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Estimation of age based on tooth cementum annulations: A comparative study using light, polarized, and phase contrast microscopy

Prabhpreet Kaur, Madhusudan Astekar[1], Jappreet Singh[2], Karandeep Singh Arora[3], Gagandeep Bhalla[4]

Department of Oral and Maxillofacial Pathology, BRS Dental College and General Hospital, [2]Department of Conservative Dentistry and Endodontics, Army Dental Corps, [1]Department of Oral and Maxillofacial Pathology, Institute of Dental Sciences, Bareilly, Uttar Pradesh, [3]Department of Oral Medicine and Radiology, Daswani Dental College and Research Centre, Kota, Rajasthan, [4]Department of Oral and Maxillofacial Pathology, Himachal Institute of Dental Sciences, Himachal Pradesh, India

Address for correspondence:
*Dr. Prabhpreet Kaur, Department of Oral and Maxillofacial Pathology, BRS Dental College and General Hospital, Panchkula, Haryana, India.
E-mail: drppk_oberoi@yahoo.com*

Abstract

Context: The identification of living or deceased persons using unique traits and characteristics of the teeth and jaws is a cornerstone of forensic science. Teeth have been used to estimate age both in the young and old, as well as in the living and dead. Gradual structural changes in teeth throughout life are the basis for age estimation. Tooth cementum annulation (TCA) is a microscopic method for the determination of an individual's age based on the analysis of incremental lines of cementum. **Aim:** To compare ages estimated using incremental lines of cementum as visualized by bright field microscopy, polarized microscopy, and phase contrast microscopy with the actual age of subject and to determine accuracy and feasibility of the method used. **Materials and Methods:** Cementum annulations of 60 permanent teeth were analyzed after longitudinal ground sections were made in the mesiodistal plane. The incremental lines were counted manually using a light, polarized and phase contrast microscopy. Ages were estimated and then compared with the actual age of individual. **Statistical Analysis:** Analysis of variance (ANOVA), Student's *t*-test, the Pearson product-moment corre (PPMCC) and regression analysis were performed. **Results:** PPMCC value $r = 0.347$, 0.542 and 0.989 were obtained using light, polarized and phase contrast microscopy methods respectively. **Conclusion:** It was concluded that incremental lines of cementum were most clearly visible under a phase contrast microscope, followed by a polarized microscope, and then a light microscope when used for age estimation.

Key words: Age estimation, annulations, cementum lines, permanent teeth, phase contrast microcscopy, polarized microscopy

Introduction

"An identity would seem to be arrived at by the way in which a person faces and uses his experience."

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Identification of an individual is based on the theory that all individuals are unique.[1] The identification of living or deceased persons using the unique traits and characteristics of teeth and jaws is a cornerstone of forensic science.[2,3] Age estimation aims to define, in the most accurate way, the chronological age of an individual for whom the age is unknown.[4]

Estimation of age plays an important role in forensic medicine, not only in identification of bodies but also in connection to crimes. Age estimation through dental parameters may also help in other situations such as determining the legal liability of teenagers and adults of unknown age, assisting adoption processes, releasing retirement funds for adults of unknown ages, and supporting research in archaeology and paleodemography.[5]

Estimating age from teeth is generally reliable as they are naturally preserved long after all other tissues have disintegrated.[6,7] Teeth can survive in most conditions encountered at death and during decomposition, even when the body is exposed to extreme forces and/or temperatures.[8] This resistance has made teeth useful indicators for age estimation.[9]

Dental age estimation methods are either based on a well-ordered cascade of changes that occur during the formation and eruption of teeth or they rely on continuous process that alter and diminish the quality of dental tissues even when individual growth is completed.[10] Tooth cementum annulations (TCA) is a microscopic method for determination of an individual's age based on the analysis of acellular extrinsic fiber cementum (AEFC).[11]

With the above background, the present study was carried out to examine the correlation between chronological age and cementum annulations of a tooth. Aim of the study was to compare age estimated using incremental lines of cementum as visualized by bright field microscopy, polarized microscopy, and phase contrast microscopy with the actual age of subject and to evaluate the accuracy and feasibility of estimated ages as assessed by three different microscopy techniques.

