ISSN 2219-6749

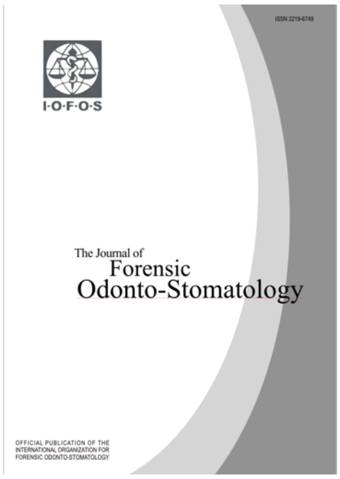
I-O-F-O-S

The Journal of Forensic Odonto-Stomatology

Volume 38, n. 3 - Dec 2020

OFFICIAL PUBLICATION OF THE
INTERNATIONAL ORGANIZATION FOR
FORENSIC ODONTO-STOMATOLOGY

JFOS
ISSN: 2219-6749



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The Journal of Forensic Odonto-Stomatology

Vol 38 n. 3 Dec 2020

CONTENTS

Child brides: the age estimation problem in young girls

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The authors declare that they have no conflict of interest.

KEYWORDS

Child brides,
Dental age estimation,
Forensic dentistry,
Third molar maturity,
Adult age,
Forensic sciences.

J Forensic Odontostomatol
2020. Dec;(38): 3-2:7
ISSN :2219-6749

ABSTRACT

The aim of this work is to study a sample of girls from 15 different countries using Third Molar Maturity Index (I_{3M}), to assess the probability that a girl has reached the legal age of 18 years. The studied sample consisted of 3228 Orthopantomograms of healthy female subjects from 15 different countries. The cut-off value of I_{3M} = 0.08 was tested to discriminate adults (≥18 years) and minors (<18 years). X-ray images were processed by computer-aided drafting program ImageJ. The information on sensitivity and specificity of I_{3M} coming from the 15 countries was pooled together using a bivariate Bayesian modeling approach. Specificity of the I_{3M} test did not change when the country was considered, and its value remains greater than 85% for each studied country. This method is useful to estimate the age of the girls involved in suspected early marriage because of the high probability of correctly identifying a minor with similar results observed among tested populations.

INTRODUCTION

There is an increasing need in estimating age for forensic purposes in recent years due to illegal immigration that not only affects Europe but countries such as the United States and more recently countries in South America such as Argentina and Colombia.[1] In this case subjects without documents can claim to be older or younger than their actual age, proof of being under or over the legally defined age limits is a requirement for several legal decisions including social benefits and protection of rights. When it is not possible to ascertain the given age of an individual, authorities can request a medical age assessment issued by an expert.[2]

According to the United Nations Children's Fund (UNICEF), 230 million children under the age of 5 still have not been registered [3] and 2018 statistics from the World Bank estimated that one billion people around the world struggle to prove who they are due to a lack of legal identification.[4]

The information suggests that this is not always the result of document falsification, but opportunistic fabrication as well. For this reason, the choice of using not only radiographic methods but interviews and social history reconstruction have prevailed to estimate whether adolescents have reached legal age.[5]

To an equal extent, the issue of a lack of documents is linked to a high number of people modifying their age in order to work in competitive sports, practising prostitution or marriage.[6,7] This last phenomenon is more commonplace than would be expected. Child marriage is defined by UNICEF as any formal marriage or informal union where one or both of the parties are under 18 years of age.[8] According to this organization, 12 million under-age girls are married every year. This is recognized as a global problem that cuts across countries, cultures, religions and ethnicities. It is not uncommon to find child brides in every region of the world.[9]

Figures from UNICEF show that in Bangladesh only 20% of children under the age of 5 are registered and 59% of women aged 20-24 years were married or in union by age 18. In Niger 64% of children under the age of 5 are registered, and 76% of women are married by age 18. In Chad only 12% of children under the age of 5 are registered, while 67% of women are married by age 18.[10,11] Although there is not a direct relationship in every country, it is evident that a lack of coverage on birth registration favours and facilitates the practice of child marriage.

Clearly there is a need to address the cultural choices that lead to these precocious marriages. However, in cases where the country has a clear legislation forbidding this practice, what is mainly required is to have the means of knowing even an approximate age of the girl. Legal age is typically reached at 18 years around the world. [12]

There are several methods that can be used to ascertain adulthood, however of the three most common - those being skeletal, psychological, and dental estimations - the latter has proven to be the most reliable. [13-15]

In 2008 Cameriere et al developed a method named The Third Molar Index (I_{3M}) for age estimation by observing the formation and closure of the apex of the third molar and established a cut-off value of 0.08 that has been tested in several populations.[16]

The aim of this work is to study a sample of girls from 15 different countries using I_{3M} to assess the probability that a girl has reached the legal age of 18 years old or not.

MATERIAL AND METHODS

The studied sample consisted of 3228 healthy female subjects from 15 countries (AL = Albania; AUS= Australia; CHN= China; CO= Colombia; DOM= Dominican Republic, ET= Egypt; F = France; I=Italy ; IND=India ; J= Japan; PL = Polonia; RCH= Chile; SRB= Serbia; TR=Turkey; ZA= South Africa) from whom a panoramic radiograph was collected (Table 1).

The inclusion criteria were as follows: age between 14 and 24 years at the time the panoramic radiographs were obtained, good-quality radiographs, and healthy subjects with known precise age and free of systemic disorders. The exclusion criteria were as follows: unclear radiographs or with radiographic distortion, gross pathology or history of orthodontic treatment, subjects of unknown age or without full dental records, and those with no third molars or third molars with developmental anomalies. Patient data was recorded in an excel file, recording patients' identification number, sex, date of birth, and date of the X- rays. The CA (chronological age) for each subject was calculated by subtracting the date of the X-rays from the date of birth and converted into decimal ages. The study was carried out in accordance with the ethical standards laid down by the Declaration of Helsinki (Finland).[17]

Measurements

As already proposed by Cameriere et al.[16] the dental maturity index (I_{3M}) of the left lower third molar was evaluated.

If the apices of the third molar were completely closed, I_{3M} is equal to = 0; if the apices were not completely closed, the sum of the distance between the inner part of the two apices are divided by the length of the third molar. The cut-off value of I_{3M} = 0.08 was tested to discriminate adults (≥18 years) and minors (<18 years). X-ray images were processed by computer-aided drafting program ImageJ.

Statistical analysis

In this work we combine the information on sensitivity and specificity of the Cameriere's test from the 15 countries pooling them using a bivariate Bayesian modeling approach.[18]

RESULTS

The frequency distribution of the individuals among the countries shows a minimum of 58 females from Australia and a maximum of 499 from South Africa (Table 1).

Table 1. Frequency distribution of individuals among the considered countries

country	AL	AUS	CHN	CO	DOM	ET	F	I	IND	J	PL	RCH	SRB	TR	ZA
frequency	152	58	99	161	285	135	85	315	114	134	435	330	271	155	499

The age distribution in the sample is almost homogeneous in the range 14 – 24 years (Table 2). On the contrary, about 45% of the individuals belong to the first I_{3M} interval [0, 0.08]. This result can be explained by considering that the first class includes all females with closed apices which are about 31% of the sample. Using the Bayesian model, we obtained a pooled estimate for both sensitivity and specificity of the test (Table 3).

Table 2. I_{3M} values and age distributions of the overall sample. The age classes are closed on the left while I_{3M} classes are closed on the right. The first class of I_{3M} is closed both on the left and right.

	Age(years)					
I_{3M}	[14,16)	[16,18)	[18,20)	[20,22)	[22,25)	total
[0,0.08]	13	61	401	600	391	1466
(0.08,0.22]	90	250	141	48	16	545
(0.22,0.4]	160	186	52	28	5	431
(0.4,0.7]	188	157	38	5	0	388
(0.7,1]	157	94	19	5	0	275
(1,4.8]	81	32	6	2	2	123
total	689	780	657	688	414	3228

There is a significant difference in the sensitivity distribution among the countries; for example, France and Italy show a significant lower sensitivity.

On the contrary, specificity of Cameriere's test does not change when the country is considered, and its value remains greater than 85% for each considered country. Its value is greater than 90% in Albania, China, Dominican Republic, Italy, India, Serbia and Turkey. A pooled analysis of the studies allowed us to summarize sensitivity and specificity of I_{3M} method as reported in Table 3.

Combining the considered studies, we obtained the sensitivity a pooled estimate for the median of 79% ($Q_{2.5\%}$ =71%; $Q_{97.5\%}$ =85%) and for the specificity a pooled median of 96% ($Q_{2.5\%}$ =93%; $Q_{97.5\%}$ =99%).

DISCUSSION

Age estimation is required to confront several social issues in many countries however despite the different situations in which estimating 18 years old is needed, it is illegal immigration which is considered most important.

To address these issues, Cameriere published several articles with samples from different countries studying the third molar through I_{3M}.[16] The results yielded by this research have been positive to the extent of attempts to establish it as regular practice used by professionals.

As in cases of illegal immigration, there are other situations that require the assistance of forensic dentistry in the determination of adult age. One of these is the problem of child brides. It is a cultural issue of great importance that sometimes clashes with national laws. In fact, some countries even allow marriage between minors[10,12,19]

Table 3. Quantile of order 2.5%, 50% (median) and 97.5% of the sensitivity and specificity distribution for each considered country.

	Sensitivity			**Specificity**		
country	$Q_{2.5\%}$	$Q_{50.0\%}$	$Q_{97.5\%}$	$Q_{2.5\%}$	$Q_{50.0\%}$	$Q_{97.5\%}$
AL	66%	76%	85%	92%	96%	98%
AUS	70%	83%	92%	85%	94%	97%
CHN	66%	76%	84%	92%	97%	99%
CO	86%	93%	97%	90%	95%	98%
DOM	85%	90%	94%	95%	98%	99%
ET	72%	81%	87%	90%	96%	98%
F	32%	47%	62%	87%	94%	98%
I	57%	65%	72%	93%	96%	99%
IND	69%	80%	88%	91%	95%	98%
J	70%	78%	85%	87%	94%	98%
PL	78%	82%	86%	90%	94%	97%
RCH	55%	63%	71%	86%	91%	94%
SRB	80%	86%	90%	93%	97%	99%
TR	78%	85%	92%	94%	98%	100%
ZA	76%	82%	86%	93%	96%	97%
pool	71%	79%	85%	93%	95%	97%

In reviewing the many factors that influence early marriage, it stands out how the lack of birth registration facilitates the persistence of the problem. As observed by the Committee on the Elimination of Discrimination against Women (CEDAW), birth registration is an important factor that can support the effective implementation and enforcement of laws on the minimum age of marriage.[7] The lack of reliable documents or birth certificate poses an overwhelming challenge even in countries where the legal age for marriage is 18 years. Without the means to estimate this, these girls cannot be protected by the law.

This study has shown how the use of the I_{3M} method, when third molar is present, can be a practical and reliable way of estimating adult age. When applying the test in the samples from 15 different countries there were not significant differences in correctly classified minors that could be associated to ethnicity or the country of origin. In fact, the specificity, number of correctly classified minors, displayed a homogeneous result with a minimum value of 91% from the Chilean sample and a maximum of 98% from Dominican Republic without evidencing a trend linked to the place of origin.

There can be different causes for the heterogeneity of specificity among nations. For example, the inter-rater variability in evaluating I_{3M} smaller than 0.08, on the contrary, it is easier to estimate I_{3M} greater than 0.08, in other words, it is easier to obtain false negative than false positive measurement.

The pooled estimate of the specificity, 95%, pointed out the low false positive frequency in using I_{3M} method. Concerning the sensitivity, number of correctly classified adults, the minimum sensitivity value was 47% from the French sample and the maximum was 93% in Colombia which emphasizes the lack of an ethnical trend. It is important to highlight that in our studies of age estimation using the third molar, it is vital to have an optimal specificity which means that I_{3M} can correctly identify a minor.

The results are in line with observations made by Liversidge et al,[20] where they found small differences among world groups when studying the timing of root development in mandibular third molars suggesting that probably it is unnecessary to have population specific reference data to estimate age of an individual using mandibular third molar root formation.

CONCLUSIONS

This method is useful to determine the adult age of girls involved in suspected early marriages because of the high probability of identifying a minor with similar results observed among tested populations.

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Evaluation of Cameriere and Willems age estimation methods in panoramic radiographs of Brazilian children

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The authors declare that they have no conflict of interest.

KEYWORDS

Age Determination by Teeth, Dental Research, Forensic Odontology, Panoramic Radiography.

J Forensic Odontostomatol 2020. Dec;(38): 3-8:15
ISSN :2219-6749

ABSTRACT

The importance of age estimation in the forensic field is inherent to the process of establishing the biological profile of children, sub-adults and adults. The established profile might be useful for the identification of deceased victims or living individuals when it comes to age of legal interest. In parallel, age estimation is also investigated for clinical purposes, especially for the diagnosis of dental and bone maturation. Several studies were developed to provide accurate age estimation methods based on skeletal and dental development. This study aimed to apply and compare Cameriere's and Willems' methods for dental age estimation in a Brazilian sample. Two examiners performed image analysis and method application in 180 panoramic radiographs of Brazilian children aged 6-14 years old. The ages estimated with both methods revealed a good correlation with the chronological ages of Brazilian boys and girls. Cameriere's method showed a slight underestimation of 0.05 years for girls and 0.03 for boys. Willems' method, on the other hand, showed an overestimation of -0.47 years for girls and -0.39 for boys. Better age estimates were obtained combining the outcomes of both methods. In practice, Cameriere's and Willems' methods reached reliable outcomes and could be applied for dental age estimation purposes.

