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The Histological Analysis of Tooth Cementum Annulations to Determine Age and Season of Death of Active Duty Military Personnel: Comparative Study Using Brightfield, Polarizing and Phase Contrast Microscopy

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The Histological Analysis of Tooth Cementum Annulations to Determine Age and Season of Death of Active Duty Military Personnel: Comparative Study Using Brightfield, Polarizing and Phase Contrast Microscopy

ABSTRACT: Quantitative tooth cementum annulation (TCA) analysis has been proposed as an accurate age-at-death marker in humans, and qualitative evaluation of the terminal band may reflect seasonality. Consolidation of preparation parameters is leading towards validation of quantitative TCA analysis, however, questions remain regarding the optical parameters that may optimize visualization. Teeth extracted from a U.S. military population as part of an oral health care plan were prepared into 100μm transverse sections and visualized using three transmission microscopy methods; brightfield, polarizing, and phase-contrast. Due to suspected modifiable sample preparation methods 20.7% of samples were devoid of sufficient annulations for accurate quantification. Two observers achieved correlations between TCA age and true age of r=0.894 to r=0.906 (n=46) and estimates were within 4 years of true age for 80.9% of samples when high quality digital microscopes were used for photomicrograph collection. Disparity between the quality of the phase-contrast microscope and other modalities precluded accurate comparison amongst all three groups. Season of extraction estimates by a single observer ranged from 58.7 to 73.9% with no significant difference between microscopy modality. Adherence to the protocol and procedural recommendations of this study could provide valuable forensic or anthropological evidence to facilitate identification through age estimation using TCA analysis. This study was unable to bolster support for season determination as an accurate TCA methodology, however, future validation may be possible using sample preparation parameters that differ from those employed for quantitative assessment

KEYWORDS: Forensic odontology, tooth cementum annulations, age determination, cementum rings, cementochronology, Dental Cementum Increment Analysis, DCIA

Throughout the lifespan of a tooth, dental cementum is deposited and mineralized in a periodic manner, such that histologically distinct layers are identifiable via optical microscopy. Tooth Cementum Annulation (TCA) analysis has been utilized in over 72 mammalian species(1, 2) and proposed as an accurate age-at-death marker in humans that may facilitate archaeological research, forensic identification, and criminal investigations(3).

Following tooth eruption, acellular extrinsic fiber cementum (AEFC) mineralization in the coronal half of the root occurs in regular phases, establishing alternating pairs of light (translucent) and dark (opaque) bands when observed by optical microscopy (3-5). The apical half of roots consists of Cellular Intrinsic Fiber Cementum (CFIC) and Cellular Mixed Stratified Cementum (CMCS), which are reactive tissues with irregular appositional growth(2) and highly variable patterns of cementum deposition that have proven less reliable for TCA analysis.

The environmental, hormonal, and biochemical factors governing banding during cementogenesis is an area of developing research, however, the optical phenomenon has been postulated to occur due to changes in collagen fiber orientation, crystallite direction or periodic changes in the degree of mineralization(3, 6). Although the exact mechanism of banding has yet to be elucidated, cementum deposition is not subject to turnover and the progressive accumulation provides chronologic markers that are hypothesized to correlate to one year of life. Furthermore, recent research has suggested the histologic appearance of the terminal cementum band may be used to identify the season of death or extraction as either summer or winter(7-9).

The first protocol for human TCA analysis was published by Stott et al. 1982 (10) and numerous TCA validation studies have been conducted with modifications to sample preparation and observation protocol. A review of 16 TCA studies show a strong correlation between the annulation ring count and true age, with an average correlation coefficient of 0.87 and range of 0.97 to 0.42 (11, 12). Despite the established correlation, TCA analysis is subject to two independent sources of variation which limit widespread acceptance by the archaeological and forensic community: first, there is an inherent variation in the individual age at tooth eruption, for which the standard deviations lie between 0.6-1.6 years(13). Second, the absence of a standardized TCA protocol results in varying degrees of observer subjectivity, and measurement errors attributable to sample preparation or microscope modality. Efforts of a working group at the 2011 European Paleopathology Association meeting culminated in the establishment of the "Cementochronology Research Program" and development of a TCA sample preparation protocol which was ISO-9001 certified in 2013 (1). Studies utilizing the Cementochronology group protocol parameters generally yield age determinations which fall within ± 0 to 4 years of true age, with a greater degree of discrepancy noted in adults >50 years of age (2, 7, 12, 14).