Materials and Methods

Study sample consisted of 60 extracted teeth obtained from the Department of Oral and Maxillofacial Surgery.

Inclusion criteria for teeth selection:

- Teeth extracted for orthodontic, prosthodontic, and periodontal reasons were collected
- No teeth with morphological/developmental abnormalities, caries, fracture/trauma, or erosion/abrasion were included in the study
- Care was taken to include only those teeth with an intact root surface without the loss of adjoining cementum.

A total of 60 teeth, from 34 male and 26 female subjects, were selected for the study. Clinical data of patient, i.e. name, age, gender, tooth number, and reason for extraction of tooth were recorded on a case history performa prior to extraction. Longitudinal ground sections in mesiodistal plane were made using a laboratory lathe under the continuous cooling of water until the desired thickness was reached. Later, sections were trimmed manually, initially on the coarse side of an Arkansas stone, followed by the finer side. Ground sections were cleaned carefully with xylene (Merck, Mumbai, India) and mounted on glass slides using DPX mountant (Merck, Mumbai, India) and cover slips (Blue Star, Chennai, India).

Incremental lines of cementum (cementum annulations) from the prepared sections were then studied using a light microscope. Photomicrographs were taken focusing on the cementum of the middle third region of the root using a Canon Powershot A95 digital camera [3x (38-114mm equiv.) F2.8-4.9 zoom lens] at 40x magnification and were then transmitted from the microscope to a laptop monitor. Cementum lines were counted manually by marking a point against each line observed and then counting the number of points marked in total per photomicrograph [Figure 1]. For each alternating light and dark band of cementum annulations, a score of 1 was noted and the total number of incremental lines was counted.

Age was estimated using the formula:

Estimated Age = Total number of cementum annulations + Age of eruption of that tooth

The number obtained was then compared with the known age of subjects. A similar procedure was followed using the polarizing microscope and phase contrast microscope, and the same comparative analysis was done [Figures 2 and 3].

Statistical analysis was performed using SPSS 16.0 software. Comparison between the three estimated ages (from the three different microscopy techniques) and actual age was done using analysis of variance (ANOVA) and the Student's *t*- test, followed by derivation of a linear regression equation.

Results

The study included 34 teeth (56.67%) from male subjects and 26 teeth (43.33%) from female subjects. Of the total sample; 2, 3, and 8 samples gave a mean age error of 0 and correlated exactly with the actual ages when light, polarized and phase contrast microscopy methods were respectively used for estimating the age. 4, 4, and 42 samples gave a mean age

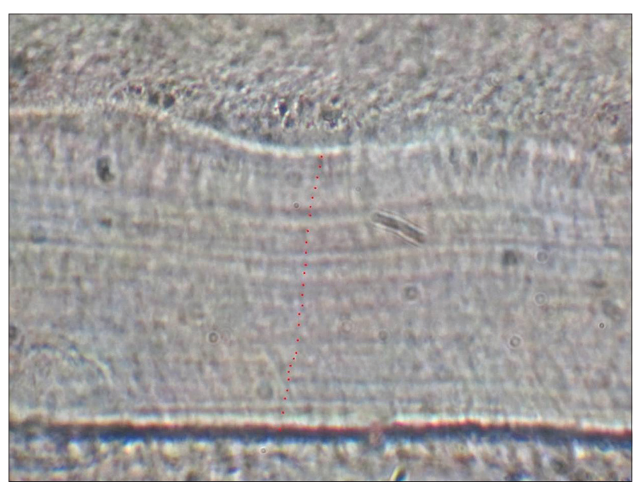

Figure 1: Photomicrograph showing incremental lines of the cementum at 40x magnification using a light microscope

