which the exact cause is unknown. For the *β*-actin pair of amplicons, the largest source of variability not due to differences in *ex vivo* ages was interperson variability (85% of the 5% because of non-age-related effects). Thus, interperson variance accounted for 4.25% of the total variance between the population samples (0.05 for total non-age-related effects times 0.85 for interperson effects = 0.0425 or 4.25% of the total variability). For the other two combinations of amplicons, residual effects contributed most strongly to the non-age-related variability. For the 18S pair of amplicons, residual effects accounted for 8.37% of the total variability ($0.14 \times 0.598 = 0.837$), while interperson accounted for 3.33% of the total and intraperson for 2.28%. For the 18S to *β*-actin pair of amplicons, residual effects were responsible for 4.76%, interperson for 1.36%, and intraperson effects accounted for 0.87% of the total. In an analysis of a large population, the effects of the age of a bloodstain clearly swamp the effects of other potential sources of differences. While our initial studies (23) with a smaller number of blood donors suggested that there might be gender-related differences in the rate of RNA degradation, the current study found no significant differences between the men and women. Additionally, RNA isolation, conversion to cDNA, and duplicate assays were not found to contribute to the random variability and were therefore factored out.

Results from the population studies on blood deposited on a non-absorbent surface are presented in Figs. 3 and 4. Figure 3 displays the results from each amplicon set on a separate graph indicating the mean and standard error for the $2^{-\Delta Ct}$ values obtained in these studies. Figure 4 integrates the results from each pair of primers and probes. The clustering of these values allows for clear separation of samples aged zero days from those aged for 6 days (with the exception of one individual) or older. Likewise, the 6-day-old samples could all be distinguished from older samples (again with one exception), while only some of the samples examined from the 30-day collection could be clearly separated from those of 90 days. The exceptional values noted earlier were all derived from the same individual.

Discussion

The results that we have presented indicate that differences in the size of the amplicons examined can be exploited to estimate the age of a bloodstain (Fig. 3*A,B*). Not surprisingly, the larger the amplicon, the more rapidly the signal derived from it disappears. Figure 3*C* reconfirms our earlier observation that 18S rRNA is more stable than *β*-actin mRNA (23). Thus, both the type (rRNA vs. mRNA) of the RNA examined as well as the size of the amplicon influences the rates of signal degradation.

Examining different-sized amplicons derived from the same RNA has the advantage of potentially minimizing any differences