INTRODUCTION

Age estimation is fundamental in civil justice, especially when it is used in adoption cases[1] or applied for asylum seekers,[2] and even for any kind of questioned civil registration.[3] In the criminal scenario, age estimation contributes to building anthropological profiles of victims[4-7] and also supports investigations of alleged minor offenders.[8]

The process of estimating age in the living increased over time given the several issues inherent to the different countries worldwide, such as authoritarian policies, civil wars and extreme poverty. Countries that offer entry to those in need have the important role of providing personal documents and possibilities to work, and access to health and education. In this context, age estimation becomes a tool to assure human rights.[9,10] In South America, Brazil is the country that shelters the highest number of refugees.[11]

Cameriere et al.[12] designed a quantitative approach through a formula based on sex and the ratio between length and apex opening measurements of each lower left tooth. Based on the staging technique developed by Demirjian et al. (based on the

classification of dental maturation stages),[13] Willems et al.[14] designed a method able to reach more accurate outcomes within a less time consuming process.
This study aimed to conduct an initial study of the application of Cameriere's and Willems' methods in a sample of digital panoramic radiographs from Brazilian children aged from 6 to 14 years. A secondary study focused on analyzing eventual differences in dental maturation between boys and girls.

MATERIAL AND METHODS

This project was approved by the Research Ethics Committee (Protocol: 06634919.7.0000.5419).
A total of 180 digital panoramic radiographs were selected: 90 girls and 90 boys from 6 to 14 years old (10 female and 10 male radiographs for each age, totaling 20 radiographs for each year) to conduct an initial study of the application of age estimation methods. The inclusion criteria were high image resolution and presence of the lower left teeth from central incisor to second molar. Digital panoramic radiographs that showed low image resolution, dental developmental changes, absence or fractures of the left lower teeth were excluded from the sample.
The radiographs were coded and tabulated in a Microsoft Excel® spreadsheet (Microsoft, Washington, USA) with respective code, gender, date of birth, date of X-ray taking and age in years. To apply the methods, the examiners had access only to the panoramic radiographs and their corresponding codes.
Both samples were analyzed by two previously calibrated examiners, who estimated the ages using Cameriere's method (metric) and Willems' method (non-metric). In addition, the estimated age by Cameriere's method was added to the estimated age by Willems' method and the final value divided by two, resulting in the third value used for statistical calculations: the mean age between the methods. The examiners repeated both methods in 30% of the sample after four weeks – radiographs from 30 girls and 30 boys, for intra-examiner analysis.
To get the necessary measurements to apply Cameriere's method (distance between the internal sides of the open apex and the total length of the dental element - reduce the possible differences between angulations and distortions of the radiographs), the software ImageJ was used (Wayne Rasband; National Institutes of Health, USA).
For statistical analysis, the following software was used: R Core Team (R Foundation for Statistical Computing, Vienna, Austria) and SAS Statistical Software v9.3 (SAS Institute, Cary, North Carolina, USA). Agreement analysis was calculated via Intraclass Correlation Coefficient (ICC) with 95% confidence interval, in order to verify if the evaluators were calibrated and if the age estimation methods were reproducible.
ICC was also used to verify the agreement between the outcomes of mean estimated age in Cameriere's method and Willems' method compared to the chronological age of the sampled children. To calculate the agreement and magnitude of the differences between the chronological and estimated ages, the Bland-Altman method and a Linear Mixed-Effects Regression were used.
The estimated differences were calculated from chronological age (chronological age less the age estimation for each method), in other words, when the estimated age was greater than the chronological age, the result was negative. The result was positive when there was an underestimation by the method, because the estimated age was subtracted from the chronological age, resulting in a positive value.

RESULTS

To verify the reproducibility of the methods and the calibration of the examiners, ICC was calculated with its respective 95% confidence interval. Considering that the ICC values approached 1: 0.99 and 0.98 for girls according to examiner 1; 0.98 and 0.96 according to examiner 2, using Cameriere's and Willems' methods respectively. For boys, the ICC resulted in a value of 1 and 0.99 according to examiner 1; 0.94 and 0.95 according to examiner 2, using Cameriere's and Willems' methods respectively.
A high correlation was detected between the ages estimated by each examiner in the main analysis and 4 weeks later. The methods were reproducible and the examiners were calibrated.
To assess whether or not the estimated age was close to the chronological age (using both methods), the ICC was calculated with its respective 95% confidence interval. The mean ages of Cameriere's and Willems' methods were considered during this analysis. The ICC values for girls and boys were close to 1: 0.94 for girls

according to examiner 1 for both methods; 0.93 and 0.94 according to examiner 2, using Camereire's and Willems' methods respectively. For boys, the ICC resulted in a value of 0.92 and 0.94 according to examiner 1; 0.93 and 0.94 according to examiner 2, using Camereire's and Willems' methods respectively. In addition, the mean presented an ICC of 0.96 for girls for both examiners and a value of 0.94 for boys according to examiner 1 and 0.95 according to examiner 2. These results suggest that both methods were able to provide age estimates that were close to the chronological age. Interestingly, the ICC was higher when the mean ages estimated from both methods were combined.

To calculate the difference between chronological and estimated ages, Bland-Altman graphs were designed, which demonstrate a statistical graphical analysis for the comparison of the two methods (in this case, Cameriere's method or Willems' method and the chronological age). The central axis is the mean of the differences between the two measurements, and thus, concordant measurements have difference values close to zero. Graphs were elaborated for girls (Figure 1) and boys (Figure 2).

Figure 1. Difference between the real ages and the estimated ages by both methods for girls, as well as their means. CM – Cameriere's method; WM – Willems' method); CMWM – Mean between Cameriere's method and Willems' method; 2SD – Two Standard Deviations.

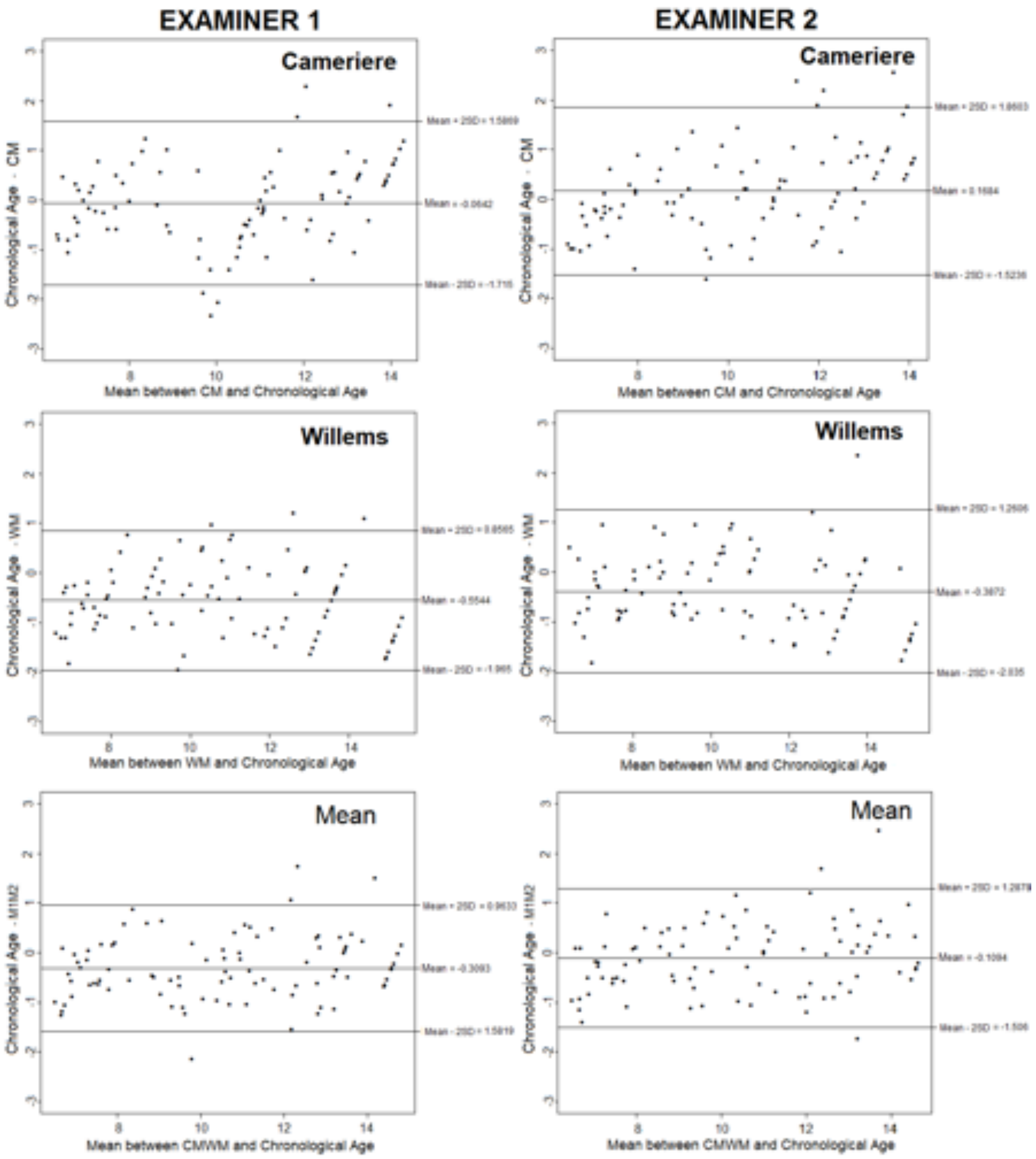
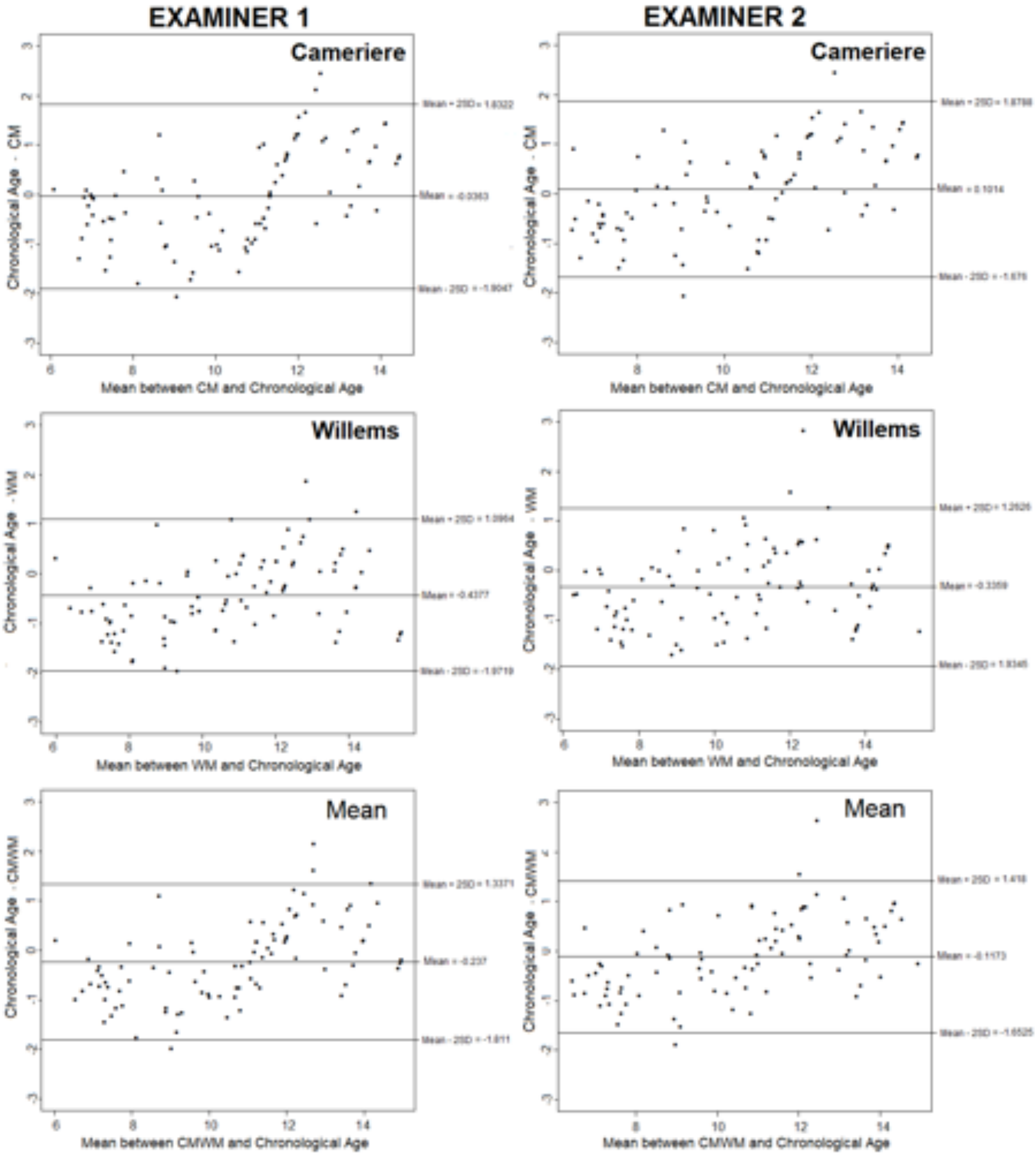


Figure 2. Difference between the real ages and the estimated ages by both methods for boys, as well as their means. CM – Cameriere's method; WM – Willems' method); CMWM – Mean between Cameriere's method and Willems' method; 2SD – Two Standard Deviations.



It is possible to observe that the graphs that represent the difference between the chronological age and the age estimates with Willems' method have the central axis towards higher negative values, a circumstance that demonstrates an overestimation of age by the method for both sexes – since the difference is calculated from the chronological age. This condition is not evidenced in Cameriere's method, because the central axis is closer to zero, showing less difference with the chronological age.