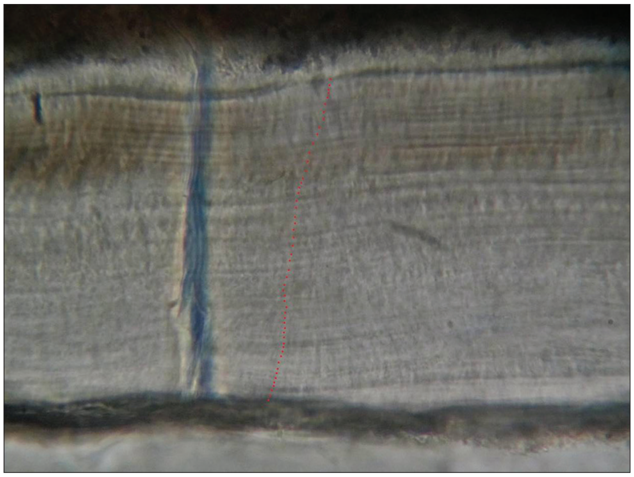

Figure 2: Photomicrograph showing incremental lines of the cementum at 40x magnification using a polarizing microscope

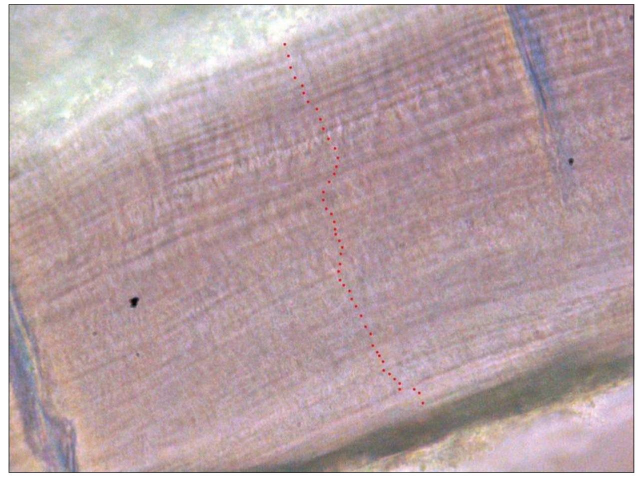

Figure 3: Photomicrograph showing incremental lines of the cementum at 40x magnification using a phase contrast microscope

error of 1-2 and 4, 25, and 9 samples gave a mean age error of 3-5 on studying the slides under light, polarized and phase contrast microscopy respectively. A frequency of 14, 15, and 1 was seen with a mean age error of 6-10; 24, 8, and 0 seen with an error range of 11-20 and 12, 5, and 0 with an error range of more than 20 when estimation of age was done using three different microscopy methods [Table 1].

ANOVA was done to analyze the differences of estimated ages obtained using three different light microscopy methods with the actual age of subjects. The *f* value obtained was 9.149. A statistically highly significant $P < 0.0001$ was derived [Table 2].

The Pearson product-moment correlation coefficient (PPMCC) was employed to examine the relationship between the ages estimated by the three microscopy methods and the chronological age individually. When the actual age and the age estimated by light microscopy method were compared, a correlation (r) of 0.347 was obtained with a highly significant $P < 0.001$ [Table 3]. Similarly, the actual age and the age estimated by polarizing microscopy gave a correlation coefficient value of 0.557 and an insignificant $P > 0.05$ [Table 4]. Also, a correlation coefficient value of 0.989 and $P > 0.05$ were obtained when chronological age and age estimated by phase contrast microscopy method were studied together which was statistically insignificant [Table 5]. It can be stated here that both the polarized and phase contrast microscopy methods gave results which had no significant differences with the actual age of subjects.

The student's *t*-test for paired samples was applied to make a comparison between methods and to assess the best method for studying cementum annulations. The three methods used in our study were now inter-compared. The light and polarized microscopy

Table 1: Frequency of observations seen with respect to the mean age errors in three different microscopy methods

Mean age error	Frequency of observations seen (%)		
	Light Microscopy	Polarized Microscopy	Phase Contrast Microscopy
0	2 (3.33)	3 (5)	8 (13.33)
1-2	4 (6.67)	4 (6.67)	42 (70)
3-5	4 (6.67)	25 (41.67)	9 (15)
6-10	14 (23.33)	15 (25)	1 (1.67)
11-20	24 (40)	8 (13.33)	0
More than 20	12 (20)	5 (8.33)	0
Total	60 (100)	60 (100)	60 (100)

Table 2: Analysis of variance (ANOVA) to analyze the difference between group means