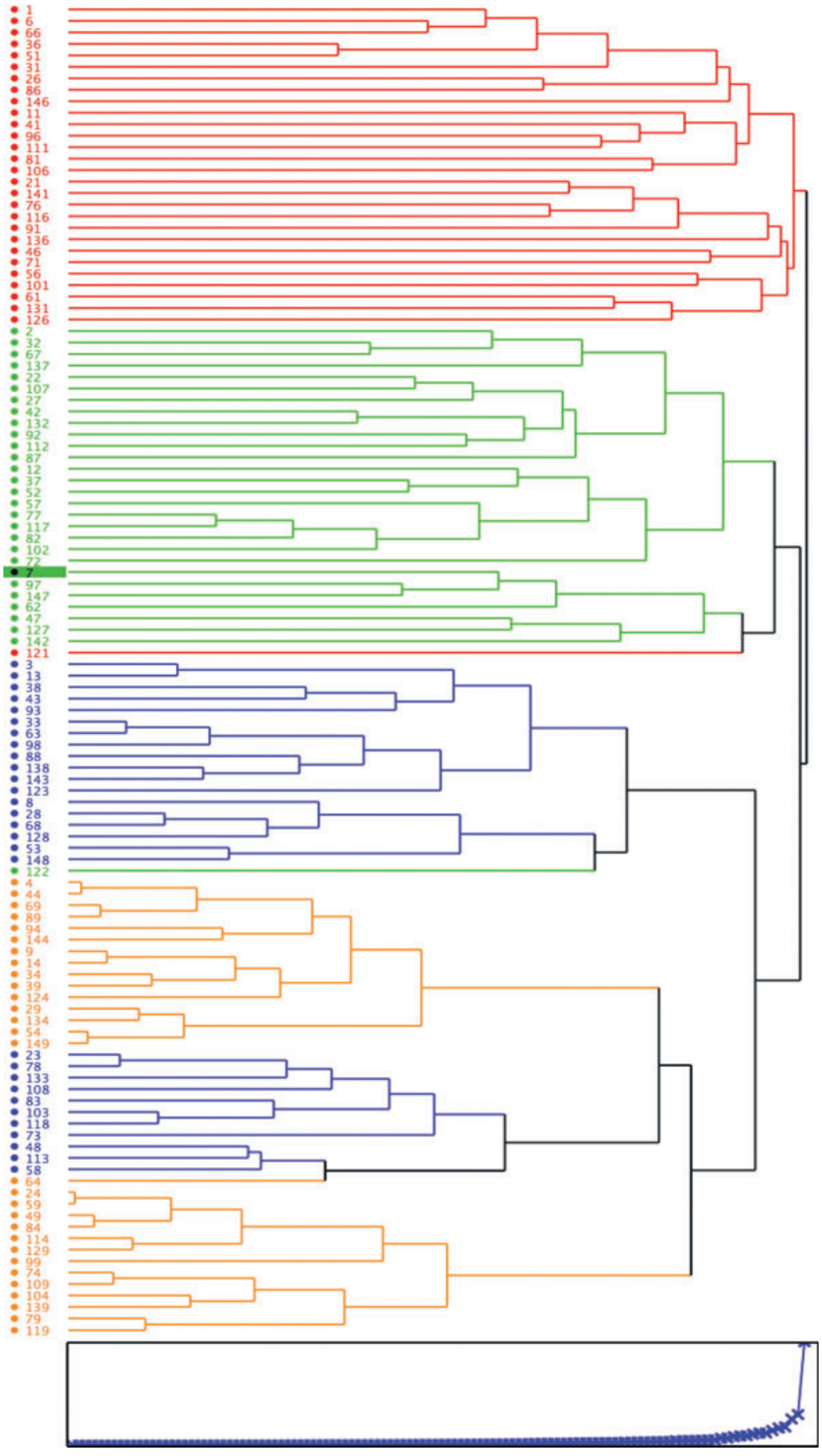

FIG. 4—*Dendrogram integrating results from all three amplicon combinations. Red lines are for bloodstains aged for zero days, green for 6 days, blue for 30 days, and orange for 90 days.*

in expression levels that might arise when comparing different RNAs, reducing both between people and within person variation. Two amplicons from the same RNA should be present in approximately equimolar amounts when the blood is first expelled from the body. The ability to examine different-sized amplicons from the same RNA coupled with differences in innate stability because of relative cellular environments provides for an almost infinite number of amplicon combinations for estimating the age of a sample. Structural RNA, such as rRNA, is more stable presumably because of the association with ribosomal proteins in the ribosome, while mRNAs are more labile presumably because of the transient association of ribosomes as they are being translated. Degradative agents would have greater access to the more exposed mRNA than the relatively protected rRNA.

The analysis of population samples for sources of variation indicated that almost all of the differences are because of the age of the bloodstains. This population analysis does not preclude differences between individuals. Such individual differences could arise because of polymorphisms in the primer- or probe-binding sites. Imprecise binding of the primers or probe could result in a delay in exponential replication of the amplicon. The impact of such a polymorphism would be dependent upon where the site is located and in which of the two amplicons it occurs. If it occurs in the more labile of the two, then the sample would appear to be older than it actually is while a polymorphism in the more stable one would result in a decreased estimate of age.

Our results also demonstrate that a multivariate analysis, incorporating results from several amplicon pairs, provides a robust way to differentiate samples of different *ex vivo* age. In 29 of 30 samples examined, fresh bloodstains could be clearly distinguished from 6-day-old samples. These 6-day-old samples can be clearly distinguished from those that are 30 or more days old. Some of the 30-day-old samples could be distinguished from 90-day-old samples, while others could not.

RNA isolated from fresh blood obtained from one donor appeared to be 6 days old in the multivariate analysis. Likewise, 6-day-old bloodstains from this same donor appeared to be 30 or more days old. These results might be obtained if the individual were polymorphic for the primer/probe of the less stable amplicon, thus reducing/delaying its signal. Because of privacy concerns, the person who donated these samples remains unknown; so verification of a polymorphism is not possible.

With the limited number of amplicons examined, RNA degradation, much like the older ABO blood testing for identity, may be useful as an exclusionary test. Excluding samples found to be deposited outside the window within which a crime has been committed will enable investigators to focus on other lines of evidence and not follow potentially false leads because of bloodstains not relevant to the case. The ability to differentiate between samples of different ages should be increased as the number and types of amplicons increases. Additional combinations would also minimize the impact of any polymorphisms. The development of additional amplicon sets, using different-sized RNA amplicons and different types of RNA (structural vs. messenger RNAs), and improvements in the optimization of existing primer/probe combinations will increase the discriminating power of the analysis and may allow for estimates of ages that go well beyond those presented in this paper. Additionally, using RNA degradation to estimate the age of a sample is not restricted to bloodstains but may be applicable to all biological tissues from which RNA can be extracted.

