Original Article

Age Estimation with Cemental Annulation Using Light, Phase Contrast and Polarized Microscopy

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Abstract

Introduction: In forensic science, the determination of age plays a vital role in the identification of bodies and persons associated with crimes. Teeth are frequently better conserved than any other human remains, so their use for identifying age at death is vital. The root portion of the teeth is covered by a thin calcified layer called cementum, the annulations of which is considered to be helpful in age estimation. The objective of the study was to ascertain and compare the accuracy and efficiency of age estimation between light, polarized, and phase-contrast microscopy in the ground and decalcified sections of the tooth stained with hematoxylin and eosin by light microscopy and picrosirius red (PSR) by polarized microscopy. **Materials and Methods:** Fifty extracted teeth were collected and stored in a solution of 10% formalin. The middle one-third portion of the root was used, later sectioned into two halves using carborundum disc. One-half of it was used to prepare ground sections, which was studied with light, polarized, and phase-contrast microscopy. The other half was decalcified with 10% formic acid, processed, and two sections of 5 -µm thickness were prepared. One was stained with PSR stain and the second section was stained with hematoxylin and eosin (H and E) stain, One was stained with PSR stain and the second section was stained with hematoxylin and eosin (H and E) stain. Statistical analysis was performed using *Z*-test and Karl Pearson's correlation coefficient. **Results:** No statistically significant difference was observed between actual and calculated age in the ground sections, while there was a statistically significant difference observed between actual and calculated age in decalcified sections stained with H and E and PSR. A strong positive correlation was observed between actual and calculated age by Karl Pearson correlation coefficient test. **Conclusion:** Cemental annulation and phase contrast microscopy can be reliably utilized in forensic science to establishing age, especially among young and middle age group individuals.

Keywords: Cemental annulations, phase contrast microscopy, picrosirius red stain, polarized microscopy

Introduction

Forensic odontology is "that branch of forensics which is in the interest of justice and deals with proper handling and examination of dental evidence and with proper evaluation and presentation of dental findings in the interest of justice."[1] Forensic odontology mainly deals with examination, evaluation, and documentation of injuries to teeth, jawbones, oral and paraoral soft tissues, thereby facilitating identification of individuals in investigations related to crime analysis, mass disasters, age estimation, etc.[2]

Forensic scientists, anthropologists as well as archaeologists need to assess the age of the humans.[3] Determination of age plays a vital role in forensics by helping in recognition of bodies, estimation of the age of the victim and/or suspect in case of crimes.[4] It aids in cases of legal liability of teenager and person of unknown age, in adoption procedure, settling of retirement funds, as well as in research related to archaeology and paleodemography.[3] Teeth

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are frequently better conserved than any other human remains, so their use for identifying age at death could be vital.

Estimation of age using dentition is classified into three stages:

- Estimation of age in prenatal, neonatal and early postnatal period
- Estimation of age in children and adolescents
- Age estimation in adults.[5]

A thin layer of calcified tissue known as cementum, which is part of periodontium, covers the root portion of a tooth and helps in attachment of the tooth to the alveolar bone. The thickness of cementum along the length of the root being thickest at apex and thinnest at cervical region. The outer surface of the cementum gives attachment to periodontal ligament fibers, while its deeper surface is firmly adhered to dentine.[6] During cementogenesis, there is the formation of alternate layers of hyperminerlized extracellular matrix and a less mineralized layer. The first layer of acellular cementum is formed before the eruption of the tooth, and subsequent layers are added up during and after the eruption of the tooth.[7,8] Dental cemental tissue shows annual deposition, which appears as incremental lines, which are formed due to morphological change occurring when teeth respond to changes in the physiological parameters. Annual incremental layers in the cementum are the most familiar dental parameters used for estimation of age in mammals.[9]