To compare the chronological and the estimated age, in general, considering the random effect, a Linear Mixed-Effects Regression model was used to analyze the variables. The estimated differences (chronological age less the age estimation for each method) are presented with their respective *p* values and 95% confidence intervals. Table 1 compares the chronological age with the estimated age of each method combining the analysis of examiners 1 and 2.

Table 1. Comparison between chronological and estimated age of each method.

SEX	COMPARISON		ESTIMATED DIFERENCE (YEARS)	*P* VALUE	CONFIDENCE INTERVAL 95%	
					LOWER LIMIT	UPPER LIMIT
Girls	Chronological Age	CM	0.05	0.4239	-0.08	0.18
	Chronological Age	WM	-0.47	0.0001	-0.60	-0.34
	Chronological Age	CMWM	-0.21	0.0014	-0.34	-0.08
Boys	Chronological Age	CM	0.03	0.5901	-0.09	0.15
	Chronological Age	WM	-0.39	0.0001	-0.51	-0.27
	Chronological Age	CMWM	-0.18	0.0035	-0.30	-0.06

It is possible to observe that the estimated differences for Cameriere's method are positive and close to zero, demonstrating a slight underestimation and small difference between the real and estimated ages. The estimated difference for Willems' method is negative and more distant from the value zero, showing an overestimation and higher difference between the real and estimated ages by the method.

Considering the confidence interval and the *p*-value, Cameriere's method includes the zero value in both sexes, has a little distance between upper and lower limits and demonstrates a *p*-value higher than 0.05. It indicates lack of statistically significant differences between chronological and estimated ages. For the Willems' method, the confidence interval is negative and does include zero values, providing evidence of statistically significant differences between chronological and estimated ages.

Willems' method influences in the overestimation when the mean age is calculated using both methods. It is confirmed because the confidence interval was close to zero and the *p* value maintained low, but the estimated differences between chronological and estimated ages were negative. In other words, ICC of the mean is close to 1, there is still an overestimation in relation to the chronological age.

DISCUSSION

Before estimating age in large samples, it is necessary to calibrate the examiners in order to analyze if the method is reproducible. This methodological set up might improve the quality of the age estimation procedure and might standardize the analysis between and within examiners.[15]

In the present study, it is possible to observe a significant correlation between the first and the second examiner analyses performed for age estimation (ICC between 0.94 and 1). Those values confirm the reproducibility of Cameriere's and Willems' methods, as well as the calibration of examiners.

Fernandes et al.[16] analyzed exclusively Cameriere's method, re-evaluating 20 orthopantomographs for intra-examiner analysis, which resulted in a p-value of 0.315 for the first examiner and 0.193 for the second examiner. These outcomes demonstrated no evidence of difference for intra-examiner reproducibility. Similarly, Galic et al.[17] analyzed the reproducibility of the Willems' method, re-examining 10% of the total sample. In their study, the intra-examiner analysis resulted in a Kappa coefficient of 0.811 – also confirming reproducibility.

It must be noted that Willems' method is based on the developmental stages of Dermijian et al.[13]

Designed as a qualitative technique, this approach is susceptible to subjectivity, interpretation and description. However, Dermijian stages are clearly defined, facilitating the classification of the development of each tooth.[15]

Another fact that justifies the high reproducibility is the quantitative characteristic of Cameriere's method, which is based on mathematical measurements and formulae with objective, statistical and numerical approach that minimize possible errors of interpretation – which might result in higher reliability.[18]

After the calibration and application of both methods in the present study, it was possible to observe high correlation values between the chronological and the estimated ages in the total sample. This outcome confirms that both age estimation methods demonstrate significant applicability in the studied sample. In addition, the mean of ages estimated by combining Cameriere's and Willems' methods revealed a maximum increase of 0.03 in the ICC.

Regarding the different correlation values between the methods, it is noted that Cameriere's method was slightly lower than the values corresponding to Willems' method. This difference is similar to the study by El-Bakary et al.,[18] which estimated age in 286 panoramic radiographs of Egyptian children from 5 to 16 years old, obtaining 98.02% accuracy with Cameriere's method and 98.62% with Willems' method. However, in this case, the difference between accuracies can be strictly correlated with the sample with subjects over 15 years of age, since the sample of Cameriere's study corresponding individuals from 5 to 15 years old.

Similarly, Rai and Anand,[19] in a study with 75 panoramic radiographs from individuals aged 5 to 14 years, in India, also concluded that the Cameriere's method has a lower accuracy compared to the Willems' method. This slight difference can be explained by the sample number used by Cameriere et al.[12] in their study, which used 455 radiographs of Italian individuals from 5 to 15 years old. This sample number differs from the research by Willems et al.,[14] performed with 2,523 panoramic radiographs of a Belgian Caucasian population. Moreover, the method designed by Cameriere et al.[12] involves more measurement and calculation steps, requiring training and experience because it is associated with a longer learning curve.[18]

Besides that, there is a better distribution of estimated ages around of chronological ages for Cameriere's method. Although the values are further from the central oblique line when compared to the Willems' method graphs in the ICC graphs, they are more evenly arranged around of the central oblique line. This result is also demonstrated by the low value of the differences between the chronological and estimated ages (0.05 years for girls and 0.03 years for boys). The *p*-values and confidence intervals do not provide evidence of difference between the chronological and estimated ages, since the *p*-values are higher than 0.05 and the confidence intervals include the zero value.

The positive value of the difference between the chronological and estimated ages demonstrates that there was a slight underestimation by Cameriere's method. Although the minimal underestimation present in this study, there are reports in the literature of higher underestimations, as Luz et al.[20] who applied Cameriere's method to a Croatian and a Brazilian sample. Similarly, Fernandes et al.[16] reported that Cameriere's method underestimated the ages of 54.4% of Brazilian individual's sample, a trend that was verified mainly from 11 years of age.

Despite the higher ICC presented in the Willems' method, it pointed to an overestimation of age when compared to the chronological age, a circumstance that can be observed by the negative results of the estimated differences. The difference for the Willems' method resulted in a value of -0.47 years for girls and -0.39 for boys. The *p*-value is less than 0.05 and the confidence interval does not include the zero value, providing evidence of differences between the chronological and estimated ages.

The overestimation of age presented by Willems' method might be justified by the original study of Willems' et al.[14] that simplified the two score tables originally created by Dermijian et al.[13] In the literature, both methods have several reports of overestimation in different populations.[17,18,21]

In the Bosnia and Herzegovina community, Galic et al.[17] demonstrated an overestimation of age of 0.25 years for girls and 0.42 for boys. Even in the Brazilian population, there are studies that present that characteristic, such as Franco et al.[21] who indicated a slight difference of -0.17 years for girls and -0.38 for boys.

On the other hand, in the study by Apaydin and Yasar,[22] the estimated ages by Willems' method had an underestimation of -0.06 years. Through the studies by Angelakopoulos et al.[23] and Halilah et al.,[24] it was possible to observe an overestimation of Cameriere's method in younger children in a sample from 6 to 14 years old from South Africa and from 5 to 16 years old from Germany.

In this context, it is essential to distinguish the possible consequences of overestimating or underestimating an individual's chronological age. In the civil sphere, false positives or false negatives tend not to have different weights because one does not have worse consequences than the other, since civil law covers numerous circumstances and each case must be assessed separately. However, in the criminal context, overestimation can have undesirable implications, because the individual can be considered imputable and legally punished for a false positive result, since it could only be targeted by educational protective measures.[8,25]

The different sexes presented high correlation coefficients. However, when the differences between the chronological and estimated ages of the female sample are compared with the male sample, Cameriere's method shows a higher tendency to underestimate and Willems' method a higher tendency to overestimate the age of girls. This tendency is evident when the values of the differences for each sex are observed: when Cameriere's method was used, the difference value for girls was 0.05 and for boys 0.03; when age was estimated by Willems' method, the difference for girls was -0.47 years and for boys was -0.39 years. Concluding that the boys' values were better distributed around the chronological age, decreasing the difference with the estimated age.

Wolf et al.[26] also found a higher difference for girls (0.08 years for girls and 0.07 for boys). However, Apaydin and Yasar[22] found a smaller error for girls when using Cameriere's method, with an underestimation of 0.603 years for boys and 0.550 for girls.

The higher overestimation for girls with Willems' method was also described by the author during his study with a sample of Belgian Caucasian children, with an overestimation for girls of 0.2 years and 0.1 for boys. Apaydin and Yasar[22] also found a higher error for girls when using Willems' method, with an underestimation of 0.062 years for girls and 0.056 for boys. However, in the study by El-Bakary, Hammad and Mohammed[18] girls had lower errors level compared to boys when using Willems' method (overestimation of 0.14 years for girls and 0.29 for boys).

Regarding the different results of the studies with higher overestimation for girls or boys, Rai et al.[19] propose that there are several genetic, environmental and geographical causes, such as the nutritional and socioeconomic status of a given population. The difference between the sexes is mainly due to advanced girls' development when compared to boys' development, especially when it comes to dental formation, since girls reach almost all stages of dental development before boys.[27,28]

It is evident that both methods are applicable to girls and boys in the studied sample, since they showed high agreement with chronological age. The mean estimated age obtained by combining both methods had a slight increase in agreement with the chronological age in relation to the mean estimated age obtained with each method separately.

ACKNOWLEDGMENTS

This work was conducted with financial support from CAPES/BRAZIL – Coordination for the Improvement of Higher Education Personnel, and Federal Police of Brazil (Prof-Forenses 001, Edital CAPES 25/2014). This study was financed in part by CAPES/BRAZIL - finance code: 001.

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Assessment of agreement between cervical vertebrae skeletal and dental age estimation with chronological age in an Indonesian population

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The authors declare that they have no conflict of interest.

KEYWORDS

Cervical vertebrae, Dental, age estimation, Panoramic, Cephalometric

J Forensic Odontostomatol 2020. Dec;(38): 3-16:24
ISSN :2219-6749

ABSTRACT

Age estimation significantly contributes to forensic medicine and law enforcement in Indonesia. However, lateral cephalometric radiographs of cervical vertebrae have not been used to estimate age in the Indonesian population. This study developed a formula to estimate the skeletal age of cervical vertebrae using multiple linear regression analyses, estimating the dental age and evaluating the agreement between cervical vertebrae skeletal-chronological, dental-chronological, and cervical vertebrae skeletal-dental ages. Several anatomical parameters were measured to obtain cervical vertebrae ratios from 100 lateral cephalometric radiographs, and followed by the calculation of dental tooth crown index data from 100 panoramic radiographs of subjects 9–18 years old. The Bland-Altman plot of cervical vertebrae skeletal and dental ages showed a mean difference of -0.094 ± 1.52 years, with upper and lower limits of 2.882 and -3.070 years, respectively. The means of the cervical vertebrae skeletal, dental, and chronological ages were 13.97 (2.67), 14.06 (2.45), and 13.97 (2.97), respectively. The mean differences between cervical vertebrae skeletal-chronological and dental-chronological ages were 0.566 (2.26) and 4.005 (2.07), respectively. Furthermore, a validation trial (group 2, n = 10, three males and seven females) was conducted to test the accuracy of the cervical vertebrae skeletal age estimation formula using consecutive sampling. The age range was 9–11 years. Cervical vertebrae skeletal age showed a better agreement with chronological age than did dental age.

INTRODUCTION

In forensic medicine, age is estimated for purposes of identification in natural disasters, criminal cases, and civil cases. Recently, several significant investigations have required age estimation. For example, the identification of tsunami victims in Aceh (2013), Palu (2018), and Banten (2018) required age estimation to distinguish between children and adults. Criminal cases of minor prostitution and the investigation of age fraud in various sports also require age confirmation, especially for those aged between nine and 18 years.

Indonesian minors and adults are classified on the basis of several laws. According to Indonesian law in the child justice system, someone less than 18 years of age is a child and cannot be convicted of a crime. The Indonesian Marriage Act stipulates that the minimum age of marriage for a female is 16 years and that for a male is 19 years. Thus, accurate age estimation in the 16 to 19 year old group is crucial in civil cases.[1–6]

Age can be determined from legal documents (chronological age) and by using biological indicators, such as body height, menarche, voice change, and skeletal and dental development.[7–9] Lamparski (1975) introduced the estimation of cervical vertebrae (CV) maturation by evaluating the shape of the inferior border of the first (atlas, C1) to the sixth (C6) CV using lateral cephalometric radiographs, but his method was deemed subjective and lacking in detail.[10,11] Mito and Sato (2002) measured the bodies of the third (C3) and fourth (C4) CV using lateral cephalometric radiographs in a Japanese population and developed a formula to estimate CV skeletal age.[12] To evaluate its accuracy, they calculated skeletal age using the Tanner-Whitehouse 2 (TW2) hand-wrist method and compared the CV skeletal and chronological ages.[12,13] The formula proved highly reliable in estimating CV skeletal age.[14–16]
Dental age estimation based on periapical, lateral cephalometric, and panoramic radiograph features can be highly accurate. According to Drusini, Ito was the first to estimate dental age by comparing the enamel and pulp chamber to dentine, but the correlation was weak.[17] Ikeda (1985) perfected the formula by including the variable of height to the crown and observed a strong correlation.[18] Nehemia et al. (2012) estimated dental age based on the radiographic pulp chamber size in the mandibular first premolar using the tooth coronal index (TCI) and analyzed histological features in the pulp chamber in an age range of 9–21 years in an Indonesian population. The results showed a strong correlation between the TCI and biological age ($r^2 = 0.6407$).[5,6,17]
CV lateral cephalometric radiographs have not been used to estimate age in the Indonesian population. We developed a formula to estimate CV skeletal age using multiple linear regression analyses of C3 and C4 bodies. Dental age was estimated by analyzing the height of the dental pulp and crown using the TCI. The extent of the mean difference between these two methods was analyzed, after which we measured the reliability of both methods in estimating biological age compared to chronological age.
All the parameters were measured twice (days 1 and 15) by one rater. To measure the intra-rater agreement, we randomly selected 15 lateral cephalometric and 15 panoramic radiographs from group 1 (100 lateral cephalometric and 100

MATERIAL AND METHODS

Study subjects

The study was approved by the Research Ethics Committee of the Faculty of Dentistry Universitas Indonesia. Medical records were used to select samples by purposive sampling. The inclusion criteria were age 9–18 years, healthy growth and development of the cervical bones and teeth, and high quality digital lateral cephalometric and panoramic radiographs to analyze C3, C4, and the mandibular first premolar. The exclusion criteria comprised CV trauma (cervical dislocations or fracture) and abnormal mandibular first premolars (attrition, caries, tooth loss, periapical lesions, post–root canal treatment, artificial crowns, fillings, and orthodontic appliances).[5,6,17] We obtained panoramic and lateral cephalometric radiographs from patients undergoing orthodontic and paedodontic treatment. We estimated the sample size and selected 100 people (*n* = 10/group; five males and five females) aged 9–18 years. They were traced using Digimizer image analysis software version 5.3.5.

