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Estimating postmortem interval: A molecular approach

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Abstract

Accurate estimates of postmortem interval (PMI), can be crucial to the successful investigation of a suspicious death. Knowing the PMI can help investigators eliminate suspects if they have a valid alibi for when the death occurred, allowing investigators to focus on other leads. Early PMIs can be determined by various physical and biochemical changes occurring shortly after death (ex. algor mortis). These changes are only reliable for estimating relatively short PMIs. Forensic entomologists can obtain longer PMIs by knowing the lifecycle of insects that colonize the corpse and the sequence of colonizing insects (which arrive first, which are secondary etc.). Forensic entomology requires extensive information on the local insect population, is subject to many environmental factors (temperature being one of the principle variables), and they need access to the bodies. We have set out to use a molecular approach similar to one we had previously used to estimate the age of a bloodstain. In an approach that is analogous to Carbon-14 dating, where the ratio of radioactive Carbon-14 to stable Carbon-12 changes in a predictable fashion over time, we compare the ratio of two different segments of RNA. One segment is relatively stable (either due to small size or nature of the RNA) while the other degrades more rapidly (typically, a larger segment). Our analysis relies upon quantitative polymerase chain reaction (qPCR) to determine the amount of one RNA segment relative to the amount of a second RNA segment. qPCR, like traditional PCR, has a forward and reverse primer to amplify the nucleic acid between the primers but also includes a probe that is complimentary to a sequence between the primers. The probe has a fluorescent molecule attached at one end and a quencher at the other so that as long as the probe is intact, no fluorescence will be detected. Each round of amplification causes the probe to be degraded, releasing the fluorescing molecule. We have developed three sets of primers and probes for both pigs and humans. We have buried both pig and human heads in a shallow grave with a few inches of topsoil covering the heads. Tooth pulp was selected as the source of the RNA. Tooth pulp is ideal for these studies since it is protected from many environmental factors such as humidity, is rarely if ever scavenged, and is often the only parts of a body remaining. Both relative RNA decay rates and a colorimetric analysis were used in the reported studies.

Previous studies indicated that humidity plays an insignificant role in the rate of RNA decay in tooth pulp but results presented here indicate that temperature plays a significant role. We have determined that a better estimate of PMI can be obtained when data are plotted according to Accumulated Degree Days (ADD) rather than calendar days. ADD is a measure of how much accumulated temperature a system has been exposed to and is often used in forensic entomology studies. Using the pig data, we show that an equation can be derived for estimating PMI within a 95% confidence interval. These equations, however, may need to be seasonal-specific (specific over a given temperature range). Unfortunately, due to limited samples, the human data are considered inconclusive. The results from pigs, however, strongly support the feasibility of developing predictive PMI equations for humans as well given sufficient samples. When fully developed, this means of estimating PMI can be used for samples collected anywhere in the world without specialized knowledge of insect fauna, is cost effective compared to other approaches, and may allow for estimations of extended PMIs over what is possible with forensic entomology.

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Executive Summary

Knowing the time of death (or postmortem interval, PMI) can prove critical to a successful homicide investigation. The correct time of death can help refute false alibis and eliminate suspects with legitimate alibis for the time of the homicide. A variety of chemical and physical means of estimating an early/recent death exist but those are only reliable for a relatively short period of time (hours to days). Forensic entomology, where knowledge of what carrion insects have colonized a body, their developmental stage, and the time required to reach that stage of maturity under the given environmental conditions, allows for PMI estimations of days to months depending upon environmental conditions. To be successful, however, forensic entomology requires detailed information on the local insect fauna and their life cycle under various different environmental conditions, knowledge of which is not always readily available. We have used a variation on an approach that we developed to estimate the age of bloodstains to obtain PMI estimates using molecular techniques. Our technique relies upon the relative concentrations of two different RNA segments within tooth pulp. One of the segments, either due to the size or the nature of the RNA, remains relatively stable while the other one decays more rapidly in a time and temperature dependent fashion. It is the changing ratio between the two segments that allows for an estimate of PMI. Using quantitative PCR (qPCR) we can determine the relative concentrations of two different non-overlapping sequences of the same gene in the same isolation and assay. By doing all manipulations in the same tube to both target segments, we can minimize experimental error. By looking at two non-overlapping segments of the same RNA species, we can eliminate differences in the levels of gene expression between individuals and within the same person depending upon health, medications or other variables. Since the segments are derived from the same species of RNA, at time zero they will be present in approximately equimolar amounts (RNA from blood has previously shown this to be true, unpublished).

We selected tooth pulp as our source of RNA. Tooth pulp is protected from many environmental factors by the tooth enamel, is rarely scavenged and is often the last part of a body to remain. Additionally, teeth allow for multiple samplings. We have buried both pig and human heads in shallow graves covered by a few inches of topsoil and secured with chicken wire to prevent larger scavengers from disturbing the sites. Teeth were collected, washed, frozen, cracked open to reveal the pulp, photographed, and a sample used to isolate RNA. The RNA is then converted to copyDNA and subjected to qPCR analysis. Two different primer and probe sets were used for both the pig and human studies. Each set consists of a forward and reverse primer to each segment to be examined as well as a probe complementary to sequences between the primers. The probe has a quencher on one end and a fluorescent molecule at the other. As long as the probe is intact, no signal will be emitted but when the PCR polymerase encounters the annealed probe while amplifying the segment, it degrades the probe releasing the fluorescent molecule from the quencher and allowing for generation of a signal.

Our results with the pig studies indicated that temperature plays a significant role in determining the rate of RNA degradation. Accordingly, PMI estimates are best obtained by expressing the data as Accumulated Degree Days (ADD). ADD is a measure of how much temperature a sample has been exposed to over time. Using both relative RNA amounts and a colorimetric analysis of pulp color, we could generate a predictive model that would allow us to estimate PMI within a 95% confidence interval. The equations for such estimates, however, are seasonal-specific (specific to a given temperature range). These equations provide a time window within which, with 95% confidence, the homicide occurred. Some windows were relatively narrow while others extended over weeks.

Unfortunately, the human studies were not so successful. Difficulties in obtaining suitable samples resulted in a small sample size, producing what we consider to be inconclusive data. The results with pigs, however, strongly suggest that a comparably large and thorough study with human samples will also result in models allowing for estimates of PMI within a 95% confidence interval.

Once fully developed, this molecular means of estimating PMI can be used on samples collected anywhere in the world (provided temperatures are not too extreme – little RNA decay occurs in frozen samples). Knowledge of local insect fauna and life cycles is not necessary. Where such information is available, our approach provides an independent means of estimating PMI, increasing the confidence of the estimates. Additionally, this technique has the potential to generate a PMI beyond the time frame forensic entomology can provide. In some of our studies, reliable RNA data could be collected long after the heads had become completely skeletonized. Upon complete skeletonization, carrion insects depart the carcass and little information using insects can be obtained. A moderately equipped molecular biology lab and a minimally trained technician is all that is required to do the analysis. The reagents themselves are relatively inexpensive, costing between $50-100 per assay.