Cementum comprises primarily of bundles of collagen fibrils, which later get mineralized by deposition of hydroxyapatite crystals, the changing orientation of which might be responsible for an optical effect, resulting in the appearance of alternate dark and translucent layers.[7,8] the alternating bands are seasonal rhythms, which occur mostly under the influence of several factors such as ultraviolet (UV) radiations, climatic conditions, and different qualities of diet and hormonal status of individuals.[8,10,11]

Till now, different microscopical methods using both ground and decalcified sections of the tooth for estimating age have been done which shows ground sections and phase-contrast microscopy have better accuracy than any other type of microscopy. The aim of the present study was to ascertaining and correlate accuracy and efficiency of age estimation by Cemental annulations between ground sections studied with light, polarized and phase-contrast microscopy with decalcified Hematoxylin and eosin (H and E) stained sections by light microscopy and decalcified Picrosirius red (PSR) stained sections by polarized microscopy.

Materials and Methods

Fifty extracted teeth were collected, which were stored in a solution of 10% formalin. The age of the individuals, any medical history, past dental history was recorded at the time of extraction of the tooth, and written consent was obtained. Each tooth was assigned a number to prevent bias during age estimation. The exclusion criteria were teeth with hypercementosis, periapical pathology, gingival recession, root caries, and third molars. Inclusion criteria were permanent teeth indicated for extraction, for any other reason (permanent lower premolars) was included in the study.

Method of preparation of sections

The teeth were first cleaned with running tap water. The middle one-third of root was used for the study, which was sectioned into two halves by using carborundum disc. One-half was used to prepare ground sections (cross-sections) manually using Arkansas stone, and other half was decalcified with 10% formic acid, rinsed, dehydrated, and embedded in paraffin. The endpoint of decalcification was checked by the chemical method. Five micron thick sections were prepared from these decalcified samples using a semi-automatic microtome. One of the sections was stained with 0.1% PSR and another section was stained with H and E.

Age estimation

The Ground sections were studied under light, polarized and phase-contrast microscopy [Figures 1-3], while the decalcified sections stained with H and E were studied with light microscopy [Figure 4] and PSR stained sections with polarized microscopy [Figure 5] under ×20 objective using Olympus research microscope (Model No: BX43). In each section area where incremental lines were clearly visible was selected and photographed. The total cementum thickness and width of clearly visible three incremental lines were measured utilizing image analysis software. (Progres, Speed XT core 3, Jenoptik).

The age estimation was obtained using the following formula,

$$\text{Estimated age} = \frac{\text{Total cementum thickness in } \mu m}{\text{Average width of incremental line in } \mu m} + \text{Eruption age of the tooth}$$

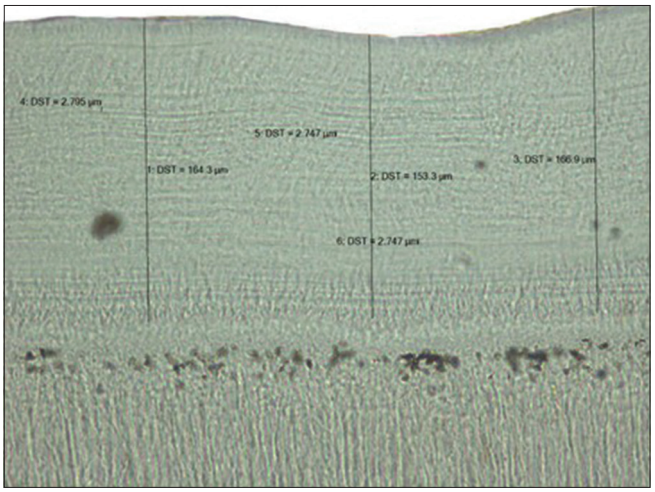


Figure 1: Photomicrograph of ground sections showing incremental lines of cementum with measurements under bright field microscopy (×20)