Estimation of season of death via TCA analysis was first published in a 2007 pilot study(7); although the reported accuracy was 99%, subsequent investigators (14, 15) reported only 61-76% accuracy and cited errors due to increased technique sensitivity, confounding optical effects inherent to transmitted light polarizing microscopy, and artifacts from periodontal fiber integration. Although the total number of pairs of translucent and opaque bands is well correlated to the age of the patient, there is limited research to establish the correlation between the terminal band and season of death or tooth extraction.

Although the key sample preparation parameters have been established(1, 16) by the Cementochronology group protocol, studies by Pundir et al. and Kaur et al. (17, 18) proposed that variations in microscope modality may enhance annulation visualization. Although both of these TCA studies reported statistically significant differences in the effectiveness of brightfield, polarizing and phase-contrast microscopes, the sample preparation and observation parameters used by the authors were inconsistent with the established standards. The presence of annulations indicate cementum is an anisotropic material giving rise to birefringent properties which may be enhanced by either polarizing or phase contrast microscopy. Polarizing microscopes limit the planes of light passing through a specimen and have the potential to enhance the distinction between materials with differing density and refraction indices(17). Phase contrast condenser and objective pairings modifies light amplitude depending on the differential phase of light passing through a transparent sample and modulate the interaction of scattered light and direct light to create a contrast effect with greater detail, a wider range of contrast in a single field of view and enhanced contrast near the edges of specimen(17). When applied to samples prepared consistent with current standards, it was the author's intent to explore whether microscope modalities impacted observer subjectivity, intra-observer agreement, age estimate accuracy, and terminal band assessment.

Aim

The aim of this study is to conduct histologic assessment of transverse, unstained, ground sections of extracted human teeth to determine the effect of varied transmission light microscopy modalities (brightfield, polarized, and phase-contrast) on inter-rater agreement and utility of tooth cementum annulation (TCA) analysis for age estimation and determination of season at the time of tooth extraction.

Materials and Methods

Specimen collection – Sixty five human teeth extracted as part of an oral health care plan were collected by Oral Surgeons at the Carl R. Darnall Army Medical Centre, Fort Hood, TX. De-identified specimens were individually stored in 10% buffered formalin and labelled with the following demographic data; biologic sex of the individual, season of extraction (winter – Nov to Mar or summer – Apr to Oct), universal numbering system tooth number, and age of the individual at the time of extraction. Inclusion criteria: human non-third molar teeth extracted from active duty military personnel aged 18-60. Exclusion criteria: damaged radicular surfaces, presence of periapical pathology, and periodontal disease extending to middle third of the root.

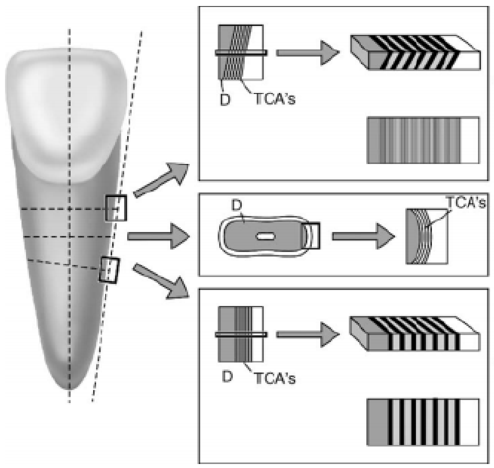

FIG. 1 – *Adapted from Maat et al., (2006). Cementum increment visibility is dependent on orientation(16); samples are mounted such that the transverse section is perpendicular to the external surface of the middle third of the root rather than long axis.*