Introduction

Statement of the problem: Knowledge of when an individual died can help determine the circumstances of their death. Time since death, or the postmortem interval (PMI), can be useful in the investigation of a suspicious death. Knowing when a suspicious death occurred can limit the number of potential suspects to those without a viable alibi for the time of the crime. Knowing the time of death can also help in the identification of unknown remains by restricting potential candidates to those known to have disappeared in the given timeframe.

Literature citations and review: Various means of estimating PMI have been developed but they can be broken down into three major categories: 1) last known activity of or contact with the decedent, 2) those that rely upon the natural process of decay that begins after death, and 3) those that depend upon knowledge of scavenging insects (forensic entomology). The first category includes nonscientific observations such as last sighting, last phone call, opened/unopened mail, etc. No further discussion of this method will be presented.

The currently accepted indicators of the second category include the presence/absence of the stiffening of the limbs (rigor mortis) (1) and/or the temperature of the body at the time of discovery relative ambient temperature (algor mortis). These two indicators can accurately estimate PMI for a period of 2-48 hours after death depending on ambient weather conditions (2). Additionally, visual identification of the stages of tissue decomposition due to autolysis and putrification, and biochemical changes within certain body fluids (for example changes in the chemistry of eye fluids) can be used to obtain an early PMI.

Biochemical means of estimating PMI have also been studies. Most techniques look for biomarkers, or recognizable byproducts of cell autolysis and putrification, such as oxalic acid, that change in concentration within human organs after death (3). Quantitation of the increase of potassium ions within the inner eye fluid was first established in 1963 as a potential indicator of time since death. Sturner declared that this method was an accurate indicator of PMI up to 104 hours after death ± 9.5 hours (4). Over the years, this claim has been frequently disputed, and many groups have worked to strengthen the discriminatory power of this indicator either through increased experimentation or through attempts to provide more powerful statistical analysis on previously obtained data (5, 6, 7, & 8). The biochemical process of decomposition continues to be investigated with the aim of extending the timeframe over which PMI estimates can be made. These studies have included both changes to the corpse itself (9, 10) as well as to the soil underneath the corpse (11, 12). The extended PMI estimates using biochemical changes, however, are highly susceptible to environmental factors such as temperature, humidity, microbial activity, pH, oxygen content, soil type, whether the corpse is clothed or wrapped in plastic, and the depth of the grave (see 13 for a review). Thus the use of biochemical signals for estimating PMI will require extensive knowledge of the local environmental factors.

Forensic entomology as an estimator of PMI is highly regarded within the forensic science community. This technique exploits the temperature-dependent life cycles of insected attracted to a body after death. Although there are several forensically significant insect orders, the carrion flies of order Diptera are most often used for PMI estimation due to their rapid colonization of a corpse (14). With a combination of information regarding environmental conditions and the life stage of carrion species present, calculated estimation of PMI can be made. Again, this method does have its limitations. Diptera remain with a decomposing body only until no flesh remains (15).

Postmortem decomposition is affected by environmental factors such as temperature, exposure to rainfall, humidity or lack thereof, composition of the surface on which the body is laid or buried, depth of burial, whether the corpse is clothed or wrapped in plastic, and the presence of scavengers (16). These factors must be taken into consideration making the calculation of exact time of death difficult. Scientific investigations continue to identify factors that can influence the rate of decomposition. Rodriguez and Bass (17) reported that burial underground can slow the decomposition process in comparison to those bodies left to decay upon the surface or in direct sunlight. Such issues as the sequence of exposure to freezing or high ambient temperatures are also critical to these analyses. Carrion insects are absent or less active during winter months. Compartmentalization of the corpse, such as placement indoors or enclosure in a plastic bag will also limit

the value of forensic entomology approaches. Continued research is needed to make these indicators of PMI more accurate.

Various groups have reported using RNA degradation as a means to estimate the age of a biological specimen. Most of these groups have looked at relatively short time frames. Inoue (18) 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 seven days postmortem. Bauer (19) 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 under certain conditions. They found that exposure to direct sunlight for two months had no significant affect on the analysis and that samples stored at 37^{o} C yielded no usable RNA. The techniques used by Bauer (19) are labor intensive and have been largely replaced by real-time RT-PCR. Sampaio-Silva (20) used quantitative real-time PCR (looking at normalized absolute RNA values) to examine murine visceral and muscle tissues to identify several RNA's degradation profiles significantly correlated with PMI over a relatively short time frame.

Our lab has previously used relative RNA decay to estimate the age of bloodstains (21, 22). In previous studies, we used real-time RT-PCR to compare the relative degradation of β-actin mRNA to that of 18S rRNA in bloodstain samples (21, 22). These publications demonstrated 18S rRNA was more stable than was β-actin mRNA, and that the relative amounts of the two RNA species changed over time in a predictable fashion confirming their utility for estimating the ex vivo age of biological specimens.

Statement of hypothesis or rationale for the research: The rationale behind our studies is that RNA degradation profiles can be used for an estimate of extended PMIs. In a process somewhat analogous to Carbon-14 dating where the ratio of radioactive carbon to non-radioactive carbon changes over time (due to radioactive decay of C14), the disappearance of a less stable molecule relative to the disappearance of a more stable one can be used to estimate the age of a specimen. The studies with RNA listed above generally looked at the relative degradation of one species of RNA (usually a "housekeeping" RNA) relative to another. Levels of different housekeeping genes' RNA, however, may vary somewhat from individual to individual or even within an individual depending upon health or other factors. In order to avoid this potentially confounding effect, we examined large and small non-overlapping segments of the same RNA species (for example 300 bp versus 71 bp of β-actin) under the expectation they would be present in equimolar amounts at the time of death. If degradation is a random process once enzymatic activity has ceased, the large fragment should be more susceptible to degradation than the smaller one simply due to target size. We chose tooth pulp as the ideal tissue for an estimate of extended PMI since it is as close to a closed system as is available in mammals (only the apex is exposed to the environment and then only after release from the jaw) and less subject to environmental effects. Additionally, scavengers do not consume teeth and they are thus often the only thing left of a body. Tooth pulps from both pigs and humans have been examined.