Radiography

Digital lateral cephalometric and panoramic radiographs were obtained from the medical records of the Dental Hospital of Universitas Trisakti, Jakarta, Indonesia. We used the lateral cephalometric radiographs to develop the CV skeletal age estimation formula based on Mito and Sato's (2002) method of measuring the parameters of anterior height (AH), posterior height (PH), antero-posterior height (AP), and body height (H) from C3 and C4 and then determining their ratios (AH/AP, H/AP, PH/AP, AH/H, H/PH, AH/PH) (Fig. 1).[12] We used panoramic radiographs to estimate dental age using the TCI, which calculates the coronal pulp cavity height (CPCH) and coronal height (CH) of the mandibular first premolar (Fig. 2). We selected the right or left region from the panoramic radiographs depending on which more clearly showed the pulp space as previously described.[5]

Statistical analysis

panoramic radiographs) using Research Randomizer[19] and analyzed them using the intraclass correlation coefficient (ICC) (an ICC >0.9 indicated excellent agreement). Multiple linear regression analysis was used to devise the

CV skeletal age estimation formula. Dental age was estimated using TCI = (CPCH × 100)/CH and Nehemia et al.'s formula (2012), which is 29.16 + (-0.4)TCI, with r^2 = 0.6407 and r = 0.8.[5,6] The Bland-Altman plot was used to evaluate the agreement between CV skeletal-chronological, dental-chronological, and CV skeletal-dental ages. One-way analysis of variance (ANOVA) was then used to measure the difference between CV skeletal, dental, and chronological ages. $P < 0.05$ was considered statistically significant.

To test the accuracy of the CV skeletal age estimation formula, we performed a trial on group 2 (10 subjects, three males and seven females, with 10 lateral cephalometric and 10 panoramic radiographs) using consecutive sampling and the same eligibility criteria. We compared the age estimation results to chronological age using unpaired t-test analysis. $P < 0.05$ was considered statistically significant.[8]

Figure 1. The third and fourth cervical vertebrae bodies from cephalometric radiographs. The parameters were AH (Anterior Height), PH (Posterior Height), AP (Antero-Posterior Height), H (Body Height)

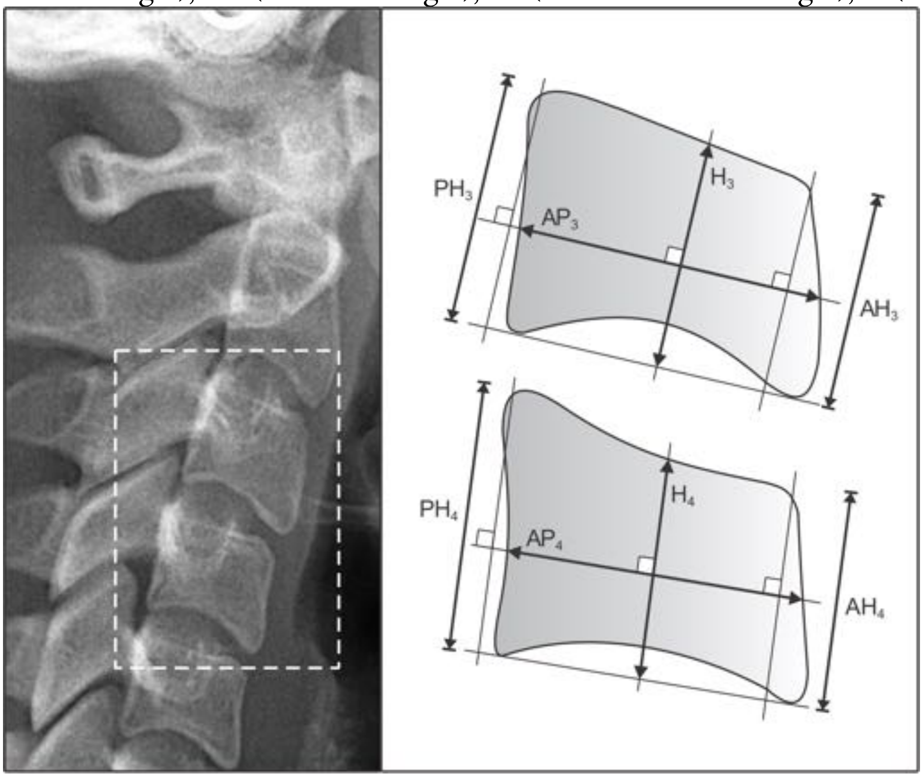
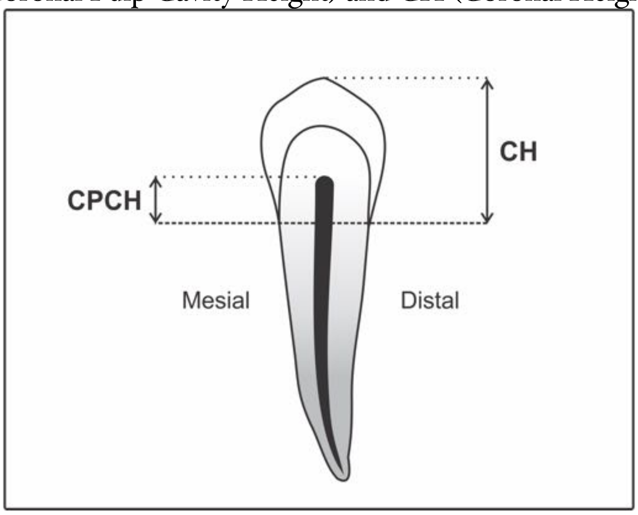


Figure 2. TCI (Tooth Coronal Index) from the lower first premolar. The parameters were CPCH (Coronal Pulp Cavity Height) and CH (Coronal Height)



RESULTS

Table 1 shows the characteristics of group 1 by chronological age and sex. The mean ICC was 0.998 (95% confidence interval [CI] = 0.995–0.999) for CV skeletal parameters and 0.975 (95% CI = 0.925–0.992) for dental parameters. These values show excellent agreement between the two measurements on days 1 and 15.

According to chronological age, the C3 and C4 measurements increased with increasing age (Fig. 3) whereas the mean TCI of the mandibular first premolar decreased (Fig. 4). The mean CV parameters increased in the age range of 9–18 years. AH, H, and PH in both C3 and C4 increased whereas AP_4 did not change. A Pearson correlation test (Table 2) revealed the correlation of all the CV parameters with chronological age: AH_3/AP_3 (r = 0.829) and AH_4/AP_4 (r = 0.834) were strongly correlated whereas H_3/AP_3, PH_3/AP_3, PH_3/AP_3, H_3/PH_3, H_4/AP_4, PH_4/AP_4, AH_4/H_4, and H_4/PH_4 were moderately correlated ($0.3 < r < 0.7$).

Table 1. Characteristics of group 1 according to chronological age and sex.

Age	Male (*n* = 50)		Female (*n* = 50)		Total (*n* = 100)	
	Mean	SD	Mean	SD	Mean	SD
9 (*n* = 10)	9.40	0.26	9.33	0.13	9.37	0.20
10 (*n* = 10)	10.33	0.28	10.32	0.22	10.33	0.23
11 (*n* = 10)	11.45	0.40	11.50	0.36	11.48	0.36
12 (*n* = 10)	12.20	0.14	12.37	0.25	12.28	0.21
13 (*n* = 10)	13.33	0.39	13.65	0.19	13.49	0.33
14 (*n* = 10)	14.56	0.25	14.52	0.29	14.54	0.26
15 (*n* = 10)	15.32	0.07	15.65	0.17	15.48	0.21
16 (*n* = 10)	16.42	0.35	16.57	0.17	16.49	0.27
17 (*n* = 10)	17.85	0.09	17.38	0.17	17.62	0.28
18 (*n* = 10)	18.58	0.22	18.60	0.22	18.59	0.21
Total (*n* = 100)	13.95	3.01	13.99	2.98	13.97	2.98

SD: standard deviation

Table 2. Correlation test between CV parameters and chronological age.

Ratio		AH/AP		H/AP		PH/AP		AH/H		H/PH		AH/PH	
		C3	C4	C3	C4	C3	C4	C3	C4	C3	C4	C3	C4
Age	*r*	0.829	0.834	0.695	0.696	0.598	0.549	0.740	0.531	0.492	0.544	0.757	0.740
	p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	n	100	100	100	100	100	100	100	100	100	100	100	100

CV: cervical vertebrae; AH: anterior height; AP: antero-posterior height; H: body height; PH: posterior height

Figure 3. Mean value of parameter C3 and C4 from the CV body by chronological age group

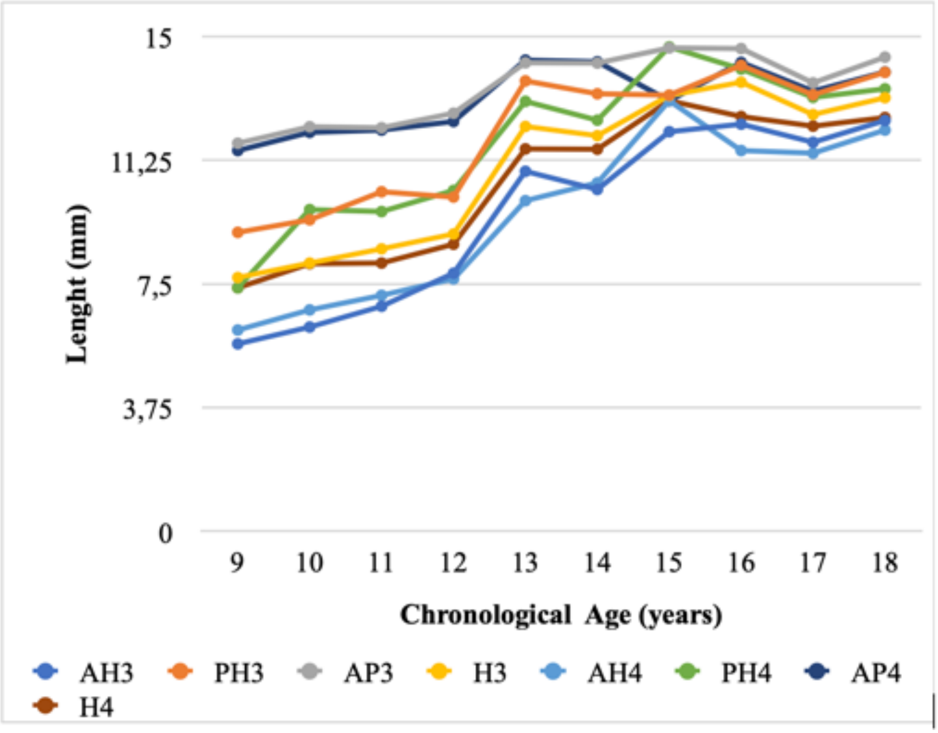
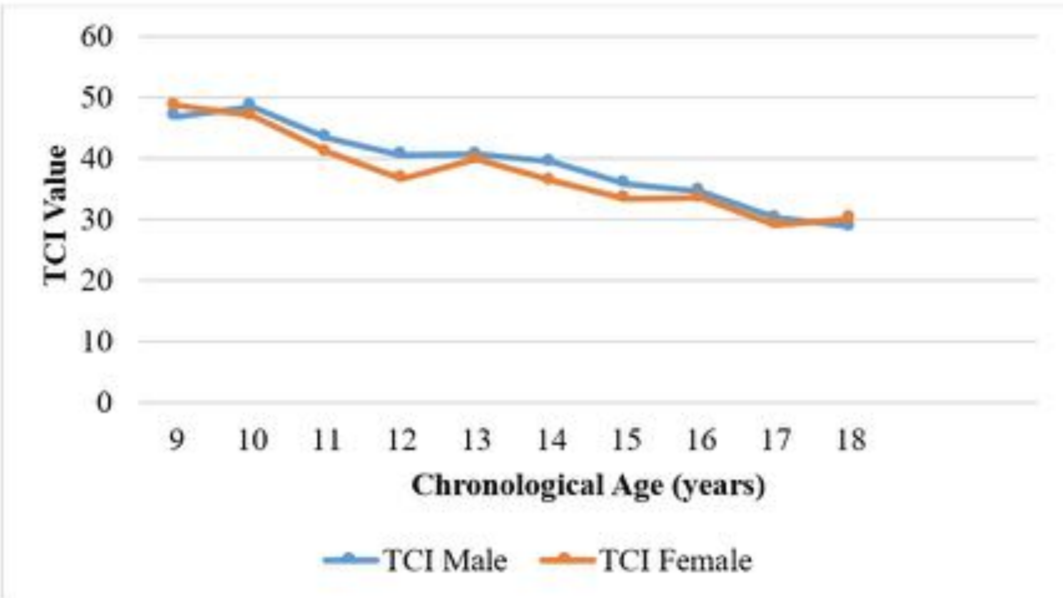


Figure 4. Mean value of TCI lower first premolar by chronological age group



The adjusted r^2 of the CV age estimation formula was 0.789, with a Durbin-Watson value of 1.192 and a one-way ANOVA of p <0.001. The average residual value was 0. The CV skeletal age estimation formula was as follows:

y = 15.827 + 14.19*(AH3AP3**) + 15.604*(**AH4AP4**) - 7.432*(**PH3AP3**) − 7.407*(**PH4AP4**) − 10.823*(**H3PH3**) + residual value**

Figure 5 shows the reliability analysis between CV skeletal and dental age estimation and chronological age. CV skeletal age was overestimated at 9–12 years and underestimated at 16–18 years. The most accurate CV skeletal age estimation was at 14 years, and the least accurate was at 18 years. Dental age estimation had the smallest mean difference at 15 years and the largest mean difference at 12 years.