Sample preparation – Specimens were gently cleaned of gross debris, rinsed with acetone, dried, then positioned in a silicone mold such that the cementum surface of the middle third of the root was perpendicular the edge of the mold. Unstained specimens were embedded in a mixed solution of resin Epoxicure and Hardener (Buehler, Lake Bluff, Illinois). Embedded specimens were assigned randomly generated sample numbers. Specimens were sectioned with a Buehler Isomet 5000 linear precision saw at a speed of 350rpm and a linear feed rate of 4-5mm/min to produce two 100µm ± 10 µm transverse root section samples for each specimen. Sections were verified by digital calipers, cleaned with acetone and mounted on microscope slides using xylene n-butyl methacrylate and Canada Balsam (Benz Microscope Optics, Ann Arbor, Michigan).

Photomicrograph and data collection – samples were evaluated under brightfield and polarizing protocols at 200-400x magnification with Olympus BX53 and Nikon Eclipse 55i microscopes fitted with digital charge-coupled devices and high resolution live monitor feedback. Phase contrast protocol observations were completed at 100-200x magnification using a Seiler Microlux IV microscope with an optical attachment for a Canon EOS 80D DSLR camera. Terminal band evaluation was conducted by a single observer via live through-focus assessment to account for artifacts due to the field of focus and optical edge effects. Season of extraction was annotated as summer for translucent/light terminal bands and winter for opaque/dark bands. All photomicrographs were captured by a single observer and stored digitally according to sample number for independent TCA counts by two observers (licensed dentists). Each pair of bands (one translucent, one opaque) was digitally marked independently by observers using Adobe Photoshop CS5 software (Figure 2) Estimated age at extraction was calculated by adding the TCA count (each pair of bands = 1 year) and the average age of eruption(18, 19). Statistical analysis completed with IBM SPSS Statistics 25 Software and hand tabulation.

TABLE 1 – *Demographic data for observed specimens*

Variable	Frequency	%
Biologic Sex		
Male	33	71.7
Female	13	28.3
Age		
20-30	38	82.6
30-40	6	13.0
40-50	2	4.4
Season of Extraction		
Summer	31	67.4
Winter	15	32.6
Tooth Type:		
Max 2nd Premolar	6	13.0
Max 1st Premolar	17	37.0
Max Central	1	2.2
Mand 2nd Premolar	9	19.5
Mand 1st Premolar	13	28.3

n=46.

TABLE 2 – *Correspondence between true and TCA estimated ages*

	Observer 1			Observer 2		
	BM1	POL1	PC1	BM2	POL2	PC2
Correlation Coefficient (r)	.906*	.906 *	.797*	.898 *	.894 *	.839*
Mean absolute difference ± SD (years)	2.7± 2.1 ^{a}	2.4± 2.0 ^{a}	4.3± 3.1 ^{b}	2.9± 2.2 ^{a}	2.4± 2.0 ^{a}	4.4± 2.8 ^{b}
TCA Ages underestimated (%)	83	86	90	88	86	92

*Statistically significant (α= 0.01), |z| <2.58

^{a,b} Groupings by statistical significance (α= 0.01)

Pearson product-moment correlation coefficient (PPMCC) measure of linear correlation between estimated age and true age for each microscope modality for both observers. Z-Test statistical analysis indicate there is no statistical difference between the correlation coefficients. Mean absolute difference via independent samples t-test statistics. *n – 46.*