Methods

Pig and human heads were buried in a shallow grave at a secure site. Several inches of dirt covered the heads and then chicken wire was staked down over the heads to keep out larger scavengers. Two teeth from each head were extracted, washed to remove dirt and any adhering tissue, frozen and cracked open with a mortar and pestle. Pulp was removed and total RNA was isolated from the specimen using an organic isolation technique (see 23). RNA was converted into copyDNA (cDNA) using reverse transcriptase (see 23). These cDNAs remain identifiable as representing a specific RNA species (for example, β-actin RNA will generate β-actin cDNA) and accurately reflect the original quantity of the RNA in the sample. In order to measure the relative amounts of the different RNA species present, we use the technique of real-time Reverse Transcriptase Polymerase Chain Reaction (real-time RT-PCR or quantitative PCR (qPCR), see 24, 25, 26, & 27 for reviews). Just as in traditional PCR, primers are designed to amplify our target cDNA segments (creating an "amplicon"), but unlike traditional PCR, qPCR includes a means of monitoring the rate of amplification. The rate of amplification reflects the amount of target RNA species extracted from the original specimen. In order to follow the rate of amplification we use the 5' nuclease assay system. This system uses a nucleic acid probe that is complementary to a section of one strand of the amplicon. Attached to the 5' end of the probe is a fluorescent label and to the 3' end is attached a molecule that acts to quench the signal from the 5' label. As long as label and quencher are together on the same molecule, no fluorescent signal can be detected. However, as the Taq polymerase replicates the target sequences and encounters the probe, it cleaves the fluorescent label from the probe thereby allowing it to be detected and quantified. At each round of replication, more of the fluorescent label will be liberated and measured. The real-time machine records the fluorescence measured generating a curve displaying the four phases of PCR amplification: 1) initial noise, 2) exponential, 3) linear, and 4) plateau. The rate at which the exponential phase is reached is a measure of how much starting target cDNA (and hence RNA) was present. The PCR cycle at which the signal reliably exceeds the initial background noise and enters the exponential portion of the amplification curve is defined as the Ct value. It is the Ct value that is used to determine the relative amount of starting RNA. Because different fluorescent molecules will be excited and fluoresce at probe-specific wavelengths, multiple probes can be monitored in the same reaction in a process referred to as multiplexing. We can thus determine the relative amounts of two different RNA species in the same reaction at the same time. By multiplexing a stable RNA with an unstable one, we can monitor how rapidly the unstable species disappears relative to the stable one over time. The differences in Ct value for the two amplicons (ΔCt) are 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). Our previously published work indicated that ribosomal RNA (rRNA) is much more stable than a typical messenger RNA (mRNA) (21). More recently, we have shown that longer stretches of RNA degrade more rapidly than shorter stretches from the same RNA species (22). That is, a 300 base pair (bp) sequence present on the β-actin mRNA will degrade more rapidly than a non-overlapping 71 bp sequence on the same β-actin mRNA. We have shown that the larger the sequence targeted for amplification, the more rapidly it disappears with time. We can therefore use both differences in stability between RNA species (ex. rRNA versus mRNA), and differences in the size of the target amplicons (ex. 300 bp versus 100 bp), to estimate PMI.

Three sets of primers and probes were designed to assay RNAs of "housekeeping" genes (genes expressed in all cell types such as β-actin mRNA, 18S rRNA, or the mitochondrial 12S rRNA) for humans and for pigs. Each set is comprised of forward and reverse primers to two non-overlapping and different sized segments of the same RNA species and probes located between the pairs of primers. Each pair has been optimized, according to the guidelines recommended by Livak and Schmittgen (24), by varying the concentration of the different primers to ensure that both the large and small amplicons are equally amplified over a broad range of concentrations. The smaller segment will serve as the internal control, being relatively stable while degradation of the larger segment will be the variable. The ratio of the unstable to stable RNA species changes in a time-dependent manner and can be used to calculate PMI. By examining this RNA ratio, the analysis is independent of the sample size (provided enough RNA is recovered to conduct the analysis). Our previous work with bloodstains indicates that only minute amounts of RNA are required for the analysis; specimens as small as 1/10 of 1 μl of fresh blood can be successfully processed. Each member of the pair was assayed in the same reaction tube,

thus ensuring that all of the experimental manipulations occur simultaneously and equally affect both target RNAs in a given incubation eliminating potential issues such as dispensing errors/variations or differences in enzyme efficiency. The target RNAs are co-isolated and converted into cDNA in the same tube, and then concurrently assayed with their respective primers and probes, in the same tube at the same time. Reaction conditions for the multiplexed amplicons in the proposed studies will be optimized. One advantage of targeting two portions of the same RNA for analysis is that since both are part of the same macromolecule, they will be present at essentially equimolar amounts at time zero. This can help to minimize complications in the assays due to variability in expression levels between different individuals. The combination of amplicons was selected to span a broad range of PMIs. Larger amplicons are useful in estimating PMIs of shorter time but are less useful over extended timeframes. Smaller amplicons degrade more slowly and can be used to estimate longer PMIs.

These primers and probes were designed following Applied Biosystems (ABI) Express software protocols. When possible, one of the primers or the probe spanned an intron-exon boundary in the DNA. This ensures that only mature processed RNA will be detected, minimizing any effects of DNA contamination of our RNA isolations. We verified that our primers and probes do not detect DNA by BLAST searches of the genome, and experimentally by testing against purified DNA (without converting RNA to cDNA). In some instances, designing primers and probes to span intron-exon boundaries was not feasible. In order to minimize the consequences of DNA contamination of target RNAs in which intron-exon boundaries do not exist or are impractical to use, we have developed protocols that effectively eliminate DNA from our RNA preparations. We have found that standard RNA isolation protocols that work well for producing RNA from blood, semen, the roots of hair, and other biological sources, resulted in a significant level of DNA contamination when the starting material was dental pulp. We found that standard treatment of samples with DNase can significantly reduce the RNA signal in qPCR assays. We have developed protocols for RNA isolation from dental pulp that effectively eliminates DNA contamination. This allows us to examine genes lacking intron-exon boundaries (such as 18S rRNA) and other potential RNAs of value to these studies. These rRNAs are particularly important since they are present at very high levels in all cell types and can be detected in samples that are more than 1.5 years old (stored at 22^{o}C, unpublished results), potentially greatly extending the timeframe over which estimates of PMI can be made.

We also photographed each tooth's dental pulp and subject it to a color analysis. Our observations on dental pulp verified those made by Duffy et al (25) that the pulp morphology changes over time. The morphology can be classified into three stages: red & rubbery; pinkish & semi-liquid; and grey & dried. Simple, visual observation of these three stages of tissue putrification can be used to form an initial assessment of the age of the sample. A more accurate and objective means of subdividing the specimens is provided by color analysis of the dental pulp. Using Adobe Photoshop we determined the amount of red, green, and blue pigments were present in three randomly selected regions of the pulp. These values, in conjunction with the RNA decay data, can help make an estimate of PMI.

Primers and probes:

See Anderson, Hobbs, and Bishop (22) for relative location of fragments on the RNAs. FB = forward primer, RP = reverse primer, and VIC or FAM refers to the labels on the probes.