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References

1. Tomellini L. De l'emplol d'une table chromatique pour les teches du sang. Arch Anthropol Crim 1907;14:580–2.
2. Schwarz F. Quantitative untersuchungen der katalase und peroxydase in blutfleck. Beitrag zur Alterbestimmung von Blutspuren Dtsch. Z. Gesamie Gerichtl Med 1934;27:1–34.
3. Sakurai H, Tsuchiya K, Fujita Y, Okada K. Dating of human blood by electron spin resonance spectroscopy. Naturwissenschaften 1989;76:24–5.
4. Fujita Y, Tsuchiya K, Abe S, Takiguchi Y, Kobo S, Sakurai H. Estimation of the age of human bloodstains by electron paramagnetic resonance spectroscopy: long-term controlled experiment in the effects of environmental factors. Forensic Sci Int 2005;152:39–43.
5. Inoue H, Takabe F, Iwasa N, Seko Y. A new marker for estimation of bloodstain age by high performance liquid chromatography. Forensic Sci Int 1992;57(1):17–27.
6. Andrasko J. The estimation of age of bloodstains by HPLC analysis. J Forensic Sci 1997;42:601–7.
7. Strasser S, Zink A, Kada G, Hinterdorfer P, Peschel O, Heckl WH, et al. Age determination of blood spots in forensic medicine by force spectroscopy. Forensic Sci Int 2007;170:8–14.
8. Bauer M. RNA in forensic science. Forensic Sci Int Genet 2007;1: 69–74.
9. Bauer M, Patzelt D. Evaluation of mRNA markers for the identification of menstrual blood. J Forensic Sci 2002;47:1278–82.
10. Juusola J, Ballantyne J. Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification. Forensic Sci Int 2003;135:85–96.
11. Nussbaumer C, Gharehbaghi-Schnell E, Korschineck I. Messenger RNA profiling: a novel method for body fluid identification by real-time PCR. Forensic Sci Int 2005;157:181–6.
12. Alvarez M, Ballantyne J. The identification of newborns using messenger RNA profiling analysis. Anal Biochem 2006;357:21–34.
13. Hanson EK, Lubenow H, Ballantyne J. Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. Anal Biochem 2009;387:303–14.
14. Haas C, Klesser B, Maake C, Baer W, Kratzer A. rnRNA profiling for body fluid identification by reverse transcription endpoint PCR and real-time PCR. Forensic Sci Int Genet 2009;3:80–8.
15. Sakurada K, Ikegaya H, Fukushima H, Akutsu T, Watanabe K, Yoshino M. Evaluation of mRNA-based approach for identification of saliva and semen. Leg Med 2009;11:125–8.
16. Inoue H, Kimura A, Tuji T. Degradation profile of mRNA in a dead rat body: basic semi-quantification study. Forensic Sci Int 2002;130:127–32.
17. Bauer M, Polzin S, Patzelt D. Quantification of RNA degradation by semi-quantitative duplex RT-PCR: a possible indicator of the age of bloodstains. Forensic Sci Int 2003;138:94–103.
18. Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ, Reed MW. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. Anal Biochem 2000;285:194–204.

19. Bustin SA. Quantification of mRNA using real-time reverse transcription PCR(RTPCR): trends and problems. J Mol Endocrinol 2002;29:23–39.
20. Saunders NA. An introduction to real-time PCR. In: Logan J, Edwards K, Saunders S, editors. Real-time PCR: current technology and applications. Norfolk, UK: Caister Academic Press, 2009;1–7.
21. Logan JM, Edwards KJ. An overview of PCR platforms. In: Logan J, Edwards K, Saunders S, editors. Real-time PCR: current technology and applications. Norfolk, UK: Caister Academic Press, 2009;7–23.
22. Lee MA, Squirrell DJ, Leslie DL, Brown T. Homogeneous fluorescent chemistries for real-time PCR. In: Logan J, Edwards K, Saunders S, editors. Real-time PCR: current technology and applications. Norfolk, UK: Caister Academic Press, 2009;23–47.
23. Anderson S, Howard B, Hobbs GR, Bishop CP. A method for determining the age of a bloodstain. Forensic Sci Int 2005;148:37–45.
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 2001;25:402–8.

Additional information and reprint requests:
Clifton Bishop, Ph.D.
Associate Professor
Department of Biology
West Virginia University
PO Box 6057
Morgantown, WV 26506-6057
E-mail: cbishop@mail.wvu.edu