TABLE 3 – *Categorical discrepancy between true and TCA estimated ages*

	% of estimates – observer 1			% of estimates – observer 2		
Discrepancy (years)	BM1	POL1	PC1*	BM2	POL2	PC2*
Category 1 (0 to ≤ 2 years)	63.04	69.57	30.43	52.17	71.74	36.96
Category 2 (>2 to ≤ 4 years)	17.39	15.21	36.96	32.61	15.22	23.91
Category 3 (>4 to ≤ 6 years)	15.22	8.70	15.22	8.70	6.52	21.74
Category 4 (>6 years)	4.35	6.52	17.39	6.52	6.52	17.39

*Statistically significant difference in categorical distribution

Chi-square test independence indicates the distribution of the discrepancy (deviation from true age) for the phase contrast age estimates are significantly different from the brightfield and polarized microscopy estimates. α= 0.05 , *n – 46.*

Results

Of the 65 specimens collected, eight were lost to avoidable sample processing errors and twelve preparations (20.7% of prepared samples) were devoid of sufficient observable annulations due to artifacts, cementum damage, presence of cellular cementum or improper angulation of transverse sectioning. Data includes observations of specimens from 46 biosamples derived from 17 unique individuals (Table 1).

Degree of correspondence between true age and TCA estimated ages – The Pearson Product-Moment Correlation Coefficients (Table 2) demonstrate a very strong degree of correspondence between the true age and TCA estimated age for BM1, BM2 POL1, POL2, PC2 and a strong degree of correspondence for PC1. The means of the absolute difference between true age and estimated ages were significantly greater for PC1 and PC2 groups compared to all other microscopy methods. The majority of all TCA ages were underestimated relative to the true age.

Differences in the degree of correspondence between true age and TCA estimated ages – Fisher's *r* to *z* transformation and subsequent z-Test statistical analyses indicate there was no statistical difference between the correlation coefficients for any experimental microscopy method, or between observers.

Categorical assessment of discrepancy between true and estimated ages – The difference in categorical distribution of the absolute difference between true age and the TCA estimate was significantly different between the phase contrast groups and all other groups (Table 3). Brightfield and polarized microscopy estimates were accurate within 4 years of the true age for 80.9% of samples, whereas the phase contrast groups achieved this degree of accuracy <70% of the time.

Inter-rater reliability for TCA counts – Intraclass Correlation Coefficient analysis (Table 4) indicated an *excellent* (r>0.86) degree of agreement between observer counts using brightfield and polarized modalities. Phase contrast microscopy had a *very good(20)* degree of reliability (0.74 < r <0.86). Despite the discrepancy in classification of the reliability, fisher's *r* to *z* transformation and z-test statistic indicates there is no significant difference between the coefficients at n – 46 and α= 0.01.

Identification of season of extraction by terminal band assessment – A single observer utilizing live microscope assessment to identify the terminal band as either light/translucent (summer) or dark/opaque (winter) 58.7% of the time with phase contrast microscopy, 73.9% with polarizing and 65.2% via Brightfield. Chi-square analysis ($\chi2$=2.38, likelihood ratio 2.416 and α= 0.30) suggests no statistical difference in the accuracy of terminal band assessment.

TABLE 4 – *Inter-Rater reliability for TCA counts*

	Microscope Modality		
	Brightfield	Polarized	Phase Contrast
ICC, r=	.926	.875	.850
Interval (0.95)	.869 - .958	.785 - .929	.744 - .914
Significance, α=	<0.01	<0.01	<0.01

Intraclass Correlation Coefficient, 95% confidence intervals and degree significance. A measure of observer agreement for TCA counts for each microscope modality.