PIGS

18S5 / 18S300

18s5

FP: GAATTGACGGAAGGGCACC

RP: AGGTTTCCCGTGTTGAGTCAAATTA

VIC: CCAGGAGTGGAGCCTGCGGC

18s300

FP: GGCGGCTTTGGTGACTCTAG

RP: GGATTTAAAGTGGACTCATTCCAATT

FAM: CGATGGTAGTCGCCGTGCCTACCA

PBA71 / PBA300

PBA71

FP: TCGCCGCCGGTCTACA

RP: GCCGTTGTCGACCACGAG

VIC: CGCCATGGATGACGATATTGCTGC

PBA300

FP: ACCGACTACCTCATGAAGATCCTG

RP: GCACTTCATGATGGAGTTGAAGG

FAM: CTGCCCGACGGCCAGGTCATC

HUMAN

18S5 / 18S300

18s5

FP: GAATTGACGGAAGGGCACC

RP: AGGTTTCCCGTGTTGAGTCAAATTA

VIC: CCAGGAGTGGAGCCTGCGGC

18s300

FP: GGCGGCTTTGGTGACTCTAG

RP: GGATTTAAAGTGGACTCATTCCAATT

FAM: CGATGGTAGTCGCCGTGCCTACCA

18SA / 18SB

18sA

FP: TTCGGAACTGAGGCCATGAT

RP: CATGCCAGAGTCTCGTTCGTT

FAM: CATTCGTATTGCGCCGCTAGAGGTG

18sB

FP: CGGAGAGGGAGCCTGAGAA

RP: CTCCAATGGATCCTCGTTAAAGG

VIC: CGGCTACCACATCCAAGGAAGGCA

Temperature:

Preliminary studies revealed that temperature plays a critical role in determining the rate of RNA decay. Temperature highs and lows were obtained either through the use of a thermometer placed in the shallow or grave or from a nearby weather station. Forensic entomologists routinely deal with temperature effects by utilizing accumulated degree days (ADD) or accumulated degree hours (ADH) in their estimates of PMI. For entomological applications, ADD are the measure of the total amount of temperature (between upper and lower viability thresholds) to which a developing insect has been exposed to reach a certain point in their life cycle. Between the threshold values, a linear relationship exists between temperature and insect developmental rate. For example, two days at 16^{o} C would be the equivalent of one day at 32^{o} C to reach the same developmental stage of the insect. In the case of RNA degradation, accumulated degree days were a sum of the total amount of temperature that a body/head has been exposed to which correlates to a particular amount of specific RNA degradation. In order to characterize the effects of temperature on the degradation of RNA within dental pulp, we applied an approach similar to that suggested by Megyesi et al. (31) for determining the relevance of ADD in non-insect model systems. In that report, levels of decomposition of human remains were assigned a numerical score, and the amount of temperature to which each body was exposed was determined. Incorporating temperature data and treating decomposition as a semi-continuous variable allowed them to calculate the impact of temperature on decomposition rates. This improved estimates of PMI when decomposition was modeled as being dependent on both time and accumulated temperature (31). In our experiments, we calculated a regression equation to account for any variation in the $2^{-\Delta Ct}$ calculations due to temperature. This allowed us to approximate ADD and provide a more precise estimate of PMI. In our studies we have observed that ambient temperature highly correlates with that in the shallow graves. Crime scene investigators can obtain the previous temperature profiles to which a specimen has been subjected from local weather stations or other sources of such information to aid in the estimation of PMI. We will, however, record both the ambient and grave temperatures in our studies.

ADD Statistical Analysis:

To determine whether the variables of RNA degradation ($2^{-\Delta Ct}$) and color (red, green, and blue) are more predictive of time in days or time in the form of ADD, the statistical analysis of multiple regression was performed. All statistical analysis was performed using JMP version 8. Factors in the model included: season; $2^{-\Delta Ct}$; red color; green color; blue color; pig nested within season: pig (season); tooth nested within pig: tooth (pig (season)); season crossed with $2^{-\Delta Ct}$: (season*$2^{-\Delta Ct}$); season crossed with red color: (season*red color); season crossed with green color: (season*green color); and season crossed with blue color: (season*blue). All factors were deemed fixed, except the nested factors of pig and tooth, which were designated as random. This model was examined twice, once with the experimental variable being the PMI of samples in days and again with the experimental variable being ADD. The resulting multiple R^2 values were used to determine the variable best predicted by the model.

To formulate an equation into which known variables can be placed to determine the PMI of a particular sample, again multiple regression was utilized. Factors in the model include predictive x-variables $2^{-\Delta Ct}$, red, green, and blue, as well as the y-variable to be predicted: ADD. To better infer an estimate of PMI from collected data, two regressions were analyzed, one containing data from the summer study alone and one from winter data. Analysis of variance (ANOVA) statistics were used to determine the statistical significance of each model. The regression supplies a prediction estimate onto which a 95% confidence interval was also placed.

Results

A total of 13 human heads and 34 pig heads were used in the study. Results from pigs will be presented first and then the human data will be provided.

Pig Data

Calendar days or as Accumulated Degree Days

To statistically determine if RNA degradation and color analysis better describe time in days or ADD, a multiple regression analysis was performed on data from a summer study and a winter study. Figure 1 shows the data for both studies presented according to calendar days while Figure 2 shows it in ADD. The resulting multiple R^2 values of the model were higher when ADD was used as the variable to be predicted (R^2=0.7759), than when time in days were predicted (R^2=0.5066). The higher R^2 designates which of the two variables were better explained by the known numerical values of RNA decay and color.

Predicting PMI

The predictive equation that we formulated to determine the age of an unknown sample is the result of multiple regression. Like linear regression, but with more than two variables, the result of this statistical analysis is the equation of a regression line that takes into account all experimental variables. In out studies, we knew the value for $2^{-\Delta Ct}$ (differences in RNAs), Red, Green, Blue, and age of the sample. We used these data to produce a regression line (and therefore an equation) that would be predictive of sample age. The confidence interval for this prediction is merely a 95% confidence interval from the regression line for the prediction of sample age. Two equations were generated, one for summer and one for winter. A 95% confidence interval was fitted to each equation. Figure 3 shows the JMP output for each model, **(A)** summer and **(B)** winter, along with corresponding ANOVA tables. In the summer months, the following equation was produced to predict the ADD that a sample must have been subjected to in order to reach the observed level of RNA degradation and coloration:

$$ADD = (1395.729 + (-1375.106*2^{-\Delta Ct}) + (-9.033*Red) + (8.094*Green) + (-1.747*Blue)) \pm 456.238$$

When a body is found in the winter months, ADD can be calculated for a particular PMI through the use of the following equation:

$$ADD = (-131.455 + (-49.563*2^{-\Delta Ct}) + (-0.346*Red) + (1.788*Green) + (0.089*Blue)) \pm 158.453$$

To determine PMI from the calculated value of ADD, past temperature history of the site in which the body was found is needed. Starting with the day the body is found, add daily average temperatures from previous day until the calculated ADD of the lower 95% confidence interval is reached. This provides lower limit of the PMI estimation range. The process is repeated for the calculated ADD of the upper 95% confidence limit. This value will give the upper limit of the estimated range of PMI. Tables 1 and 2 hold estimates of PMI calculated using the provided equations for summer and winter respectfully.