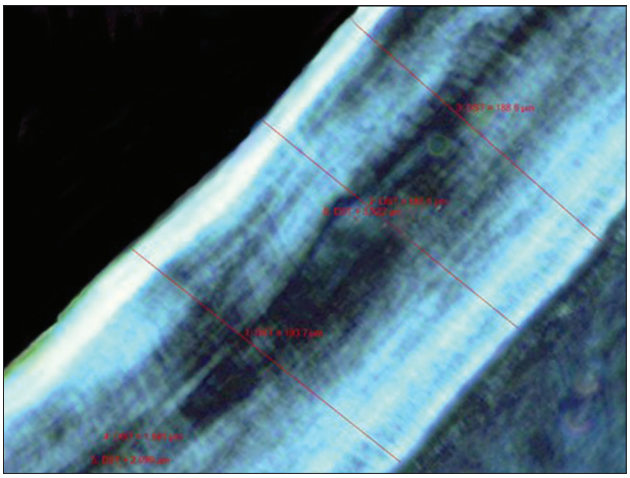


Figure 2: Photomicrograph of ground sections showing incremental lines of cementum with measurements under polarized microscopy (×20)

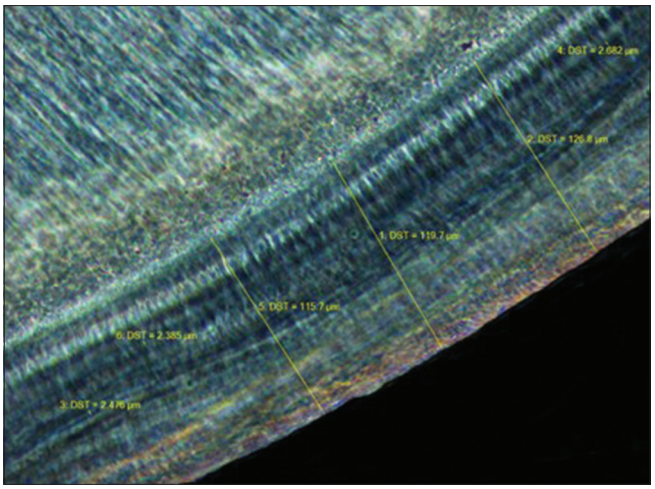


Figure 3: Photomicrograph of ground sections showing incremental lines of cementum with measurements under phase contrast microscopy (×20)

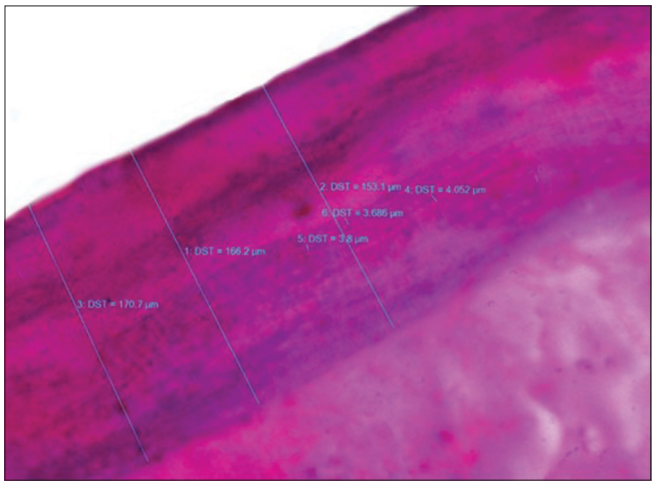


Figure 4: Photomicrograph of decalcified H and E stained sections showing incremental lines of cementum with measurements under bright field microscopy (×20)