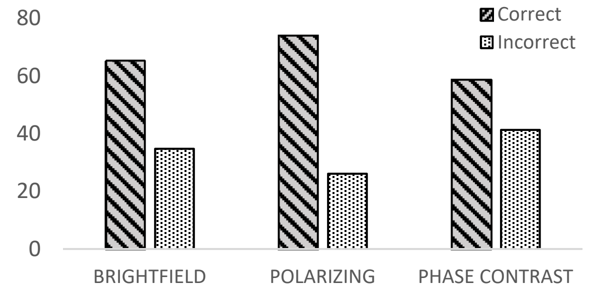

Figure 1 – Season of extraction identification (%) by microscope modality (single observer). No statistically significant difference ($\chi 2=2.38$, df=2, $\alpha= 0.30$)

Discussion

This study bolsters support for the use of TCA analysis for age determination but was unable to reproduce reliable season determination. Procedural challenges and discordance of equipment quality underlined the sensitivity of the technique; a major barrier toward adoption of cementochronology for routine forensic and archaeological applications.

Specimen preparation procedural errors:

The specimen preparation and mounting protocol resulted in the loss of eight specimens due to operator error or material contamination. Previous studies demonstrated specimen processing success using a variety of embedding mediums, including xylene based glue, epoxy resins or physical supports via silicone tubing (2). Supply limitations prevented acquisition of manufacturer recommended molds and release agents for pairing with the epoxicure and hardener (Buehler, Lake Bluff, Illinois). The first batch of 8 specimens was removed from formalin, rinsed with distilled water, dried for 24 hours then positioned in a silicone mold with release agent and embedded in mixed epoxicure and hardener. Suspected contamination from residual formalin, inadequate mixing, or contamination from the mold/release agent resulted in incomplete curing of embedding medium and the specimens could not be cleanly salvaged for re-processing. Subsequent batches were cleaned with acetone prior to drying and placed in a new silicone mold devoid of release agents and no further specimens were lost.

Technique sensitivity of sample preparation:

Of the 57 specimens utilized for sample preparation, 12 (20.7%) were devoid of sufficient observable annulations; these samples were not included in the statistical analysis or reporting tables. The percentage of unreadable samples was higher than other studies using similar sample preparation protocols; 10% by Mallar et al. (21), 14% by Wilson et al.(14) and 16.2% by Wittwer-Backofen et al. (13). Increased operator experience and procedural modifications have the potential to reduce the incidence of sample exclusion:

Cutting Angle – As confirmed by Maat et al. (16) the visibility of annulations is enhanced by ensuring the cutting angle is perpendicular to the exterior of the root surface to be analyzed. Cellular cementum is frequently found in furcations or areas of significant root morphology variegation(2), therefore, appropriate areas of interest were identified and the tooth embedded in resin such that the cutting angle would be aligned in a perpendicular fashion. The 100 µm transverse sections were batch processed without microscopic cutting angle analysis or adjustments in between sections that could have increased annulation clarity.

Precision saw settings and polishing – A significant number of sections were sacrificed to determine optimal precision saw linear feed rate and RPM to minimize section fragmentation and produce 100µm sections free of excessive cutting marks. While some authors report excellent success with un-polished samples(2, 14), cutting marks obscured incremental lines in several samples in our study when those lines ran parallel to the area of interest. Some investigators recommend polishing samples with abrasive papers on a polisher-grinder to achieve the desired final section thickness (12, 15) whereas others recommend cutting to desired thickness then lightly polishing with aluminum oxide particles to remove cutting lines(1). Further studies could mitigate the interference of cutting marks by polishing or simply rotating the embedded samples such that the wafer blade cutting marks don't run in parallel to the area of interest.

Sample count – Isolating and preparing cementum for TCA is very technique sensitive; although a minimum of two cross section samples were prepared per specimen, generally only one very limited microscopic area of cementum was deemed suitable for evaluation. To improve the number of specimens yielding observable TCA counts, it may be prudent increase the number of samples to 5 sequential sections with intervening microscopic assessment, as proposed by Colard et al.(1) to mitigate the challenges of cutting angle and blade mark optimization.

Discordance of microscope quality:

The clarity and ability to distinguish individual annulations with the Olympus BX53 and Nikon Eclipse 55i microscopes fitted with digital charge-coupled devices and high resolution live monitor feedback was far superior to the optics of the phase contrast microscope arrangement available to the researchers. The disparity was deemed so great that the primary objective of drawing conclusions regarding efficacy of phase contrast over polarized or brightfield was deemed impossible.