Table 1: Estimates of ADD and PMI for Randomly Selected Samples Obtained During Summer Analyses

Subject	Actual ADD	Predicted ADD	Actual Date of Death	Predicted Date of Death Lower 95%	Predicted Date of Death Upper 95%
1	0	-216.87	3/25/2008	2/2/2008	3/25/2008
2	167.22	-82.89	3/25/2008	2/4/2008	4/8/2008
3	803.06	520.41	3/25/2008	2/9/2008	5/15/2008
4	803.06	516.33	3/25/2008	2/20/2008	5/16/2008
5	1041.67	672.36	3/25/2008	3/10/2008	5/23/2008

Table 2: Estimates of ADD and PMI for Randomly Selected Samples Obtained During Winter Analyses

Subject	Actual ADD	Predicted ADD	Actual Date of Death	Predicted Date of Death Lower 95%	Predicted Date of Death Upper 95%
1	5.0	-91.72	11/18/2008	11/2/2008	11/25/2008
2	12.5	36.08	11/18/2008	10/30/2008	12/2/2008
3	12.5	-93.94	11/18/2008	11/5/2008	12/18/2008
4	115.83	138.74	11/18/2008	10/30/2008	12/20/2008
5	147.22	217.49	11/18/2008	10/7/2008	12/27/2008

The large values used to place a 95% confidence interval around estimates do at times make for rather large approximations of PMI. This of course depends on the magnitude of daily temperatures recorded for the days prior to the discovery of a body.

Combining data points based upon ADD values

Figure 4A. displays the data obtained using 18S 5/300. Figure 4B. illustrates the results with PBA171/300. The PBA171/500 amplicon combination did not yield any useful data due to the early degradation of the 500 bp β-actin

fragment. In Figure 4A and B samples with ADD values within 100 were combined and the mean with standard error were graphed.

Human Data

Graphs of the results of the human study are presented in Figure 5. All involve the 18S rRNA; the β-actin sets proved to be non-informative.

We would be remiss as scientists if we did not elaborate more on the human data. We suspect the data we generated is more a reflection of the age of our donors rather than the technical approach. Our samples were all obtained from individuals donating their bodies to medical research. As such, all but one of our samples was over 55 years old, with the majority being much older. Our pigs are much younger, more the equivalent of teenage or young adult humans. We did obtain samples from an 18 and 90-year old male humans on the same day. The morphology of the two tooth pulps was dramatically different. The youth's pulp was a bright red/pink much like our pig samples while the older person's was nearly completely white. The collection and assays from these two individuals continues.

Conclusions

Discussion of Findings

The results of the pig studies show that measureable changes in postmortem tooth pulp can be used to estimate PMI. Temperature has been shown to be an important variable in using tooth pulp for estimating PMI. The accumulated time since death plus the aggregate of the temperature to which the sample has been exposed, in the form of Accumulated Degree Days (ADD) provides the most accurate estimate of PMI. We have shown that using individual amplicon pairs, a window of time within which death occurred can be generated with a 95% confidence. Additional studies and additional statistical analysis will be required before this means of estimating PMI is implemented in crime scene investigations. The results presented in this report clearly demonstrate the potential for developing this approach to augment existing means of estimating PMI. We will continue to collect and analyze data for the duration of the award and beyond, resources permitting. For a discussion of the anomalous values obtained ~day 20 in Fig. 1, see reference 23.

Pig Studies

When tracking RNA decay over time, two differently sized portions of the same β-actin mRNA were statistically analyzed from two seasons: summer and winter. Although samples were collected from pigs in the summer study for 140 days, the larger portion (300 bp) of RNA could no longer be amplified by Real-Time PCR after day 84 in the summer, making calculation of $2^{-\Delta Ct}$ impossible after this point in time. Winter studies were completed on day 126 after all teeth had been sampled. The vast differences in RNA degradation between seasons, based on time in days, indicated that temperature plays a major role in the degradation of RNA in postmortem pulp. Additional data sets/graphs are presented in Figure 3 A and B but have not yet been included in the statistical analysis.

When the same RNA degradation measurement, $2^{-\Delta Ct}$, is plotted by ADD, in place of time in days, it can be seen that although the winter study occurred over a longer period of absolute time, it did not contain the range of ADD covered in the summer study. In fact, at approximately 400 degree days, both studies had reached the same the level of RNA degradation. These results, along with results of multiple regression analysis, gave evidence that PMI estimations made using our technique should utilize time in the form of ADD, not time in the form of days.

The models into which the preceding data were fit allowed for the creation of predictive equations to derive ADD. Upon further analysis of the ANOVA accompanying each model, it can be seen that numerical values of color are significant indicators, as well as RNA degradation, to the prediction of ADD in summer months. This is intuitive as all four measurable variables ($2^{-\Delta Ct}$, red color, green color, and blue color), vary throughout the tooth pulp degradation process. In the winter study, the only variable of significance is green color. As very little measurable changes occur in pulp at relatively low temperatures, it is understandable that RNA degradation was not considered to be significant. Through visual analysis alone, the red color of pulp at early ages did not seem to differ greatly from pulp of older ages as well.

Upon the discovery of a corpse within a shallow grave, PMI can be estimated using the described method by measuring two factors: RNA decay and the quantification of color from postmortem tooth pulp. Results from qRT-PCR would be in the form of $2^{-\Delta Ct}$. Color values can be obtained from a digital photograph of the same tooth sampled from the body for RNA analysis. These values would then be inserted into one of two equations based on the time of year the body was unearthed. The resulting values are the amount of time in temperature needed to reach this sample's specific stage of decomposition. Starting with the calendar date of the body's discovery, adding all positive daily average temperatures of the past days, until the value determined by the equation is met, will provide the estimate of PMI. By determining the upper and lower confidence limits provided by the equation, the estimate provided will be accurate 95% of the time it is

utilized. Some estimates of PMI using this method will be more precise than others. Higher temperatures of the days prior to the estimates make for smaller values of ADD needed to satisfy confidence intervals.

For these studies performed at both high and low environmental temperatures, it has been determined that temperature plays a major role in either progressing or retarding the stages of RNA decomposition and the morphology of tooth pulp. These observed changes are more related to temperature in terms of ADD, than to absolute time in days. With a better understanding of the stages of decomposition of RNA and coloration that occur in postmortem tooth pulp, an assay to estimate the accumulated temperature to which samples had been exposed in the form of ADD has been illustrated. By estimating ADD, this can be used to determine the PMI of a certain individual. Although information can be gained through the use of this analysis in temperate regions, where estimates can be made for longer periods of time than present estimators, its best use at this time may be in locations in which warm ambient temperatures dominate the climate. Our results suggest that seasonal-specific equations may be the most accurate means of estimating PMI with a 95% confidence.

Additional studies

Figure 4A presents data from a 66 bp segment versus a 301 bp non-overlapping segment of 18S. The R^2 value of 0.34 indicates that this amplicon pair may provide useful information in predicting PMI. The R^2 value in Figure 4B of 0.4 suggests the 71 bp versus 300 bp segment of β-actin may be more valuable than that provided by the 18S pair in Figure 4A.