The Bland-Altman plot showed that the mean difference between CV skeletal age and chronological age and between dental age and chronological age was 0.0000 ± 1.34 years and 0.0937 ± 1.37 years, respectively (Fig. 6). The upper and lower limits of CV skeletal age were 2.63 and -2.63 years, respectively, while those of dental age were 2.78 and -2.59 years, respectively. The Bland-Altman plot between CV skeletal age and dental age showed a mean difference of -0.094 ± 1.52 years, with upper and lower limits of 2.882 and -3.070 years, respectively. Therefore, compared to dental age estimation, CV skeletal age estimation conformed better to chronological age.

Figure 5. Difference in CV skeletal age and dental age estimation with chronological age per age group (n = 100)

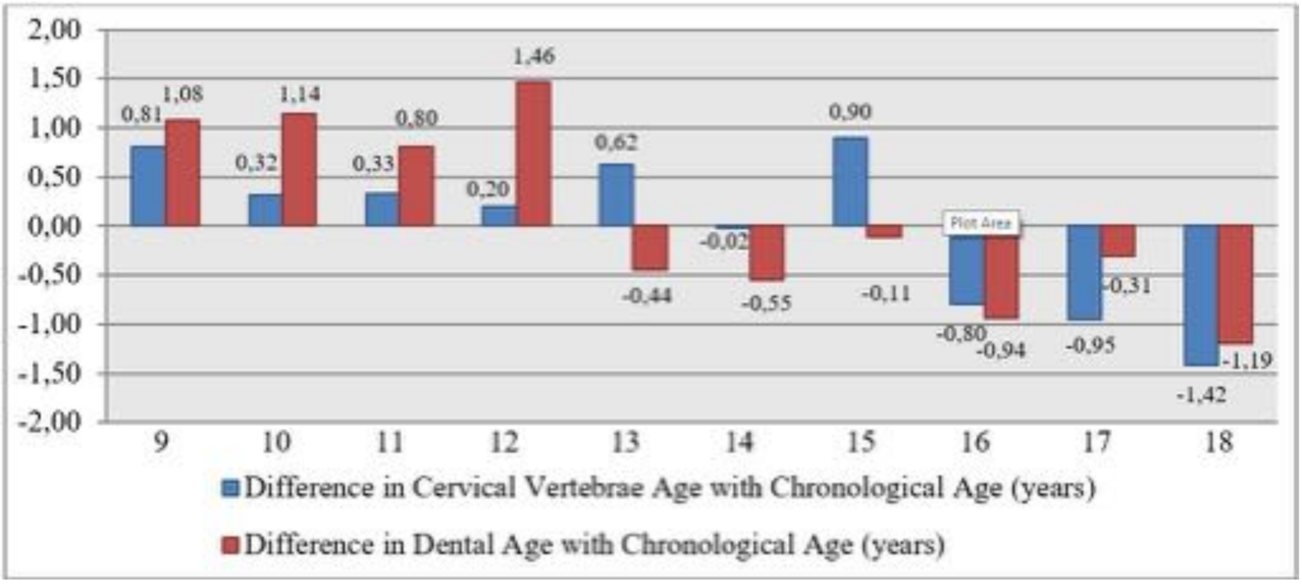
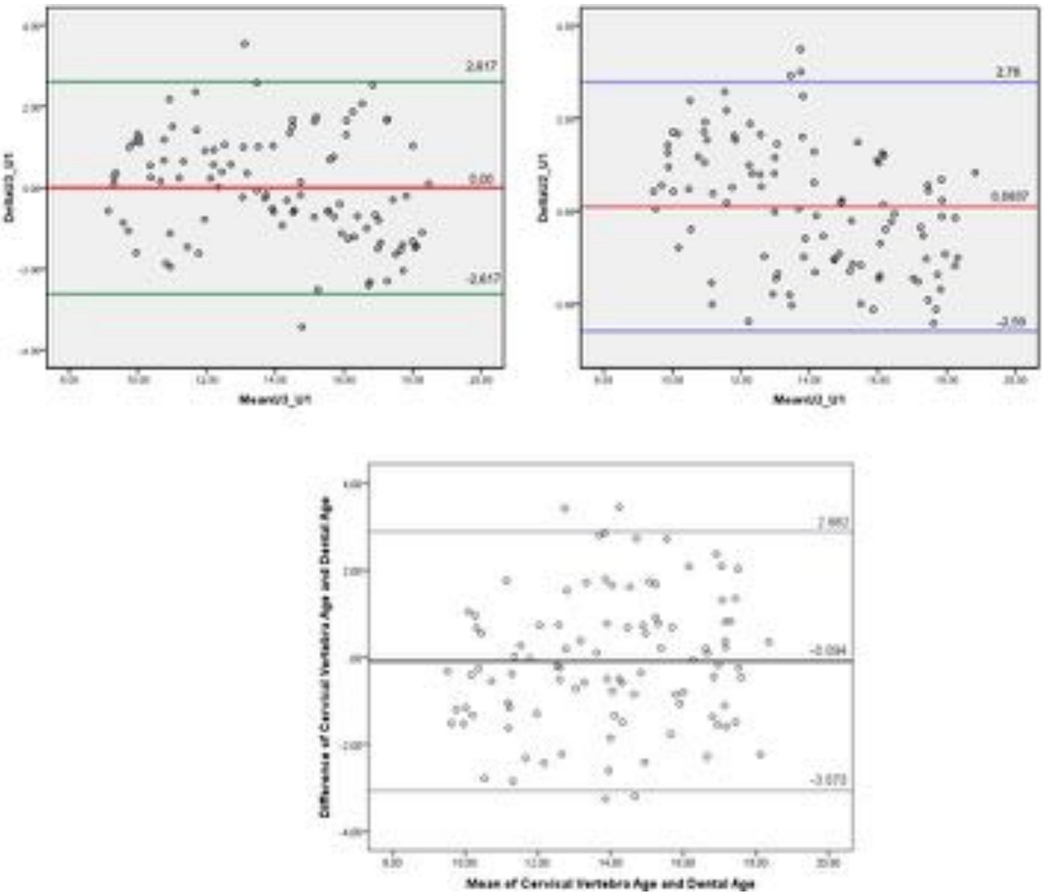


Figure 6. The agreement Bland Altman plot between CV skeletal age (left) and dental age (right) estimation with chronological age



We found no statistically significant differences (p >0.05) between CV skeletal, dental, and chronological ages using one-way ANOVA (Table 3), which is consistent with previous studies.[12,14,16]

Table 3. One-way ANOVA for CV skeletal, dental, and chronological ages.

Age	Mean (SD)	*P*-value
CV skeletal age	13.97 (2.67)	0.961
Dental age	14.06 (2.45)	
Chronological age	13.97 (2.97)	

ANOVA: analysis of variance; CV: cervical vertebrae; SD: standard deviation

Table 4. Characteristics of group 2 according to chronological age and sex.

Age	Male (n = 3)		Female (n = 7)		Total (n = 10)	
	Mean	SD	Mean	SD	Mean	SD
9 (n = 6)	9.58	-	9.45	0.25	9.47	0.23
10 (n = 3)	10.33	-	10.38	0.18	10.36	0.12
11 (n = 1)	11.17	-	-	-	11.17	-
Total (n=10)	10.36	0.80	9.72	0.50	9.7	0.64

SD: standard deviation

Table 5. Unpaired t-test analysis of the mean difference between CV skeletal and chronological ages and between dental and chronological ages (group 2).

Age	Mean (SD)	*P*-value
Skeletal-chronological age	0.366 (2.05)	0.002
Dental-chronological age	3.805 (2.26)	

CV: cervical vertebrae; SD: standard deviation

In the validation trial with group 2 (Table 4), the age range was 9–11 years, with a mean SD of 9.707 (0.64) years. Using the CV skeletal age estimation formula for group 1, the CV skeletal age estimation for group 2 was 10.273 (1.59) years while the dental age estimation was 13.712 (2.32) years. Table 5 shows the mean difference between CV skeletal and chronological ages and between dental and chronological ages; the CV skeletal age result was more accurate (p = 0.002).

DISCUSSION

Lateral cephalometric radiographs are often used to assess bone maturity level and determine skeletal age. However, CV skeletal age can be calculated objectively and reliably as in the TW2 hand-wrist method.[11] There is a significant correlation and no significant difference between hand-wrist skeletal age and the estimation of CV skeletal age from morphological CV parameters;[13,15] CV can replace the gold standard of hand-wrist bone for skeletal age estimation.[10,13,16] The CV body has a strong relationship with the development process in shape and size changes, is clearly visible, and can be measured using lateral cephalometric radiographs. C3 and C4 are measured because C1 and C2 do not show absolute changes and C5 and C6 are often invisible on lateral cephalometric radiographs.[20–22]

Teeth are often used to predict age with excellent accuracy.[1] The mandibular first premolar is highly accurate in age estimation, having the smallest standard of error among incisors, canines, and molars.[1,5,6,23] Nehemia et al. (2012) used panoramic radiographs for dental age estimation,[5] and Nurfitria et al. (2018) found no significant difference in age estimation using either periapical or panoramic radiographs.[6] Therefore, the TCI can be used with periapical or panoramic radiographs.[5,6,17]

In Figure 3, as previously reported, AP_4 remains constant, probably because of the AP dimensions of the vertebral body, which tend to develop in a superior-inferior manner with age.[12,13,24] Figure 4 illustrates the downward trend in TCI scores with increasing age group. This result indicates the decreased size of the pulp chamber with age, which can be caused by physiological factors such as the formation of secondary dentine, which starts after the crown and roots are fully formed and continues to develop as long as the tooth is viable. This secondary dentine deposit causes the pulp chamber to decrease in size and narrows the existing root canals. This narrowing tends to occur more quickly in the pulp horn region than in other regions. Some researchers argue that changes in pulp chamber size can also occur due to pathological stimuli, including caries and traumatic occlusion.[5,20]

All the CV parameter ratios showed correlations with chronological age (Table 2). While the strength of the correlation is categorized as weak (0 <*r* <0.3), moderate (0.3 <*r* <0.7), or strong (*r* >0.7),[25] Mito, Caldas, and the present research found a diverse strength of correlation between the ratios. The correlation strength differs because of differences in the subject population and race.[10,12]

Age estimation using CV skeletal is more accurate than dental age estimation and can be used as a chronological age estimation tool. The CV skeletal age estimation formula from lateral cephalometric radiographs is limited to the 9–18 year age group of both sexes with no C3 or C4 abnormalities. Bones change with age; thus, the accuracy of the formula in other age groups needs to be tested further.

The limitation of this study is that the CV skeletal age estimation formula was validated only by using consecutive sampling on 10 subjects aged 9–11 years. The testing of the formula in this age group does not provide a perfect analysis.

CONCLUSIONS

Both CV skeletal and dental age estimation proved effective as tools for estimating chronological age, but CV skeletal age showed better agreement. We recommend estimating CV skeletal age in a larger sample differentiated according to sex for improved estimation.

Acknowledgments

We thank the Dental Hospital of Universitas Trisakti, Jakarta, Indonesia for the digital radiograph samples and Denys Putra Alim, MD as the statistics mentor in this study.

Financial support

This study was funded by Universitas Indonesia PITTA A Research Grant Year 2019 No. NKB-0416/UN2.R3.1/HKP 05.00/2019.

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Volume analysis of second molar pulp chamber using cone beam computed tomography for age estimation in Egyptian adults

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The authors declare that they have no conflict of interest.

KEYWORDS

Forensic Odontology,
Anthropology population data,
Cone beam computed tomography,
Second molar,
Pulp chamber volume

J Forensic Odontostomatol
2020. Dec;(38): 3-25:34
ISSN :2219-6749

ABSTRACT

Aim: The present work aimed to evaluate age-related variations in the dental pulp chamber volume of second molars using cone beam computed tomography (CBCT) imaging, in order to establish a specific mathematical model for second molars and measure its accuracy, especially in the case of Egyptian adults. **Subjects and methods**: From 187 subjects between 21–50 years of age, CBCT images of 257 maxillary and 248 mandibular second molars were included. A mathematical model for human age estimation was established. An independent additional set of CBCT images was obtained to test the model's accuracy. **Results**: For maxillary and mandibular teeth, R^2 for the pooled sexes were 0.51 and 0.52, and SEE were 5.92 and 5.71, respectively. A model for each sex was established, due to the significant difference between them, where R^2 was equal to 0.668 and 0.650 in males and 0.46 and 0.48 in females, concerning maxillary and mandibular teeth, respectively. When testing the validation samples, the mean absolute error (MAE) between the actual and estimated ages from the pooled sex model were 4.89 and 4.61 for maxillary and mandibular teeth, respectively. **Conclusion**: The pulp chamber volume of second molars is a relatively accurate indicator for age estimation in Egyptian adults.