Age	Mean±SD	*n*	*F*	Significance
Actual age	42.8±11.22	60	9.149	*P*<0.0001 Highly significant
Age estimated by light microscopy	32.7±11.68			
Age estimated by polarized microscopy	38.77±11.54			
Age estimated by phase contrast microscopy	41.42±11.37			

Table 3: Comparison of mean and standard deviation (SD) values of estimated age by light microscopy and actual age in years

Age	Mean±SD	*n*	*P*	*r*	Significance
Actual age	42.8±11.22	60	<0.001	0.347	Highly significant
Age estimated by light microscopy	32.7±11.68				

Table 4: Comparison of mean and standard deviation (SD) values of estimated age by polarized microscopy and actual age in years

Age	Mean±SD	*n*	*P*	*r*	Significance
Actual age	42.8±11.22	60	>0.05	0.557	Not significant
Age estimated by polarized microscopy	38.77±11.54				

methods gave statistically significant results ($P < 0.05$), i.e. results obtained by these two methods had significant differences [Table 6]. Also, on comparing ages estimated by light and phase contrast microscopes, results were highly significant ($P < 0.001$) indicating differences in the values obtained by these two methods [Table 7]. However, when polarized and phase contrast microscopy methods were compared, results were statistically insignificant ($P > 0.05$) [Table 8]. It can be concluded that the ages estimated by these two methods individually did not have significant differences and thus both these methods could be used for estimating age. However, correlation values of 0.557 [Table 4] and 0.989 [Table 5] clearly determined the phase contrast microscopy as a better method over the polarizing microscopy.

Regression analysis was performed with the actual ages of the subjects at the y-axis and the age estimated by the light microscopy method at the x-axis among the study group of 60 subjects. A regression equation of $y = 0.3336x + 31.89$ was obtained. Further, the regression coefficient, i.e., age change expected in years for unit change in the cementum annulations, was 0.3337. Similarly, linear regression graphs were plotted for the polarized microscopy and the phase contrast microscopy methods where regression equations $y = 0.5424x + 21.77$ and $y = 0.9761x + 2.374$ and regression coefficients, 0.542 and 0.989, were obtained, respectively.

Table 5: Comparison of mean and standard deviation (SD) values of estimated age by phase contrast microscopy and actual age in years

Age	Mean±SD	*n*	*P*	*r*	Significance
Actual age	42.8±11.22	60	>0.05	0.989	Not significant
Age estimated by phase contrast microscopy	41.42±11.37				

Table 6: Comparison of mean and standard deviation (SD) values of estimated age by light microscopy and polarized microscopy in years

Age	Mean±SD	*N*	*P*	*r*	Significance
Age estimated by light microscopy	32.7±11.68	60	<0.05	0.386	Significant
Age estimated by polarized microscopy	38.77±11.54				

Table 7: Comparison of mean and standard deviation (SD) values of estimated age by light microscopy and phase contrast microscopy in years

Age	Mean±SD	*N*	*P*	*r*	Significance
Age estimated by light microscopy	32.7±11.68	60	<0.001	0.338	Highly significant
Age estimated by phase contrast microscopy	41.42±11.37				

Table 8: Comparison of mean and standard deviation (SD) values of estimated age by polarized microscopy and phase contrast microscopy in years

Age	Mean±SD	*n*	*P*	*r*	Significance
Age estimated by polarized microscopy	38.77±11.54	60	>0.05	0.547	Not significant
Age estimated by phase contrast microscopy	41.42±11.37				

Discussion

In the present study, we used incisors, canines, premolars, and molars for our analysis. Similar to our study, Jankauskas *et al*. (2001) typically used incisors, canines, premolars, and molars to count cementum layers.[12] Dias *et al*. (2010), Avadhani *et al*. (2009) and Aggrawal *et al*. (2009) also studied all the maxillary and mandibular teeth.[5,9,13] In contrast to our study, Condon *et al*. (1986), Charles *et al*. (1986), and Renz *et al*. (1997) in their studies indicated that premolars are a more reliable age indicator.[14-16] Lipsinic *et al*. chose to use maxillary bicuspids; others such as Solheim stated that mandibular second bicuspids and central incisors have the best correlation for annulations count.[17-19] Radovic (2012) in his study examined samples consisting of only permanent premolars from the upper and lower jaws.[11]