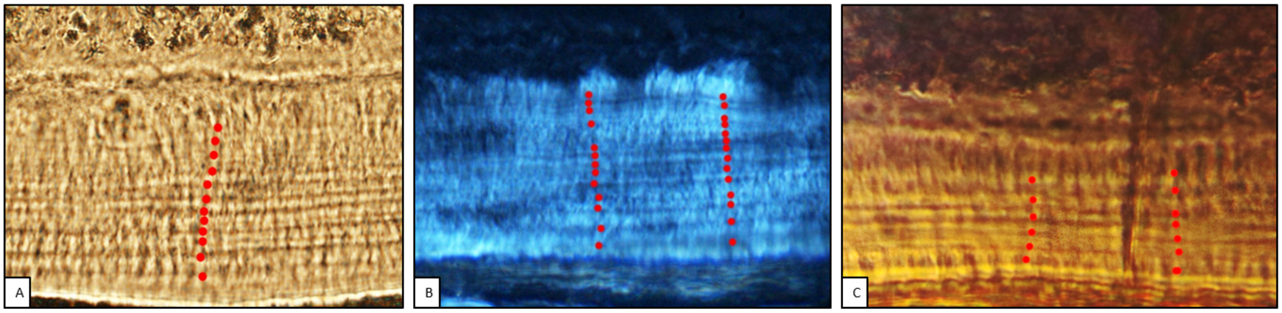

Figure 2. Quantitative TCA Analysis, 100um transverse sections, extracted human teeth. Each dot represents one pair (light and dark) of cementum bands and is estimated to be one year of life. A,B –Brightfield, polarizing microscopy 200x, Olympus BX53 digital microscope with digital CCD. C-Phase contrast Seiler microlux IV with Canon 80D camera attachment.

TCA for Age Estimation

The high degree of correspondence between TCA age estimate observed in this study is consistent with similar Human TCA validation studies (16 studies) that show an average correlation coefficient of 0.87, with a range of 0.97 to 0.42 (11). With the use of high quality microscopes with CCD digital imaging on high resolution screens, the BM and POL estimates were accurate within 4 years of true age for 80.9%, a finding which is similar to the 84-86% reported by Wilson et al. (14). Considering the TCA age estimates are subject to an inherent error of ± 0.6-1.6 years(13) due to the variability in age of eruption, the remaining error attributed to TCA analysis is small. The high accuracy in this study is in part due to adoption of the procedural recommendations of the Cementochronology group, but also potentially impacted by the narrow age range and young age of the sampled military population. At higher ages, TCA counts become challenging due to compacted lines(4), decreased cementum deposition (22) and the resultant correlations between TCA estimates and true age in older individuals are weaker (2, 22). As an individual ages, there is a greater probability for variability in cementum annulations(23) as the subject cementum is exposed to a greater range of functional stimuli, hormonal changes and potential for pathology. This study also excluded third molars due to a lower correlation between TCA count and true age (24). The impact of periodontal disease on TCA counts is unclear based on the current body of research; multiple authors provide data suggesting periodontal disease has a significant adverse effect on TCA accuracy (25, 26) but a large study by Wittwer et al.(13) corrected for age and concluded TCA estimates are independent of the severity of periodontal disease. Due to the divisive nature of periodontal disease implications in TCA analysis, affected teeth were excluded from this protocol and it seems prudent for future analyses to select transverse sections devoid of pathology whenever such selection would still enable sampling of acellular extrinsic fiber cementum.