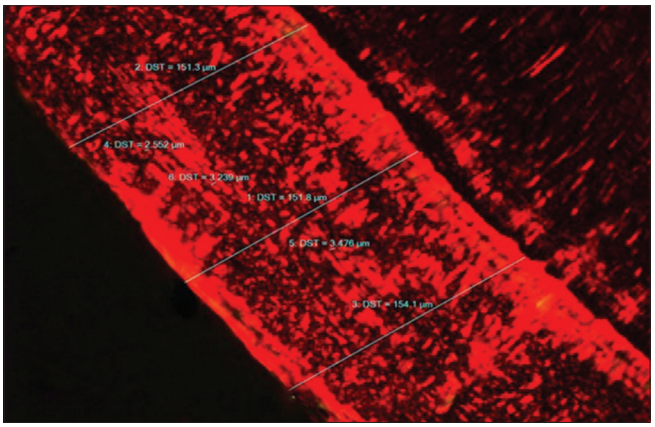


Figure 5: Photomicrograph of decalcified Picrosirius red stained incremental lines of cementum with measurements under polarized microscopy (×20)

The obtained data were tabulated and subjected to statistical analysis using *Z*-test and Karl–Pearson correlation coefficient between actual and estimated age.

Results

Fifty ground sections were studied under light, polarized, and phase contrast microscope for evaluating incremental lines of cementum to estimate the age. *Z*-test was applied to compare mean and standard deviation (SD) values of calculated age with actual age, which indicated that there was no statistically significant difference observed between actual and calculated age in ground sections [Table 1].

Fifty decalcified sections prepared from the same teeth were stained with H and E and PSR stain and were also studied under light and polarized microscope respectively to evaluate age from incremental lines of cementum. *Z*-test was applied to compare mean and SD values of calculated age with actual age, which indicated a statistically significant difference between actual and calculated age in decalcified H and E and PSR stained sections [Table 1].

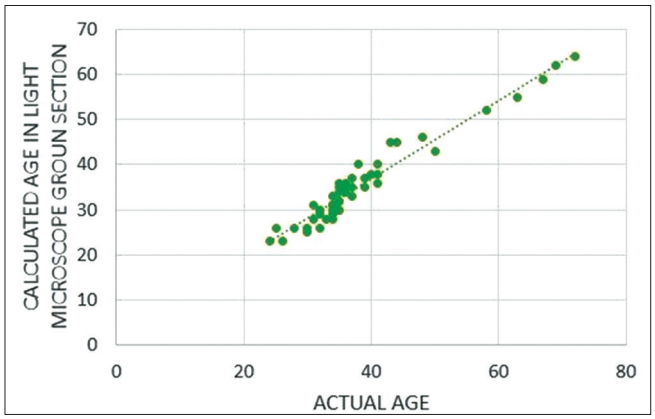
Correlation co-efficient was estimated for both ground sections of teeth by light, polarized and phase-contrast microscopy [Graphs 1-3] and decalcified sections stained with H and E under light microscopy and PSR stained sections under polarized microscopy [Graphs 4 and 5] using Karl Pearson correlation coefficient test which indicated a strong positive correlation between actual and calculated age [Table 1].

Discussion

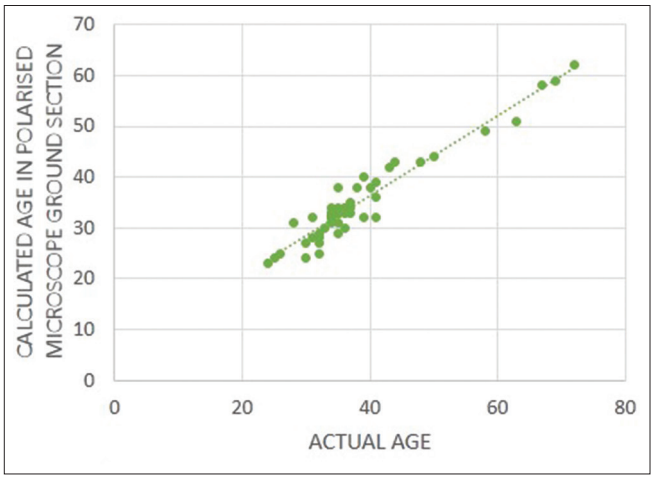
Person identification is a challenging task in situations where dead bodies are destroyed beyond recognition. Science and technology is being used by the forensic branch of medicine for dealing in such crime investigations and delivering justice. In