In the present study, we used unstained ground sections to study incremental lines of cementum. Foster (2012) focused on the techniques useful for study of tooth root cementum. Comparison of multiple histological stains revealed that while commonly applied hematoxylin and eosin (H and E) and toluidine blue (TB) stains offer adequate results, these are not necessarily optimal for cementum visualization. The infrequently used Alcian blue with nuclear fast red counter stain was found to provide excellent contrast for both acellular and cellular cementum in human molars. While no truly unique extracellular matrix (ECM) markers have been identified to differentiate cementum from the other hard tissues, immunohistochemistry (IHC) for bone sialoprotein (BSP) and osteopontin (OPN) that localize to both acellular and cellular cementum layers is a reliable approach for visualizing cementum and providing insight into its developmental biology.[20] Shukla *et al*. (2012) in their study concluded that cresyl violet showed better contrast of cementum than toluidine blue, periodic acid Schiff (PAS), and H and E in decalcified and ground sections under light and fluorescence microscopy.[21]

There has been no unanimity in the sectioning method used. Many authors prefer sections to be longitudinal, whereas others prefer cross-sections. Both methods are seen to have their own advantages and limitations. In the present study, 60 longitudinal ground sections were prepared for age estimation, which gave an opportunity to count lines in both the cellular and acellular cementum on the same tooth. Aggarwal *et al*. (2008) and Joshi *et al*. (2010) in their studies also used longitudinal ground sections similar to our study.[9,22] Klevezal and Kleinenberg in their study advocated use of longitudinal sections for viewing the whole root surface,[23] whereas Stott *et al*. (1982) preferred cross-sections that allowed a series of observations.[24,25] Avadhani *et al*. (2009) studied 25 teeth, half of which were sectioned longitudinally and the other half group were cross-sectioned.[13]

In the present study, we used the middle third region of the root for counting cementum annulations. In the mid-root region of a tooth, the cementum present is usually acellular, undisturbed and even in growth such that annulations can be counted easily without any hindrance. Lieberman and Meadow (1992) in their study stated that annulations of acellular cementum are more easily microscopically resolved because in this region cementum is less compressed than the cementum near the cementoenamel junction (CEJ) and contains lesser cellular cementum than the root apex.[26-29] According to Huffman *et al*. (2010), the apical region of root proved to be the best area to observe and count the cementum layers. The more rapidly growing cellular cementum found at the apex of root showed the clearest layers, whereas the slower and thinner acellular cementum layers found in the middle and cervical regions were difficult to observe.[14]

In the present study, we stated that mean age error of majority of ages estimated by light microscopy method (63.33%) were within 6-20 years of the actual chronological age [Table 1]. Our findings were on a higher range when matched with those published by Stott *et al*. (1982). When the computer software was used for counting the annulations by Wittwer-Backofen *et al*. (2004), the variation between the actual and estimated age was found to be in the range of 2-3 years.[13,30] Rao *et al*. (2008) used closed-circuit television (CCTV) for counting the annulations, showing variation of 1-2 years from actual age.[13] Of the 60 specimens examined, it was stated that when phase contrast microscope was used for age estimation, majority of the subjects (70%) showed a variation of 1-2 years from the actual age [Table 1]. However, no relevant data was available in the literature to quote this parameter of our study.

In the present study, correlation of ages estimated by light, polarized, and phase contrast microscopy with actual age of subjects gave correlation values (r) as 0.347, 0.557 and 0.989 respectively [Tables 3-5]. The PPMCC is +1 in case of a perfect increasing (positive) linear relationship (correlation), and -1 in case of a perfect decreasing (negative) linear relationship. Kasetty *et al*. (2010) and Lipsinic *et al*. (1986) in their studies observed a positive correlation between estimated age and known age as $r = 0.42$ and 0.51, respectively. However, in a study by Stein, a much higher correlation between predicted age and known age was seen ($r = 0.93$).[31] Wittwer-Backofen *et al*. (2004), Solheim (1990) and Kvaal *et al*. (1995) derived correlation of cementum annulations and age as $r = 0.98$, 0.63 and 0.84 in their respective studies.[17,26,32]