Human Studies

Figure 5 A and B present data for a 66 bp versus a 301 bp non-overlapping segment of 18S (5A) and a 171 bp versus a 501 bp non-overlapping segment of 18S. Although R^2 values were generated, the usefulness of these values is questionable due to the small sample size. The curve in Figures 5A and B show a trend opposite that seen in the pig studies. The larger amplicons in those pairs appear to be the more stable one. This may be due to residual enzyme activity preferentially digesting one end of the 18S molecule or to differences in accessibility of the 18S rRNA within the ribosome. Residual RNase activity may be present if the samples were still partially hydrated.

Unfortunately, the human studies were problematic from the start. Due to relatively recent mishaps in WVU's human donor system, causing unwanted public relation issues, no human samples were provided for the initial ~half of the award period. When a system was worked out to provide greater access to such samples, the number of samples was still too small to allow for a meaningful statistical analysis. The samples we received were from person's donating their bodies to science that meant that the samples were exclusively from elderly people (with one exception), many of whom had few if any natural teeth remaining. We rejected those samples with less than 8 teeth. At this point, we can only conclude that the human studies were unsuccessful. With the remaining funds and any funds that we can obtain through local (i.e., WVU) or other means, however, we will continue to expand the human data samples. Since the draft report was submitted, we obtained a sample from an 18-year old male. The youth's pulp was a bright red/pink similar to that of our pigs while the older peoples' pulp was generally gray-whitish from the start. We suspect that either new primer/probes need to be used on older individuals or that pulp from older individuals may not be a good indicator of PMI. Bone marrow might be a better source for such studies.

Implications for Policy and Practice

Once sufficient studies have been completed, estimating PMI using RNA decay and colorimetric changes in tooth pulp will allow for an extended estimate of PMI, going beyond that provided by forensic entomology. These tooth pulp analyses are more cost effective than hiring a forensic entomologist. Additionally, PMI estimates can be made that are

independent of knowledge of local insect fauna meaning that PMIs can be obtained from samples collected anywhere in the world. Finally, when other means of estimating PMI are available (time frames over which physical/chemical changes and/or forensic entomology can be utilized) this approach provides an independent means of validating estimates made by other approaches.

Implications for Further Research

Additional work, using multiple amplicon primers and probes and over more variable environmental conditions needs to be undertaken before this approach can be implemented in the crime lab. The human data sets especially need significantly more samples before this approach can be used to reliably estimate PMI. We believe that this approach could be reliably used to estimate PMI in young to early middle age humans (that needs to be verified with more samples) but our results suggest that either new primer/probe combinations be used with older individuals or that bone marrow be used as the source of RNA instead of tooth pulp. Like tooth pulp, bone marrow provides a relatively isolated environment and may be amenable to such studies. While not ideal, these approaches provide the only means of estimating PMI once insects have left the bodies. In instances where forensic entomology cannot be used (insects have departed or not enough is known about local insects' life cycles), this approach may be the only alternative.

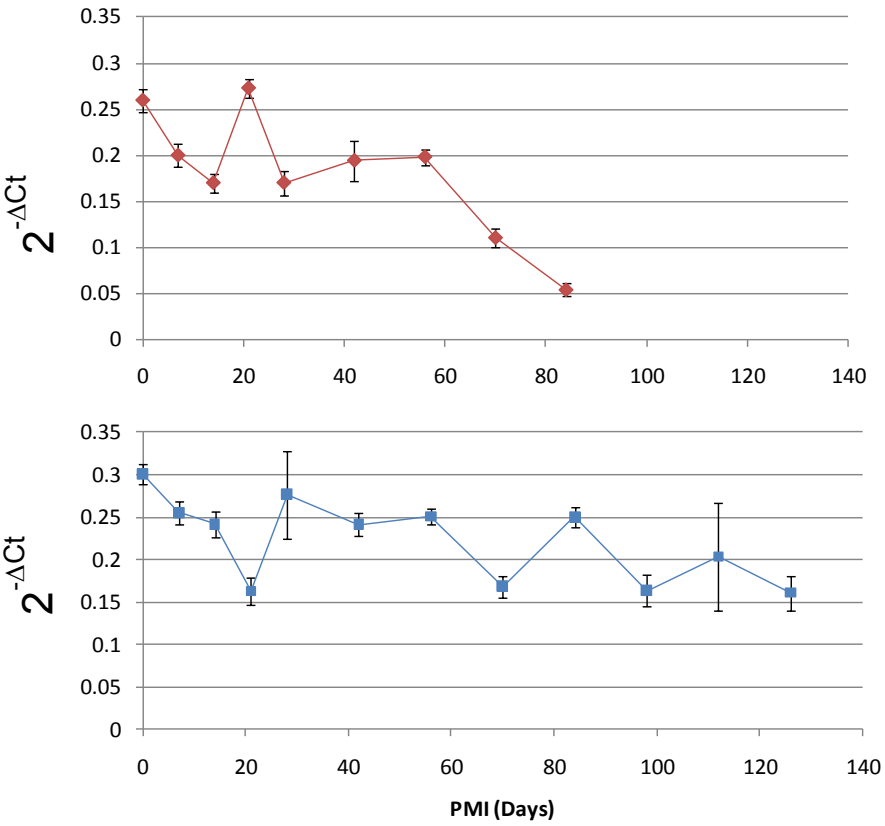


Figure 1. Relative concentration of non-overlapping β-actin 71 bp versus 301 bp calculated according to calendar days. Summer (192 teeth, upper graph) versus winter (288 teeth, lower graph). Error bars indicate standard error.