INTRODUCTION

Forensic odontology deals with the proper evaluation and presentation of dental findings for many purposes, including the identification of living persons and corpses, mainly for age, sex, and race identification.[1,2] Teeth have gained the attention in personal identification as they follow a well-defined sequential developmental pattern. In addition, they are highly resistant to mechanical, chemical, and physical impacts and time.[3,4]

Estimating the chronological age of unidentified individuals is a practice based on gradual structural changes in enamel, dentine, and cementum, which occur in teeth throughout life. Factors like attrition and cementum apposition are highly influenced by the life-style of an individual, so they cannot be regarded as reliable parameters. However, secondary dentine deposition and root dentine translucency have been found to be more reliable by many authors.[5]

Secondary dentine formation by odontoblasts starts when the apical part of the root has completely developed, and it is regular when not under the influence of dental caries or other physical/chemical insults to the given tooth. Secondary dentine formation produces age-related decrease in pulp chamber volume that is variable among different populations and both sexes.[6,7,8] Many radiological techniques have been introduced for quantification of dental pulp chamber volume, where some of them have been two-dimensional studies (e.g., common periapical X-rays and orthopantomograms—OPGs) and others have been three-dimensional studies (computed tomography—CT, micro-CT, and cone beam CT, and x-ray free imaging like Magnetic Resonance).[9] CBCT offers the advantage of causing no magnification errors due to geometric distortion, giving more detailed information, less artefacts, and having a lower radiation dose than CT. [10] Quantification of the pulpal chamber volume from a dental modelling based on CBCT analysis uses a geometrical approximation of the different parts of the tooth, namely root, pulp and crown, which can be assimilated to elliptical-based cones (root and pulp) or elliptical-based truncated cones (crown). This allows a quick evaluation of the volumes of interest on the examined tooth.[11]

On analysis of previous studies, most of them focused on single rooted teeth for assessment of secondary dentine deposition for dental age estimation. In addition, the previous studies on an Egyptian population were performed mostly using a two- dimensional technique and no Egyptian specific equation was established for any type of tooth.

AIM OF THE WORK

This study aimed to evaluate the age-related variations in the dental pulp chamber volume of second molars using CBCT imaging, in order to establish a mathematical model specific for second molars, especially in the case of Egyptian adults, and measure its accuracy.

SUBJECTS AND METHODS

Sample size calculation:

Sample size calculation was performed using G power 3.1.9.2 software with linear multiple regression model, study power 95%, α value 0.05, the R^2 value for maxillary and mandibular second molars (0.498, 0.487 respectively).[12] The sample size determined was 40 cases.

Subjects:

Archived CBCT scans from 101 females and 86 males whose age ranged between 21-50 years old were collected retrospectively in duration from January 2018 till January 2019 (table 1). All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The work has been approved by the Institutional Review Board (R.19.12.692). All the CBCT images were taken for diagnosis or treatment purposes, thus, there was no unnecessary or additional radiation exposure to the subjects. All participants have given informed consent to conduct radiological examination for different diagnostic reasons. To observe ethical considerations, all information provided was treated as confidential by the researchers, and the names of individuals were not released at the analysis and reporting stage.

The inclusion criteria of the second molars were completely formed roots, no caries, minimal tooth attrition or abrasion, and no pulpal calcification. [4] The exclusion criteria of the second molars were fractured roots, teeth with any signs of periapical surgery, endodontic access, crown preparation, and fixed prosthodontics. To ensure minimal abrasion or attrition to teeth, all tooth surfaces were covered by enamel on the radiograph. Both maxillary and mandibular second molars were included for analysis. Although some authors used only one tooth per subject [13] others have selected more than one tooth of the same type per subject. [14]

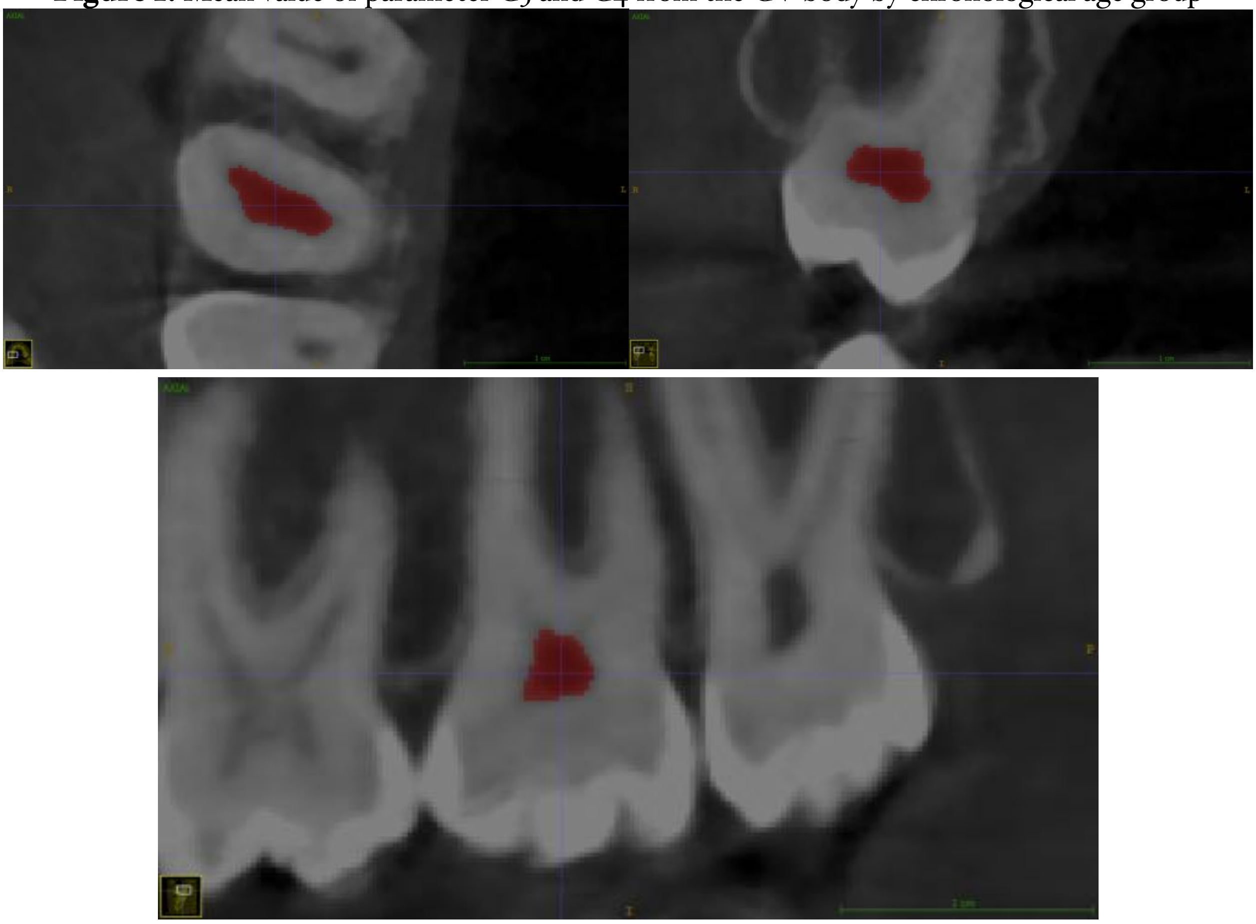
Table 1. Age and sex distribution of the samples used for the establishment of the method.

Age (years)	Male	Female	Maxillary Second Molar			Mandibular Second Molar		
			Male	Female	Total	Male	Female	Total
21–30	38	56	63	70	133	59	65	124
31–40	24	28	27	42	69	34	37	71
41–50	24	17	30	25	55	30	23	53
Total	86	101	120	137	257	123	125	248

Image acquisition:
CBCT unit Planmeca (Quantitative Radiology, Helsinki, Finland) was used for obtaining the image. The exposure parameters of the CBCT image were 120 kVp, 4.19–107.39 mAs, in accordance with the subjects' size and field of view (FOV). The selected FOV was 5x5 cm. The acquired images were subsequently reconstructed with a voxel-size of 0.15 × 0.15 × 0.15 mm and exported as DICOM datasets.

Image segmentation:
The software ITK-SNAP 3.8 (open source software, www.itksnap.org) was used for measuring pulp chamber volumes using automatic segmentation or seed region-growing To apply it, the observer sets up a "seed" inside the structure to reconstruct, in this case the pulp chamber. This seed grows and a volume is finally obtained.[15]
Segmenting the chamber space is based on difference of the gray levels corresponding to surrounding dentine tissue (high intensity structure) and the present pulp tissue (low intensity tissue). Succeeding the image opening in software, the intensity of gray level values of the pulp chamber space and the surrounding dentine was checked at different levels and places to determine the least value at both structures to determine "lower and upper thresholds". For the lower threshold, the darkest point at the pulp chamber was selected as it represents the area with no calcification and represents the gray level of the soft tissue. For the upper threshold, the lowest value at dentine that visually surrounds the pulp space at the CBCT was recorded. Determination of thresholds allows the software to select voxels with gray levels lying in between the lower and the upper thresholds automatically which is corresponding only to pulp chamber space. To decrease the calculation errors, the "regions of interest" (ROIs) tool was used to limit the image boundaries to the lateral walls, pulp floor, and pulp roof only to prevent the segmentation tool to select voxels at the radicular pulp space in case of presence of wide radicular canals. After proper evaluation, each segmented area was three-dimensionally reconstructed and the volume was digitally calculated by the software (figure 1).

Figure 1. Mean value of parameter C3 and C4 from the CV body by chronological age group



Model establishment:
In order to establish an Egyptian specific mathematical model for the estimation of human age, linear regression analysis was conducted with age as the dependent variable and pulp chamber volume as the independent variable, The age used in the model was approximated to a lower age if less than 6 months and to higher age if more than 6 months.

Inter-observer and intra-observer variability:
All the measurements were carried out by the same examiner. To test inter-observer reproducibility, random samples of 40 teeth were re-examined by another examiner. To test intra-observer reproducibility, random samples of 40 teeth were re-examined by the same examiner after a one month interval.

Model validation:
Another group of CBCT images of 38 maxillary and 28 mandibular second molars (age 23-49 years old) was collected in order to validate the established models. After obtaining the pulp chamber volume, the mathematical model was used to obtain the estimated age. From both the estimated and actual age, the mean absolute error (MAE) and root mean square error (RMSE) were calculated to assess the accuracy of the model.

Statistical analysis:
The data were analyzed using SPSS program Standard version 21. The normality of the data was first tested with a one sample Kolmogorov–Smirnov test. Continuous variables were presented as mean ± SD (standard deviation). The intra-class correlation coefficient (ICC) was calculated for assessment of the inter- and intra-observer variability. A paired t-test was used to compare the pulp chamber volume between the right and left second molars in the same subject. Pulp chamber volumes were compared with Student's t-test for the two groups (i.e., between both sexes) or via a one-way ANOVA for multiple groups. Pearson correlation was used to assess the relationship between dental pulp chamber volume and age. The significant variables of the analysis were entered into linear regression models to predict the most significant determinants and to control for possible interactions and confounding effects. For all the statistical tests mentioned above, the threshold of significance was fixed at a 5% level (p-value).

RESULTS

The ICC was strong (0.917, 0.979) for the inter-observer and intra-observer cases, respectively. No significant differences were found between the right- and left-sided teeth (p-values of 0.333 and 0.731 for maxillary and mandibular second molars, respectively), thus, both of them were included.
Results showed significant difference in pulp chamber volume between both sex (P-value 0.001 in maxillary teeth and 0.004 in mandibular teeth) and between different age groups (p-value 0.001) (tables 2 and 3)

Table 2. Mean value of the pulp chamber volume of maxillary second molars.

Age (years)	Volume of Maxillary Second Molar (mm³)						P-value
	Male			Female			
	Min	Max	Mean ± SD	Min	Max	Mean ± SD	
21–30	19.46	46.63	29.35 ± 5.21	13.00	41.98	25.25 ± 5.79	0.002*c
31–40	18.00	29.03	22.91 ± 3.48	13.38	32.14	19.83 ± 4.45	
41–50	10.47	23.34	15.6 ± 3.17	7.43	19.67	13.44 ± 3.50	
P-value	<0.001*a			<0.001*b			

(a) Comparison between different age groups in males (ANOVA test); (b) comparison between different age groups in females (ANOVA test); (c) comparison between both sexes (t-test). * significant results were found where p <0.05.

Table 3. Pulp chamber volume of mandibular second molars.

Age (years)	Volume of Mandibular Second Molar (mm^3)						P-value
	Male			Female			
	Min	Max	Mean ± SD	Min	Max	Mean ± SD	
21–30	20.40	48.39	29.59 ± 5.84	13.89	40.55	25.24 ± 6.154	0.004*c
31–40	14.94	30.63	20.82 ± 3.56	13.61	31.42	18.14 ± 3.97	
41–50	10.26	24.24	15.1 ± 3.12	7.68	22.61	13.24 ± 4.00	
P-value	<0.001*a			<0.001*b			

(a) Comparison between different age groups in males (ANOVA test); (b) comparison between different age groups in females (ANOVA test); (c) comparison between both sexes (t-test). * significant results were found where p <0.05.

The r value for maxillary teeth were −0.82, −0.69, and −0.71, and for mandibular teeth they were −0.81, −0.70 −0.72 in males, females, and the pooled sex, respectively (Table 4).