Table 1: Calculated age of ground and decalcified sections under different microscopy

	Calculated age by light microscope in ground sections	Calculated age by polarized microscope in ground sections	Calculated age by phase contrast microscope in ground sections	Calculated age by light microscope in decalcified h & e stained sections	Calculated age by polarized microscope in decalcified psr stained sections
CALCULATED AGE (MEAN±SD)	35.46±9.51	35±8.66	37.8±9.54	33.16±8.86	34.44±9.21
ACTUAL AGE (MEAN±SD)	38.34±10.60	38.34±10.60	38.34±10.60	38.34±10.60	38.34±10.60
Z TEST VALUE	-1.43	-1.73	-0.27	-2.65	-1.96
P VALUE	0.15	0.08	0.79	0.01	0.05
SIGNIFICANCE	Not significant	Not significant	Not significant	significant	Significant
KARL PEARSON'S CORRELATION COEFFICIENT (R)	0.9728	0.9617	0.9863	0.9652	0.9705
(correlation between calculated age & actual age)	Strong positive correlation	Strong positive correlation	Strong positive correlation	Strong positive correlation	Strong positive correlation



Graph 1: Correlation coefficient between actual age and calculated age in light microscopy of ground sections



Graph 2: correlation coefficient between actual age and calculated age in polarized microscopy of ground sections

recent years, significance of utilization of dental identification methods in forensic is on the rise.[2]

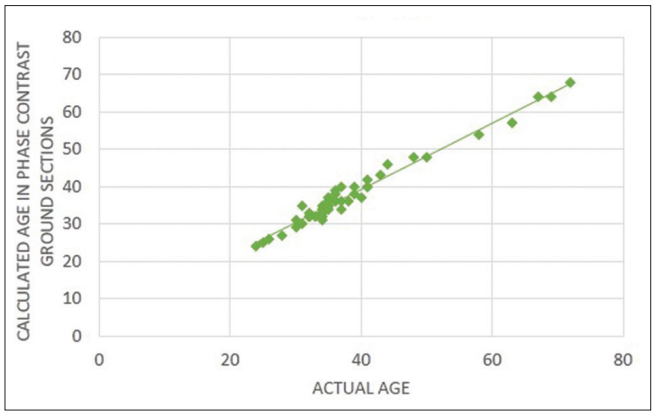
Studies show that cementum annulation is more reliable in age estimation than skeletal and morphological characteristics.[2] Cementum is a unique avascular mineralized tissue that facilitates anchoring of periodontal ligament fibres to the root. It is believed that the cementum grows continuously thick during lifetime.[10]

The thickness of cementum increases around three times in the age between 16 and 70. In acellular cementum, incremental lines are thin, even and have a tendency to be closely organized. The cellular cementum is formed more rapidly, and the incremental lines here are thick, irregular, and farther apart. Alteration in the degree of mineralization is the main reason for the appearance of incremental lines in cementum.[10] Acellular extrinsic fiber cementum varies in thickness from 20 to 250 μm depending on the age.[3]

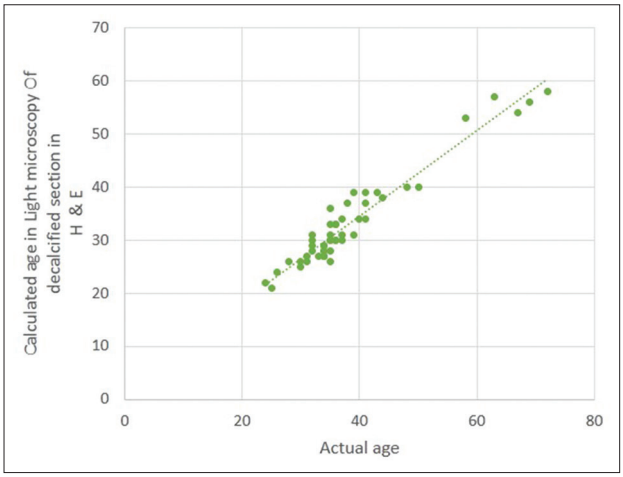
Pair of alternate light and dark bands depicts an incremental line which is deposited annually.[9] Variation in cementogenesis could lead to a change in the appearance of annulations, which might be induced due to reasons such as changes in ecological condition, i.e., altitude, temperature, humidity, UV light, pollution, biomechanical force, nutrition, and hormonal changes.[8,10,12] Different species worldwide show the appearance of cemental annulations mainly due to metabolic rhythm associated with seasonal variations. The main reason behind this is parathyroid hormone metabolism, which in association with Vitamin D, in turn regulates the calcium resorption.[8]

