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.

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Meeting Presentations

a. 2010 Estimating Postmortem Interval: A Molecular Approach. Young, S.T. and Bishop, C.P. Bioinformatics and Forensics Annual Summit, San Diego, CA.
b. 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
c. 2012 Extended Postmortem Interval Estimates. Young, S.T. and Bishop, C.P. 2012 National Institute of Justice Conference, Alexandria, VA
d. 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
e. Online presentations of the above talk on April 10, 16, and 30, 2013
f. 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
g. 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