Inter-rater reliability

Due to microscope access limitations, only one observer was able to conduct the live through-focus assessment for terminal band assessment / season of extraction estimation. Both observers had similar difficulties assessing TCA counts using the lower quality phase-contrast microscope images, as demonstrated by the statistically significant differences in the mean absolute differences (>4 years of true for PC groups) and the lower percentage of age estimates (<37%) that were within 0-2 years of true. Despite the differences between microscope modalities, the overall TCA age estimate inter-rater reliability correlations in this study were at the upper end (ICC r= 0.85 to 0.93) of the correlation ranges (r= 0.70 to 0.94) reported in other multi-observer studies (1, 2, 13, 25). The low inter-rater agreement correlation of 0.70 reported by Dias et al. (25) was observed when analyzing a population with a mean of 44.2 years and a range extending from 17 to 77 years, therefore, the discrepancy with our study may be attributed to the greater degree of observation difficulty and annulation variegation in older individuals. Collard et al.(1) report higher inter-rater agreement , perhaps due to implementation of observer calibration training as well as the acquisition of 4-10 photographs per tooth to establish mean counts that minimize both intra and inter-observer errors. Our study, as well as those referenced, assessed the agreement between observers counting annulations on the same photomicrographs. While this is expected to increase the concordance, future studies may consider the agreement amongst observers if they independently acquire photomicrographs from shared samples such that the degree of agreement accounts for the fundamental difficulties associated with area of interest selection and optical variability during image acquisition.

Season of extraction

Since Wedel's 2007 pilot study (7) demonstrating 99% correct season of extraction identification using 500µm transverse sections and brightfield microscopy, no study has replicated this degree of success. A cadaveric sample study by Ralston (15) demonstrated 61.5 to 71.2% correct season identification but a low inter-observer agreement of 28.8%. Ralston noted significant challenges identifying the true terminal band based on the microscopic focus plane, cofounding optical edge effects and inconsistent terminal band presentation at different observation sites from the same sample or specimen. In our study, these challenges were apparent to such a degree that still-photomicrograph assessment of terminal bands was deemed impossible and only live through-focus determinations were made. Even with live terminal band assessment, this study only successfully identified the correct season of extraction in 58.3 to 73.9% of samples with no significant difference between microscope modality. This was lower than the 72-76% correct with 78% inter-observer agreement demonstrated by Wilson et al.(14) who utilized the same sample preparation protocol and static photomicrograph assessment. In 2016, Meckel (27) utilized the preparation technique by Wedel to assess terminal cementum bands of human bodies exposed to decomposition but was unable to establish a correlation or achieve strong intra-observer agreements across multiple observations; caution should be exercised when assessing terminal bands subject to decomposition or taphonomic processes. Until protocol modifications to enhance terminal band visualization are elucidated, the authors of this study do not support the adoption of terminal band assessment to determine season of death.

Barriers to Adoption

Despite the accuracy and inter-rater reliability reported by multiple TCA validation studies, forensic anthropologists preferentially utilize sternal rib end, cranial suture analysis, auricular surfaces or pubic symphysis evaluation methods of age estimation, despite standard errors generally greater than ± 7 years (2, 28).

Protocol variance – Reluctance to adopt TCA analysis is likely in part due to the absence of protocol standardization prior to the Cementochronology research group efforts. Several studies diverging from these recommended parameters reported variable inter-observer agreement and weaker correlations between TCA estimates and true age(11); Dias et al. (r=0.58 and mean error of 9.7 years) used transverse sections of only 30µm and Kvaal et al. (r=0.73) demineralized and stained the cementum prior to observation(25, 29). The authors recommend adherence to the protocol parameters outlined by the Cementochronology(1) group, as well as the procedural/equipment recommendations made by Wilson et al.(14) and this study to achieve reliable TCA age estimates.