Alternate dark and light are layers with the difference in optical properties, which is mainly due to variation in cementum formation at different phases. The optical effects of alternating dark and translucent layers in cementum could be due to changes in the orientation of uncalcified dense bundles of collagen fibers.[13]

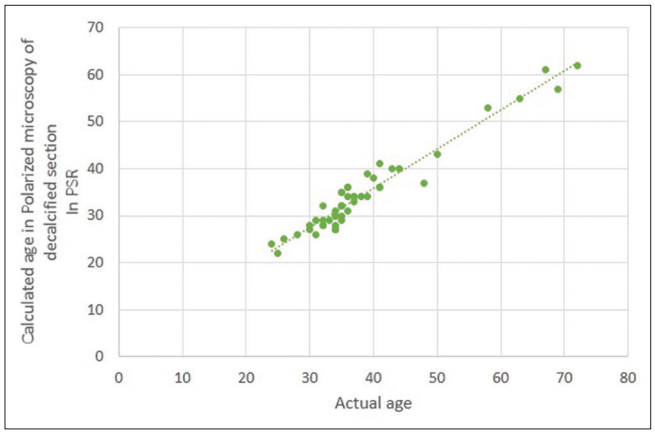
In the past decades, different researchers have used these optical properties of cemental annulations for establishing age in various animals such as otter, caribou, moose, squirrel, bear,



Graph 3: Correlation coefficient between actual age and calculated age in phase contrast microscopy of ground sections



Graph 4: Correlation coefficient between actual age and calculated age in light microscopy of decalcified sections stained with H and E



Graph 5: Correlation coefficient between actual age and calculated age in polarized microscopy of decalcified sections stained with Picrosirius red

dear, and bat.[4,15] Since then, various researchers used cemental annulations for establishing the age in the human population and suggested that tooth cementum annulations method can be used to establish age in humans.[4,9,13-18]

In our study, among the different microscopy used to study ground sections, phase contrast microscopy was better than other types of microscope, which showed a mean difference of 1 year between actual age and calculated age, while light and polarized microscopy showed a mean difference of 2.88 and 3.34, respectively. The Karl Pearson's correlation coefficient was 0.97, 0.96, and 0.98 for light, polarized, and phase-contrast microscopy, respectively, suggesting a strong positive correlation between actual and estimated age similar to study done by Condon *et al*. In their study, higher correlation was not because of the wider age range of study population (13–79 years). The exclusion of the study population younger <18 years and >70 years also produced a correlation coefficient of 0.93.[18]

Various investigators such as Pundir *et al*.,[18] Joshi *et al*.[13] reported cemental annulations to be more clearly evident with phase-contrast microscopy than any other types of microscopy for estimating the human age. In the present study, two cases showed age estimation differences of 6 and 5 years, which were of elderly individuals of 63 and 69 years, respectively. These can be due to periodontal problems of the individuals which occur due to aging that may interfere with the cementum deposition.[3] With increasing age, higher is the inaccuracy of age estimation in the case of both males and females. As the cemental annulation is interrelated with sharpey's fibers, with declining in periodontal health, it is possible that sharpey's fibers lose their functional importance and degenerate due to reduced alveolar bone which may, in turn, cause arrest in cemental annulation deposition.[4,19,20]