The regressions analysis was statistically significant (<p = 0.001). Figure 2 and 3 shows a scatterplot distribution between age and the pulp chamber volume of maxillary and mandibular second molars. In the establishment of the linear regression model, the coefficient of determination (R^2) and standard error of estimate were calculated R^2 was for maxillary teeth were 0.68, 0.46, and 0.51, and for mandibular teeth they were 0.65, 0.48, 0.52 in males, females, and the pooled sex, respectively (Table 4). The established mathematical models are shown in Table 5.

After the establishment of the mathematical models, another group of CBCT were used for their validation. The MAE and RMSE of the estimated and actual ages are shown in Table 6.

Table 4. Correlation coefficient (r), coefficient of determination (R^2) and standard error of estimate (SEE) for the male, female, and pooled sex samples.

	Pooled Sex			Male			Female		
	r	R^2	SEE	r	R^2	SEE	r	R^2	SEE
Maxillary	-0.71	0.51	5.92	−0.82	0.67	4.89	- 0.68	0.46	6.20
Mandibular	-0.72	0.52	5.71	−0.81	0.65	4.87	-0.70	0.48	5.87

Table 5. Established mathematical model for age estimation via the pulp chamber volume of maxillary and mandibular second molars.

Maxillary Second Molar	Unknown sex	Age = 50.985 − 0.846 × pulp chamber volume
	Male	Age = 55.626 − 0.956 × pulp chamber volume
	Female	Age = 49.375 − 0.851 × pulp chamber volume
Mandibular Second Molar	Unknown sex	Age = 49.261 − 0.783 × pulp chamber volume
	Male	Age = 52.933 − 0.858 × pulp chamber volume
	Female	Age = 47.697 − 0.798 × pulp chamber volume

Table 6. Mean absolute error (MAE) and root mean square error (RMSE) between the actual age and estimated age, using established models.

		Maxillary Second Molar	**Mandibular Second Molar**
Pooled Sex	Number (teeth)	38	28
	MAE (mm^3)	4.89	4.61
	RMSE (mm^3)	6.08	5.97
Male	Number (teeth)	15	9
	MAE (mm^3)	3.40	1.33
	RMSE	4.24	1.94
Female	Number (teeth)	23	19
	MAE (mm^3)	4.77	4.89
	RMSE (mm^3)	5.86	5.871

Figure 2. Scatterplot distribution between age and the pulp chamber volume of maxillary second molars.

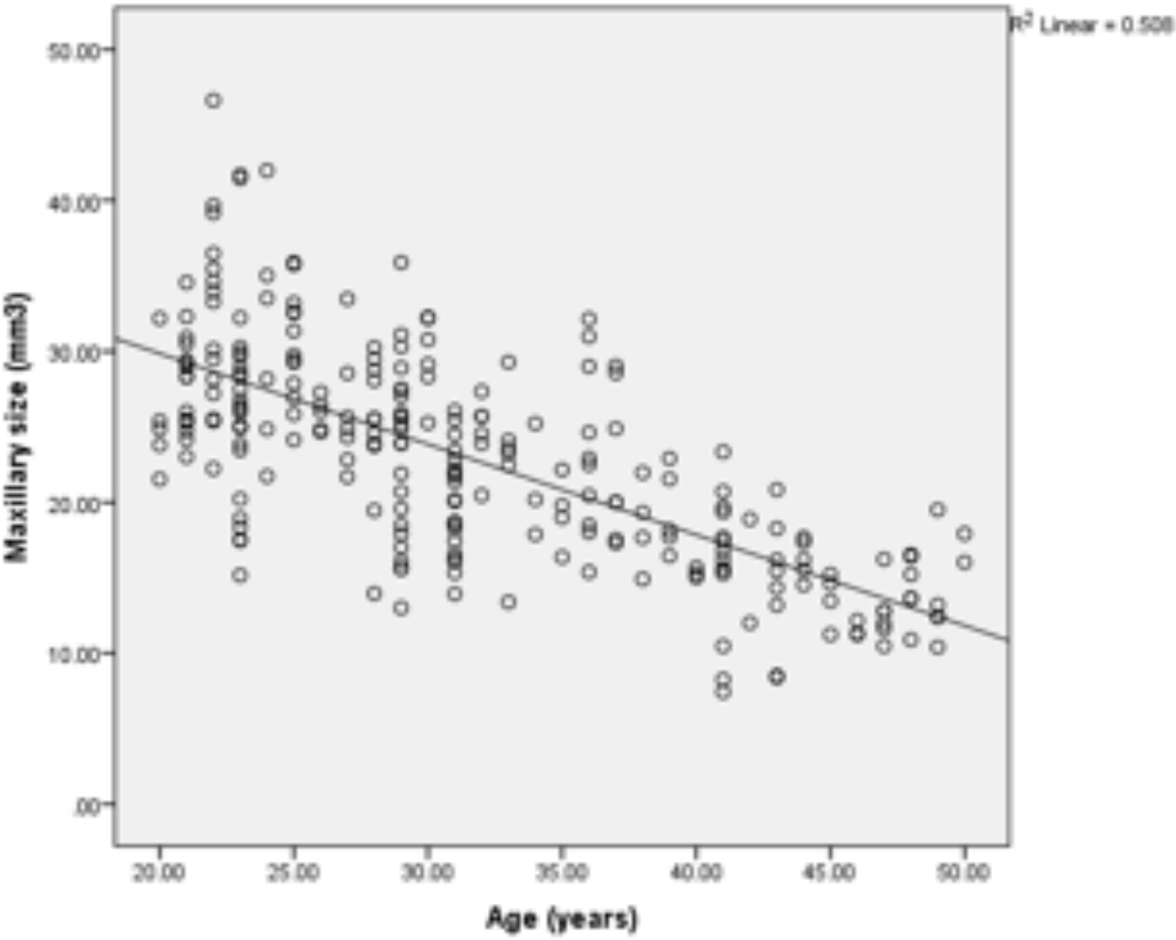
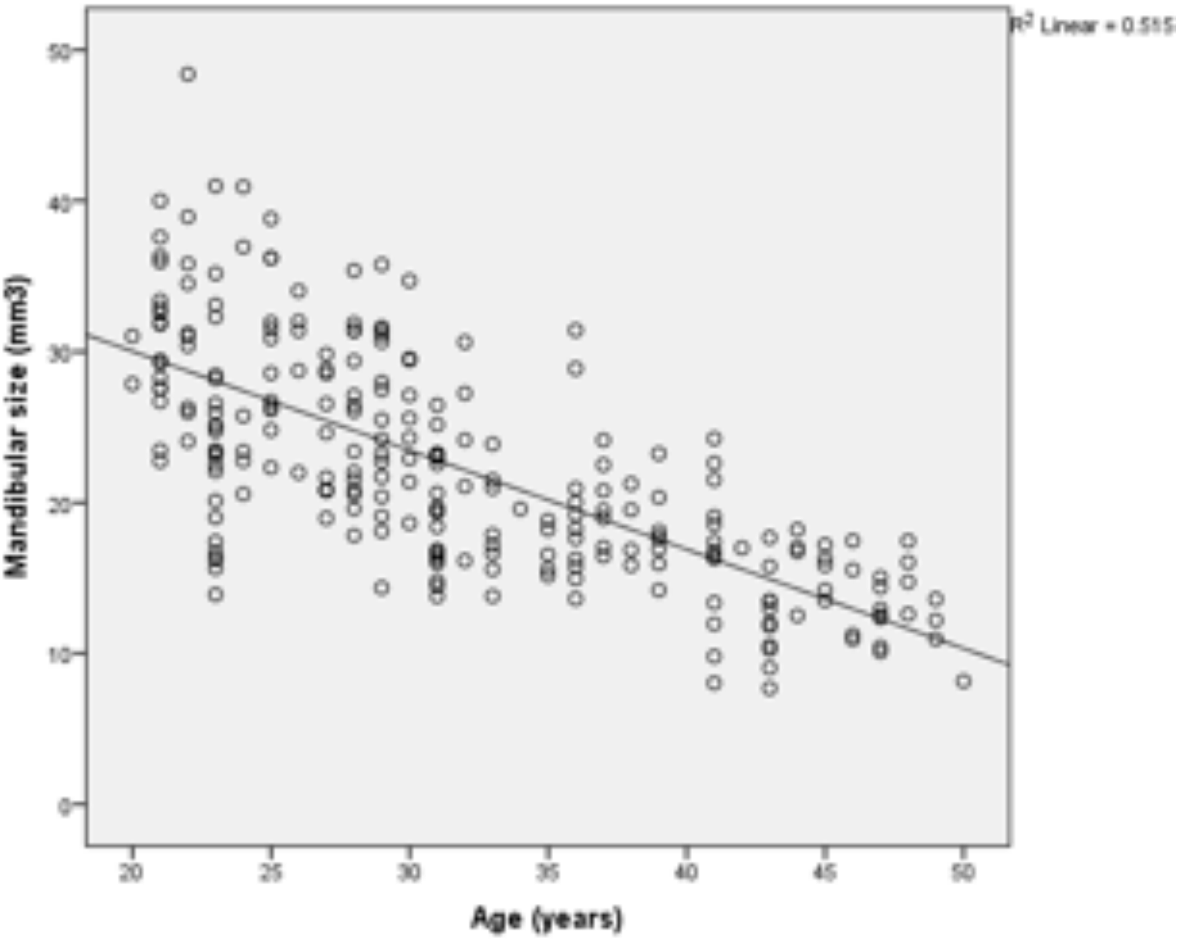


Figure 3. Scatterplot distribution between age and the pulp chamber volume of mandibular second molars.



DISCUSSION

From human teeth, molars were selected in the present study as they have a wider pulp chamber cavity, allowing better delineation of the pulp chamber borders in three-dimensional image-based segmentation as compared to other teeth. Moreover, the anatomical position of molars at a planar field in the posterior part of the mouth enables the attainment of clear and accurate three-dimensional images after reconstruction. Specifically, the second molar was chosen due to its morphological stability and less common cases of caries and attrition when compared to first molar. [16] In addition, in the study conducted by Ge et al. in 2016, they found that among the 13 types of teeth, second molars were the best for age identification.[12]

When assessing a pulp chamber, volume is more preferred over assessing area or linear measurements, as secondary dentine apposition is not homogenously distributed along all pulp surfaces. In molar teeth, it appears in greater amounts on the roof and floor of the coronal pulp chamber. [17] When the pulp chamber and tooth volumes were examined in relation to age in a previous study, pulp chamber volume variability was found to better correlate with age than tooth volume.[18]

Regarding the selected age group, previous studies have reported that a change in pulp chamber volume is significant in subjects below 50 years old, after which change becomes relatively slow and insignificant. [16] Therefore, the age group selected was 21–50 years old. In addition, statistically significant shrinkage in pulp chambers occurs after every 10 years. [4] Thus stratification on a 10-year age difference in the present study was performed.

In regard to the assessment method, a three-dimensional technique is preferred for volume assessment. CBCT was selected as it is an easy, low cost, low radiation dose, accurate, and highly reproducible approach that allows for the accurate calculation of tooth volumes. In addition, the technique provides a larger scanning area, in the form of a multi-planar cross-sectional dataset and three-dimensional reconstructions (from a single scan).[14,19,20]

According to our knowledge, there are few studies in Egypt assessing dental age estimation and this is the first study that uses pulp chamber volume changes in second molars as an indicator of secondary dentine apposition in relation to age.

The pulp volume was ranging between 7.43-48.39 and SD 3.12-6.154. these values are less than that recorded by Ge et al., 2016 but this is due to the more confined age group selected in our study.[12]

When assessing differences in pulp chamber volume by sex, the mean value was higher in males in both maxillary and mandibular teeth, and this is explained by the fact that the size of the teeth may be smaller in females, which could result in a smaller pulp chamber size.[16] In addition, hormonal differences in both sexes may be a reason for such variations.[21] The difference was statistically significant here, necessitating the derivation of a sex-specific equation to predict age. This difference can be explained by Stroud et al., 1994, who found that there is a degree of sexual dimorphism in dentine thickness controlled by the Y chromosome.[22] Our finding is consistent with previous studies that have found a difference between sexes,[4,12,16,23] but goes against other studies [10,13,17,24] which have stated no sex difference.

The previous studies on Egyptian subjects [25-28] reported no sex difference, unlike the present research. This could be attributed to the imaging techniques used, as they used two-dimensional radiographs. When the three-dimensional pulp is reproduced in a two-dimensional radiograph, the edges of the pulp may be blurred due to the cylindrical shape of the pulp of multi-rooted teeth.