Nature of the phenomenon: Despite the undeniable correlation between annulations and age, resistance to widespread adoption of TCA analysis may be due to the uncertainty regarding the physiological processes responsible for the banding. Acellular extrinsic fiber cementum (AEFC) growth is the progressive mineralization of PDL sharpey fiber mineralization (3) and occurs at a slower rate than cellular cementum growth, such that the cementoblasts remain at the surface and do not form cementocytes/lacunae(4). In the 1990's, Liebermann et al(4) postulated banding patterns were due to seasonal changes in the orientation of fibers and subsequently formed crystallites. Liebermann proposed the effect may be impacted by occlusal force discrepancies due to seasonal diet variation, but the presence of the effect in humans, domesticated animals (5), equatorial species and impacted teeth (24, 26) suggests there are complex metabolic and environmental etiologic factors. Proponents of the fiber orientation theory were relying on early microprobe studies which suggested the ratio of calcium to phosphorus across increments is relatively constant (5). Recent studies using x-ray fluorescence and diffraction mapping downplay the role of fiber orientation in light of evidence there is a significant difference in carbonated hydroxyapatite content (30) between the light (hypomineralized) and dark (hypermineralized) bands. Laser raman microspectrometer analysis by Colard et al. in 2016 (3) confirmed that variations in mineral to organic content ratios corresponded to the observed banding patterns. Although there is likely a complex interplay of physiological processes contributing to cementum mineralization, new research points towards parathyroid hormone as one potent regulator of cementogenesis (6, 31), and the seasonal fluctuations of PTH (32) may be an etiologic factor for the seasonal phenomenon. Several researchers have noted qualitative differences in band widths or degree of mineralization and proposed that certain irregularities reflect stress markers consistent with significant hormonal events. Although these irregularities were associated with pregnancy, skeletal trauma and renal disease (13, 26), research efforts to systematically identify the qualitative features of the irregularities and observer reliability have only recently emerged(23). Due to the lack of validation, random fluctuations in the formation process or local stress processes cannot be excluded as potential etiology of these stress markers and further investigation is needed.

Military impact and applicability

In their effort to provide the fullest accounting for over 81,900 missing DOD personnel from prior conflicts(33), the forensic anthropologists and odontologists of the Defense POW/MIA Accounting Agency (DPAA) often utilize dental identification methodologies. TCA analyses facilitate the post-mortem to ante-mortem reconciliation process by narrowing the age range of possible candidates while preserving two-thirds of the tooth for additional forensic methods such as isotope ratio mass spectrometry or nuclear/mitochondrial DNA analysis. TCA analysis has been successfully applied to excavated human remains subjected to taphonomic changes due to diagenesis, soil erosion, and partial mechanical destruction (34-36); however, the applicabilty to samples that have been subject to blast events or aviation accidents is an area for further development. A recent study by Oliveira-Santos et al.(37) demonstrated a low correspondance between TCA age and true age for samples exposed to temperatures of 400 and 900 degrees celcius for three hours, however, future studies could investigate TCA anaylsis validity for specimens subjected to enviromental exposures or trauma consistent with military conflict.

Conclusions

This study successfully demonstrated equivalent, high accuracy TCA analysis of cementum counts using high quality digital microscopy with polarized and brightfield transmission methods. Although the operators preferred the contrast range visible in a single field of view with the phase-contrast microscope, the disparity between the quality of optics and image capture devices was too great to render any judgement regarding phase-contrast efficacy relative to the other light transmission modalities tested.

Terminal band assessment for season of extraction/death estimate was deemed unreliable with static photomicrograph evaluation of samples prepared according to the parameters in this study. Although live through-focus microscopy improved the observer's confidence in terminal band assessment, this study was unable to bolster support for season determination using TCA. Further elucidation of the effect of taphonomic changes on terminal banding as well as the parameters that will optimize terminal band visualization may be required prior to future validation

The protocol employed in this study achieved a high level of correspondence between TCA estimate and age. Although the accuracy is a consequence of excluding 20.7% of the samples with unreadable cementum annulations, it is important to recognize that there is a high degree of probability that greater operator experience and implementing the aforementioned protocol revisions could reduce sample exclusion. More importantly, TCA analysis remains a valuable forensic and anthropological tool *if* inappropriate specimens are excluded.

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