Aggarwal *et al*. (2008), Dias *et al*. (2010), Jankauskas *et al*. (2001), Kvaal and Solheim (1995), Meinl *et al*. (2008) and Stein and Corcoran (1994) in their studies stated that estimates of age using cementum annulations correlated significantly with the known age in individuals under the age of 35. They also concluded that studying cementum annulations for age estimation reported a significant decrease in accuracy of the method with age, especially after the age of 40.[26] Pilloud (2004) found a correlation ($r = 0.85$) between counted lines and known age in a group of individuals aged from 21-45 years, but no correlation ($r = 0.029$) in a group of individuals aged from 57-90 years.[16]

Charles *et al*. (1986), Condon *et al*. (1986), Maat *et al*. (2006), Nagesh Kumar and Nirmala (1998), and Wittwer-Backofen *et al*. (2004) reported a well-correlated connection between cementum layering and chronological age in their studies.[10] However, Lipsinic *et al*. (1986), Lucas and Loh (1986) and Miller *et al*. (1988) did not reveal a relationship between chronological age and count of tooth cementum annulations in their studies.[16]

Some authors like Beasley *et al*. (1992), Lieberman (1994), Klevezal and Shishlina (2001), Jankauskas *et al*. (2001), Stutz (2002), Hillson and Antoine (2003), Maat *et al*. (2006), and Roksandic *et al*. (2009) have applied or tested the TCA method on archaeological material in their studies.[14] Burke (1993), Klevezal (1996), Lieberman (1994), and Wall-Scheffler (2007) in their studies stated that TCA are not only used in archaeofaunal analysis of archaeological sites to determine the age at death, but also the season of death.[26] Wedel (2007) in his study evaluated the seasonal apposition of cementum and its layered appearance.[33]

In the previous studies observers either had to count directly while looking through the microscope or had to use simple photographs. Features crucial to improvement of results include the use of various microscopic methods, like polarizing and phase contrast microscopy and digital image enhancement procedures.[22]

In the present study, we estimated the age using light, polarized, and phase contrast microscopy. It was seen that cementum annulations were more clearly visible under the phase contrast microscope as compared to the light microscope and polarizing microscope. The polarized microscope was preferred over the light microscope as it showed better discernability of the annulations.[31] Aggarwal *et al*. (2008) also found similar results in their study.[9] Joshi *et al*. (2010) and Pundir *et al*. (2009) in their study concluded that among the methods of counting incremental lines by various types of microscopy, phase contrast microscopy was more reliable for age estimation than polarizing microscope.[22,34] Kaur *et al*. (2008) found that the age estimated using polarizing microscopy showed a strong correlation with the chronological age of the individual. When cemental annulations were further studied under different imbibing media like quinoline and distilled water, they found that the visibility of cemental annulations was enhanced under the quinoline.[25] However, we did not use any imbibing media to study tooth sections under polarized microscope in our study.

Minimum errors in estimating age by our present study could be explained as follows: Method of counting cementum annulations is subjective making it prone to interobserver error; clinical data for knowing the actual age was collected from patients who may not be reliable; age of eruption of a tooth may show minor variations genetically from individual to individual; to improve distinction between tooth cementum annulations in unstained, undecalcified microscopic sections of teeth, it is recommended to cut ground sections perpendicular to the exterior of root, not perpendicular to root axis;[35] the field of focus for a specimen may vary when different microscopes are used which may induce errors in the results. Further studies can be carried out with a larger sample size to validate results of the present study.

Conclusion

In our study, we estimated the age of a subject using a light microscope, a polarized microscope, and a phase contrast microscope. A positive correlation was seen between the number of the cementum annulations and the chronological age of an individual. The PPMCC for the actual age and estimated ages suggested that incremental lines were most clearly visible under a phase contrast microscope followed by a polarized microscope and then a light microscope.

Keeping in view the limitations, we conclude from our study that the TCA method for age estimation is a promising and valuable aid for forensic identification. Further, the use of a phase contrast microscope improves the accuracy of individual age estimation, and hence its applicability has a wide potential for forensic and other legal purposes.

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