Studies show that after 60 years of age, the cemental apposition diminishes by one third.[21] Michaela Huffman reported that the estimation of age in older individuals by cemental layers is not possible as it underestimates the age of the individuals.[22] In the present study, specimens from middle age group adults showed better age estimation than that from the elderly age group, which was also reported in other studies.[14,23]

In the present study, under phase-contrast microscopy, 10 cases showed an average of 2 years of overestimation of the age than the actual age. Such deviation in age estimation can be due to early or late eruption of teeth and disturbances in calcium metabolism. Such alterations could affect whole dentition, which can be observed by comparing the results of different teeth from the same individual.[8,13]

In the present study, demineralized sections from the mid-root comprising acellular cementum was stained with H and E and PSR to estimate individuals age, which showed $r = 0.96$ and 0.97, respectively, suggesting that PSR stained sections were better than H and E stained sections to estimate the age of the individual.

Cementum is more intensely birefringent than dentin and less compared to enamel under polarized microscope.[24,25] The organic portion of the cementum consist of mainly type I collagen.[26] Collagen being birefringent, and this property is

mainly due to ground substance acid mucopolysaccharides, which are also anisotropic.[24] Collagen consists of a basic amino group that reacts with acidic dye exclusively. It is seen that Sirius red dye has anisotropic molecular structure and when it binds in an orderly fashion with collagen, it enhances its birefringence.[27] Thus PSR staining in conjunction with polarizing microscopy is of value in histopathological studies, as PSR greatly enhances the birefringence of birefringent structures.[25]

Conclusion

Recent studies indicate that the dental cementum annulations could be used reliably than any morphologic or histologic methods using adult skeleton for estimation of age. Hence, on the basis of the present study, it is indicative that phase-contrast microscopy in ground sections can be more reliable to estimate the age of the humans in the field of forensics and in the demineralized sections, PSR stained sections showed better results than the H and E stained sections; however, further studies have to be done to prove the reliability of estimating the age from PSR stained sections.

Ethical Approval

The study is approved by research ethical committee, Sri Siddhartha Dental College and Hospital, Agalakote, Tumkur, Karnataka, India on 20/10/2014. The written informed consent was obtained by the participants for participation in our study and use of patient data for research and educational purposes. We would also state that the procedures in our study have not violated the guidelines laid down in the Declaration of Helsinki (2013).