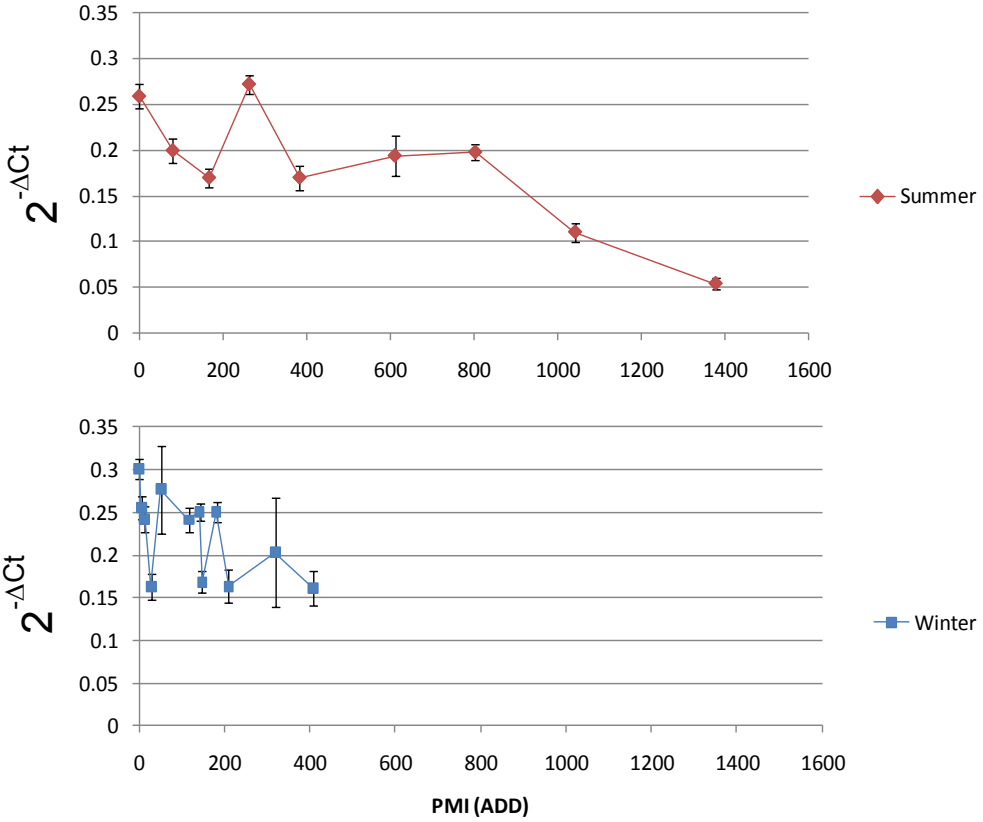
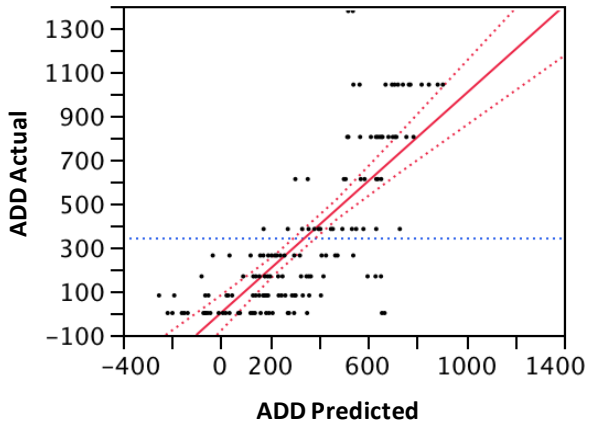
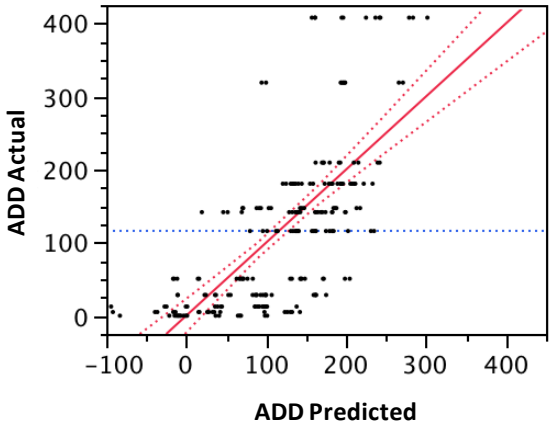


Figure 2. Relative concentration of non-overlapping β-actin 71 bp versus 301 bp calculated according to Accumulated Degree Days. Summer (192 teeth, upper graph) versus winter (288 teeth, lower graph). Error bars indicate standard error.

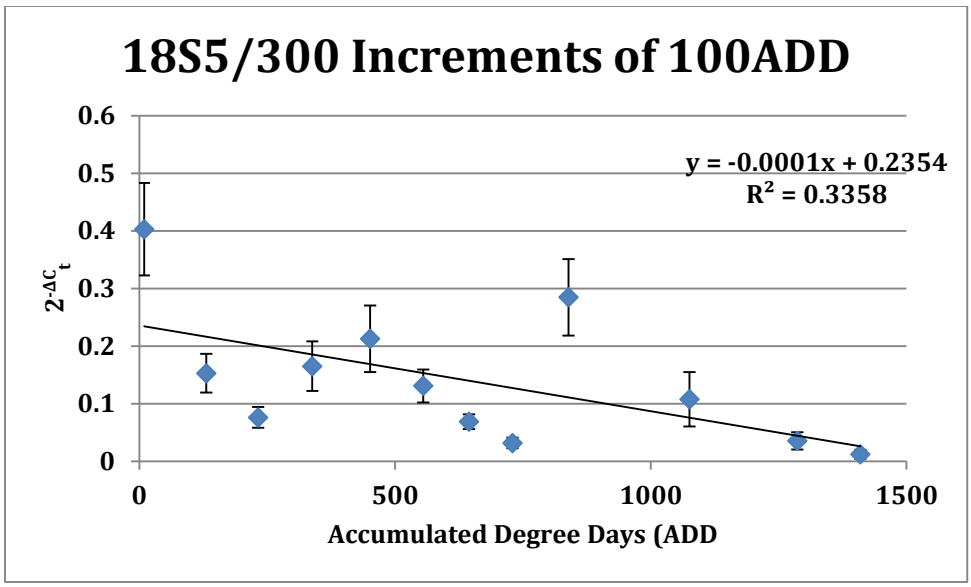




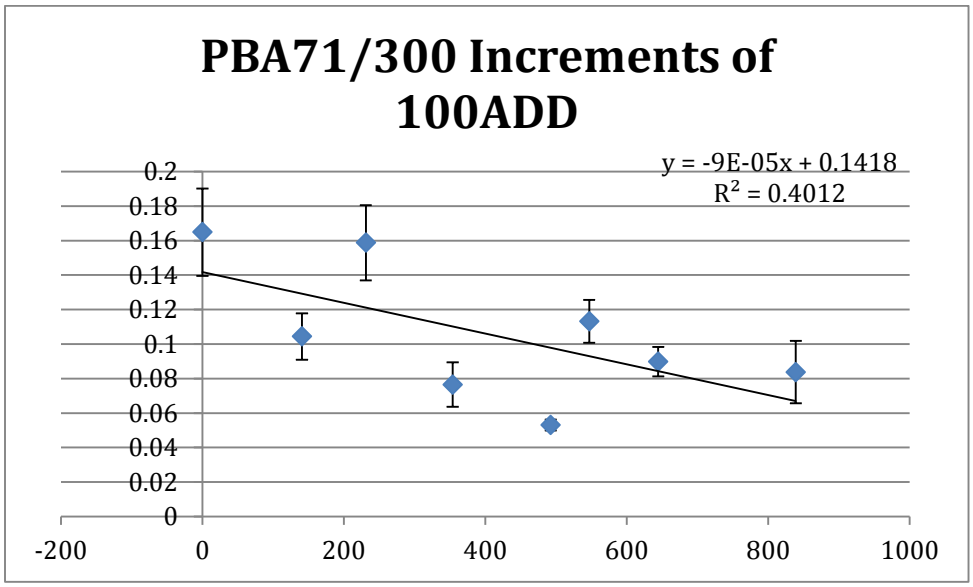
Source of Variation	df	Sum of Squares	F	p
$2^{-\Delta Ct}$	1	1,676,970.5	31.9653	<0.0001
Red Color	1	2,569,049.5	48.9695	<0.0001
Green Color	1	3,919,053	74.7024	<0.0001
Blue Color	1	266,457.6	5.0790	0.0256
Error	153	8,026,718		

Source of Variation	df	Sum of Squares	F	p
$2^{-\Delta Ct}$	1	12179.2	1.9035	0.1688
Red Color	1	15222.6	2.3791	0.1241
Green Color	1	1067268	166.8001	<0.0001
Blue Color	1	3000.1	0.4689	0.4941
Error	275	1759583.1		

Figure 3. JMP Multiple Regression Analysis and Corresponding ANOVA Table for Models Predicting ADD. To construct predictive equations to estimate ADD from the known variables of RNA degradation and color, multiple regression analysis was performed in JMP. Results for each model, (A) summer and (B) winter, are shown. The horizontal blue line indicates the mean of actual ADD measurements for all samples within the model. The solid red line in each graph represents the modeled space where predicted ADD and actual ADD are equal. Dashed red lines indicate confidence limits. Below each graphical representation are the ANOVA results for each factor within the model. Factors were considered to significantly affect the prediction of ADD if p-values of less than 0.05 were reported.