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Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Rakesh G, Singh A. Age estimation by Gustafson's method and its modifications. J Indo Pac Acad Forensic Odontol 2010;1:12-9.
2. Saxena S, Sharma P, Gupta N. Experimental studies of forensic odontology to aid in the identification process. J Forensic Dent Sci 2010;2:69-76.
3. Dias PE, Beaini TL, Melani RF. Age estimation from dental cementum incremental lines and periodontal disease. J Forensic Odontostomatol 2010;28:13-21.
4. Aggarwal P, Saxena S, Bansal P. Incremental lines in root cementum of human teeth: An approach to their role in age estimation using polarizing microscopy. Indian J Dent Res 2008;19:326-30.
5. Acharya AB, Sivapathasundharam B. Forensic odontology. In: Acharya AB, Sivapathasundharam B, editors. Shafer's Textbook of Oral Pathology. 6th ed. Delhi: Elsevier; 2009. p. 883-9.
6. Charan Gowda BK, Reddy S, Kokila G, Pradeep L. Cemental annulation and phase contrast microscope; tool for age estimation. J South India Medico Legal Assoc 2014;6:9-13. Available from: http://v5.books.elsevier.com/bookscat/samples/9780723431817/9780723431817.pdf. [Last accessed on 2020 Jun 04].
7. Rai B, Dhattwal SK, Bhardwaj DN, Anand SC. Coronal displacement of cementum in impacted teeth in age determination. World J Med Sci 2006;1:117-8.
8. Wittwer-Backofen U, Gampe J, Vaupel JW. Tooth cementum annulation for age estimation: Results from a large known-age validation study. Am J Phys Anthropol 2004;123:119-29.
9. Stott GG, Sis RF, Levy BM. Cemental annulation as an age criterion in forensic dentistry. J Dent Res 1982;61:814-7.
10. Lieberman DE. The biological basis for seasonal increments in dental cementum and their application to archaeological research. J Archaeol Sci 1994;21:525-39. Available from: https://scholar.harvard.edu/files/dlieberman/files/1994b.pdf. [Last accessed on 2020 Jun 04].
11. Radović MB. Ageing in the Danube gorges population (9500-5500 BC): Tooth cementum annulation method. Starinar 2012;62:9-18 Available from: https://pdfs.semanticscholar.org/c454/8fe70c634ddbad5dc286d6810ee6f10e6a66.pdf?_ga=2.19260728.1428945003.1578981461-428727317.1557882185. [Last accessed on 2020 Jun 06].
12. Kagerer P, Grupe G. Age-at-death diagnosis and determination of life-history parameters by incremental lines in human dental cementum as an identification aid. Forensic Sci Int 2001;118:75-82.
13. Joshi PS, Chougule MS, Agrawal GP. Comparison of polarizing & phase contrast microscopy for estimation of age based on cemental annulations. Indian J Forensic Odontol 2010;3:87-95.
14. Lipsinic FE, Paunovich E, Houston GD, Robison SF. Correlation of age and incremental lines in the cementum of human teeth. J Forensic Sci 1986;31:982-9.
15. Avadhani A, Tupkari, JV, Khambaty A, Sardar M. Cementum annulations and age determination. J Forensic Dent Sci 2009;1:73-6.
16. Charles DK, Condon K, Cheverud JM, Buikstra JE. Cementum annulation and age determination in Homo sapiens. I. Tooth variability and observer error. Am J Phys Anthropol 1986;71:311-20.
17. Condon K, Charles DK, Cheverud JM, Buikstra JE. Cementum annulation and age determination in Homo sapiens. II. Estimates and accuracy. Am J Phys Anthropol 1986;71:321-30.
18. Pundir S, Saxena S, Aggrawal P. Estimation of age based on tooth cementum annulations using three different microscopic methods. J Forensic Dent Sci 2009;1:82-7.
19. Jagannathan N, Neelakantan P, Thiruvengadam C, Ramani P, Premkumar P, Natesan A, *et al*. Age estimation in an Indian population using pulp/tooth volume ratio of mandibular canines obtained from cone beam computed tomography. J Forensic Odontostomatol 2011;29:1-6.
20. Shukla D, Shukla D, Vinuth DP, Sowmya SV, Jeevan MB, Kale AD, *et al*. Cementum made more visual. J Forensic Odonto Stomatol 2012;30:29-37. Available from: http://www.iofos.eu/Journals/JFOS%20Jun12/44comp.pdf. [Last accessed on 2020 Jun 06].
21. Soleim T. Dental Cementum opposition as an indicator of age. Scand J Dent Res 1990;98:510-9.
22. Huffman M, Antoine D. Analysis of cementum layers in archaeological material. Dent Anthropol 2010;23:67-73.
23. Miller CS, Dove SB, Cottone JA. Failure of use of cemental annulations in teeth to determine the age of humans. J Forensic Sci 1988;33:137-43.
24. Wolman M. Polarized light microscopy as a tool of diagnostic pathology. J Histochem Cytochem 1975;23:21-50.
25. Roush JK, Breur GJ, Wilson JW. Picrosirius red staining of dental structures. Stain Technol 1988;63:363-7.
26. Kumar GS. Cementum. In: Orban's oral Histology & Embryology. 14th ed., Ch. 7. New Delhi: Elsevier; 2015. p. 116-32.
27. Whittaker P, Kloner RA, Boughner DR, Pickering JG. Quantitative assessment of myocardial collagen with picrosirius red staining and circularly polarized light. Basic Res Cardiol 1994;89:397-410.