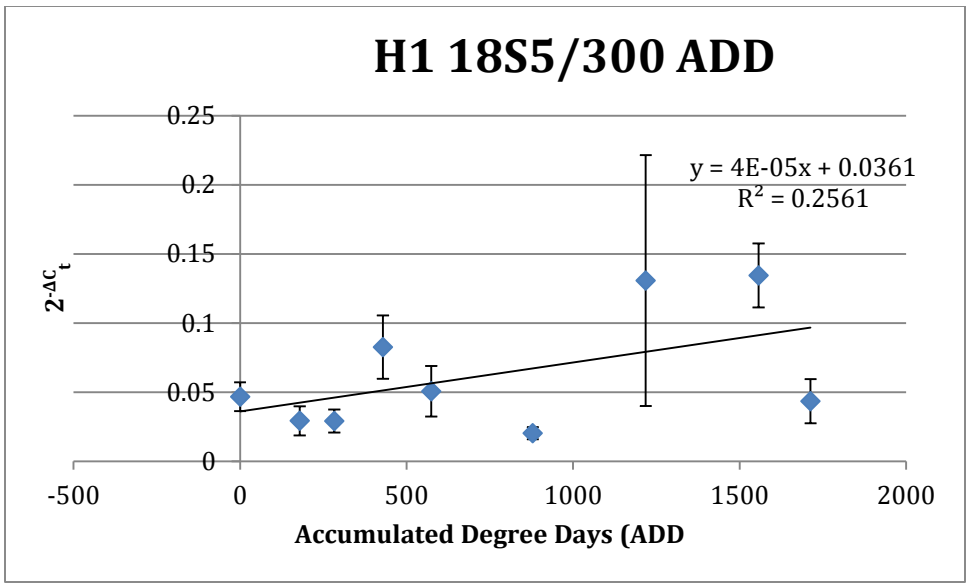


A,

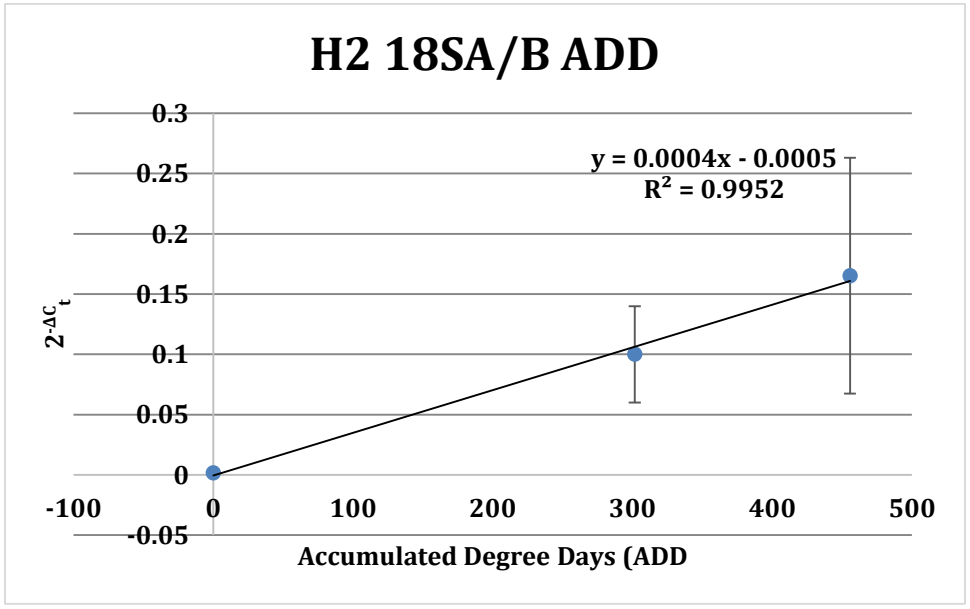


B.

Figure 4. Pig data for two amplicon pairs. n = 216 teeth. A. The relative concentration of a 66 bp 18S fragment is compared to a non-overlapping 301 bp 18S fragment. B. The relative concentration of a 71 bp β-actin fragment is compared to a non-overlapping 300 bp β-actin fragment. Values within 100 ADD were averaged and the mean plus standard error are presented.



A.



B.

Figure 5. Data from human studies. N = 24 teeth were assayed. A. The relative concentration of a 66 bp 18S fragment is compared to a non-overlapping 301 bp 18S fragment. B. The relative concentration of a 171 bp 18S fragment is compared to a non-overlapping 501 bp 18S fragment.

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Dissemination of Research Findings

Publication

Young, S. T., Wells, J.D., Hobbs, G.R., and Bishop, C.B. (2013). Estimating Postmortem Interval Using RNA Degradation and Morphological Changes in Tooth Pulp. Forensic Sci. International **229:** 163 e1-6.

Meeting Presentations

2010 Estimating Postmortem Interval: A Molecular Approach. Young, S.T. and Bishop, C.P. Bioinformatics and Forensics Annual Summit, San Diego, CA.

2011 A Molecular Approach for Extended Postmortem Interval Estimates. Young, S.T. and Bishop, C.P. 19th International Association of Forensic Sciences, Funchal, Madeira, Portugal

2012 Extended Postmortem Interval Estimates. Young, S.T. and Bishop, C.P. 2012 National Institute of Justice Conference, Alexandria, VA

2013 A Molecular Approach for Extended Postmortem Interval Estimates. Young, S. T., Moore, J. R., and Bishop. 2013 National Institute of Justice Conference. Washington, DC

Online presentations of the above talk on April 10, 16, and 30, 2013

2013 A Molecular Approach for Extended Postmortem Interval Estimates. Young, S. T., Moore, J. R., and Bishop. International Association of Bloodstain Pattern Analysis Conference, San Diego, CA. September

2014 Analysis of RNA Degradation in Tooth Pulp to Determine PMI. Balasko, A., Young, S.T., Moore, J.R. and Bishop, C.P. Mid-Atlantic Association of Forensic Scientists. State College, PA