As regards pulp chamber volume, the present study showed statistically significant changes in pulp chamber volume with increasing age with moderate to strong negative correlation (r value for maxillary teeth were −0.82, −0.69, and −0.71, and for mandibular teeth they were −0.81, −0.70 − 0.72 in males, females, and the pooled sex, respectively). This finding is higher than the previous study on the Davangere population, assessing pulp to tooth area ratio in mandibular second molars using a two-dimensional imaging technique, where r = −0.441, −0.406, and −0.419 among males, females, and pooled sexes respectively.[16] Also, it is higher than a previous study assessing the tooth coronal index (TCI) of mandibular second molars in Egyptian adults using a two-dimensional technique, where r ranged between 0.079–0.17 in different sexes. [27]

The correlation was found to be more relevant in males than females and this is similar to previous studies on mandibular second molars in the Davangere population.[16]

The R^2 is often used to indicate the association between chronological age and pulp chamber volume. In previous studies, R^2 was highly variable depending on the tooth type, sample size, age distribution range, imaging system, and population source. In the present study, the R^2 is relatively large, ranging between 0.46-0.68. These values were very close to the previous study that assessed the pulp chamber volume of 13 types of teeth and reported that the second molars showed the best R^2 value with respect to age (0.458–0.642), especially in the case of the maxillary teeth.[12]

The observed R^2 was stronger for males than females. These findings are similar to the previous study on mandibular second molars in the Davangere population, in which the R^2 of the pulp to tooth area ratio in second molars was 0.19, 0.16, and 0.174 for males, females, and pooled sexes, respectively.[16] However, the relationship goes against the other study by Ge et al., 2016, in which males showed a higher R^2 value in both mandibular and maxillary second molars, where the R^2 values of maxillary teeth were 0.498. 0.491, 0.642, and for mandibular teeth they were 0.487, 0.458, and 0.614, in pooled sexes, males, and females, respectively. [12]

In the present study, the R^2 of mandibular teeth was better than maxillary teeth. This goes against the previous study assessing the pulp chamber volume of second molars.[17] However, other studies assessing other types of teeth are similar to our study, with mandibular teeth showing a better R^2. Kazmi et al., 2019, found that the R^2 values of mandibular and maxillary canine pulp chamber volumes of identified sexes were 0.33 and 0.31, respectively. [23] Also, Biuki et al., 2017, assessed the pulp chamber volumes of anterior teeth and reported an R^2 value ranging from 0.65 to 0.75 for maxillary teeth and from 0.60 to 0.76 for mandibular teeth.[29] Similarly, another previous study on first molars reported an R^2 value of pulp chamber volume with age of 0.586

and 0.609 for maxillary and mandibular teeth, respectively. [30]
The SEE predicts the deviation of estimated age from the actual age. In the present work, the SEE was calculated and was around 6 years for maxillary teeth and around 5 years for mandibular teeth. These findings are much better than the SEE reported for the pulp to tooth area ratios of mandibular second molars in the Davangere population, which were 11.9 and 12.0 years in males and females, respectively. [16] Also, this is much lower than the SEE reported by Ge et al., 2016, in maxillary (6.75–8) and mandibular (7–8.11) second molars. [17] This difference in SEE could be attributed to the localized age group selected in the current study (21–50 years).
After the establishment of mathematical models, another group of CBCT images was used for validation. The MAE between the actual and estimated ages from the regression model was less than 10, which is accepted in forensic practice. However, the MAE of mandibular teeth in males was relatively small. The RMSE is the standard deviation of the residuals (prediction errors). Residuals are a measure of how far from the regression line data points are found. The RMSE was very close to that of the SEE from regression model, except for mandibular teeth in males, in which the RMSE was relatively small. This extremely low value of both the MAE and RMSE in mandibular teeth in males can be attributed to the relatively small number of teeth used from this group in the validation procedure.
One of the major limitations of the present study is unavailability of micro-CT for the assessment of the segmentation accuracy of CBCT. In addition, a smaller number of teeth was obtained from older people due to tooth loss and difficulty in retrieving teeth meeting the inclusion criteria. However, this selection was necessary as the volume measurement is the sole purpose. Another limitation was the small number of teeth in the validation sample; thus, it is recommended to perform validation of the established model on a wider scale.

CONCLUSIONS

The present study has investigated the relationship between age and the pulp chamber volume of maxillary and mandibular second molars. There was significant difference between both sexes and between maxillary and mandibular second molars. The established model, intended for age estimation in Egyptian adults, has reasonable accuracy, with a relatively large correlation coefficient. It is recommended to use a sex specific equation if possible due its higher accuracy. The study of the pulp chamber volume of multi-rooted teeth, specifically second molars, for age estimation is promising and is worthy of analysis using a larger sample size.

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Comparative forensic analysis of reverse root canal filing and conventional method for DNA isolation from extracted teeth under different environmental conditions: A prospective study

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The authors declare that they have no conflict of interest.

KEYWORDS

DNA isolation,
Forensic odontology,
Reverse root canal,
Teeth

J Forensic Odontostomatol
2020. Dec;(38): 3-35:41
ISSN :2219-6749

ABSTRACT

The grinding of a whole tooth specimen has been considered the conventional method to extract genomic deoxyribonucleic acid (DNA) in forensic science. However, we have tried the less destructive reverse root canal filing (RRCF) method without disturbing the morphology of the tooth to achieve competent amplifiable DNA. A total of 27 pairs of bilateral intact extracted teeth from the same subject were used in three different simulated environmental conditions for the respective RRCF and conventional methods: (a) soil burial for six months, (b) incineration at 200° C for four minutes, and (c) immersion in water for two months. Qualitative agarose gel electrophoresis assessment and downstream amplification were performed. The results showed significantly higher mean DNA concentration for the RRCF method in all three environmental conditions (p value = 0.008) in comparison to the conventional method. However, comparable qualitative results were found in both methods for the mean DNA concentration for incinerated (159.49 ng/ml), soil (119.52 ng/ml), and water (108.60 ng/ml) samples. It was concluded that the RRCF method is better quantitatively (ng/ml) and comparable in terms of quality with respect to the conventional method, with the added advantage of preservation of the tooth morphology.

INTRODUCTION

Calcified tissues like teeth and bone are a major source for forensic research, which are accessible and useful even after exposure to extreme conditions (e.g., mass disaster, fire, blast, decomposed body). Teeth are most likely to be contaminated when exposed to the environment.[1] Gaytmenn and Sweet showed that DNA quantity extracted from the root is 10 times greater than that retrieved from the crown.[2] The dental tissues comprising the dentine and pulp contain the majority of the cells, as the enamel and cementum are mostly acellular and contain relatively small amounts of DNA. The complete powdering of the tooth specimen has remained the gold standard for DNA extraction from samples of teeth.[3,4]

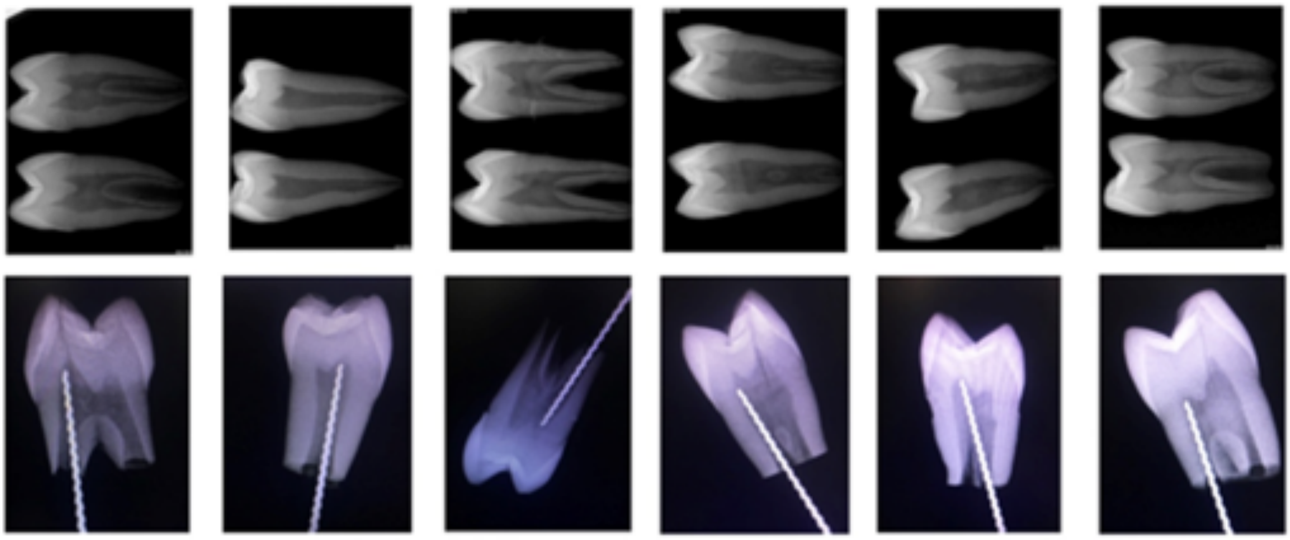
A tooth, when chosen as forensic evidence, needs to be preserved for reference as well as for further analysis. But the standard method of complete pulverisation of the tooth results in loss of the specimen. With the evolution of newer techniques,[5] alternative methods of DNA extraction have been tried and tested in order to prevent the loss of the

specimen.[6] Numerous techniques for sampling teeth are described in the literature, including pulverisation of the tooth[7] and root,[8] vertical sectioning of the tooth, [9, 10]horizontal sectioning at the enamel–cementum (crown–root) junction,[11] endodontic filling method,[12]and non-powdering digestion buffers method.[13] Harvella et al. proposed that occlusal perforation, cervical perforation, and the cervical cut method allowed preservation of the tooth sample. They found that a satisfactory amount of DNA was obtained from 30 different ancient samples.[14]
The amount of DNA in the tooth depends upon various factors like tooth type, age, and individual variations. Thus, bilateral teeth of the same individual were chosen to eliminate variation in the amount of DNA within the tooth.[15]Premolars were chosen among other teeth due to availability. Garriga et al. ascribed the use of premolars to the fact that bilateral tooth extraction was primarily for orthodontic purposes (23 pairs). The most common extracted teeth for orthodontic reasons are maxillary and mandibular first premolars.[16, 17] A female predilection was also noticed with a male/female ratio of 3:4.[16]
A method using a reverse root canal filing (RRCF) technique by endodontic files for retrieving dentine and pulp for DNA analysis was first demonstrated by J.C. Cobb.[18] This method used H files to retrieve the dentine and pulp tissue in powdered form and therefore allowed a substantial amount of preservation of the tooth structure. Cobb demonstrated the superior results of the RRCF method over the conventional method.
The current study aimed to compare the efficacy of DNA extraction from teeth sampled by two different methods. Teeth were subjected to different simulated environmental conditions to evaluate the effect on the sampling process. Comparison of DNA yield between the two sampling methods would allow for the evaluation of the RRCF method.

MATERIALS AND METHODS

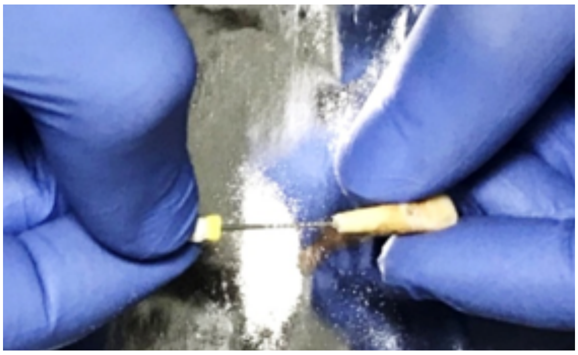
A two year prospective study was conducted using 27 pairs of teeth from patients undergoing bilateral extraction of permanent teeth. Bilateral healthy intact teeth from the same individual of the same type, devoid of caries, restorations, and root canal treatment, were extracted. The samples were then categorised and subjected to three simulated environmental conditions: (a) soil burial for a duration of six months,[15] (b) incineration at 200 °C for four minutes,[16] and (c) immersion in fresh water for two months. Two teeth from the same arch of the same subject were divided to be assessed by the RRCF and conventional grinding methods, respectively. We obtained approval from the institute's ethics committee, along with informed consent from the volunteers.
Extracted teeth were physically cleaned from attached soft tissue using a BP blade with handle (Amkay, India). They were soaked in 2% sodium hypochlorite (Chemdent, India) for 5 minutes and then 100% ethanol for 5 minutes, followed by drying at 30 °C overnight. For samples buried in soil, plastic containers containing soil with teeth placed at a depth of 20 cm were used and labelled for each sample. These containers were then exposed to normal weather conditions for six months (soil and water). For incinerated samples, all the samples were heated to a standing temperature of 200° C in an electric furnace for four minutes. For samples exposed to water, plastic containers containing fresh water with teeth placed at a depth of 30 cm were used and labelled for each sample. Fresh water was changed weekly for a total duration of two months.
After completion of the different environmental exposures, the teeth went through physical cleaning, followed by chemical cleaning with 1% hypochlorite. Finally, UV irradiation was applied for 30 minutes to achieve maximum neutralisation of exogenous contamination.
Grinding of tooth samples:
A: Conventional method: The tooth was ground to a powder mechanically using a sterile mortar and pestle. This powder was then collected over a sterile sheet of foil and transferred to a microcentrifuge tube (Eppendorf, Germany) of 2 ml capacity.
B: RRCF method: Pulp cavity was accessed from the apical portion of the tooth after removing the apical root portion of 2 to 3 mm. Hedstrom endodontic files (Mani, Japan) (sizes 08–80) were used to powder the dentine and collected on sterile sheets of foil. Radiovisiography (RVG) of each tooth was performed before and after filing in order to assess the condition of the root and pulp chamber. (Figures 1 and 2).

Figure 1.



Case number1 Case number2 Case number7 Case number8 Case number13 Case number14

Figure 2.



Organic extraction by standard phenol-chloroform method (Fisher, BP17521-400) was used uniformly in both the groups and the three environmental conditions. The quantity of extracted DNA was evaluated by Nanodrop, BioPhotometer (Eppendorf, Germany). This involved the standardising of the cuvette with 2 µl of buffer (molecular grade water). After standardisation, 2 µl of extracted DNA was used to determine the concentration and optical density (OD) value of each sample. Quality assessment was performed by 1% agarose gel electrophoresis (Gel Electrophoresis unit, Vari-Gel Maxi system - Scie-Plas, England). One percent agarose was used in TAE buffer to cast the gel along with 5 µl of ethidium bromide. Then, 7 µl of DNA sample with 3 µl of loading dye was placed in the well. The gel was run at 200 kvh for thirty minutes. The image was captured using a gel documentation system (Gel-Doc™ EZ Imager - Bio Rad, California, USA). The same purified extracted product was amplified using a known primer to ascertain its reliability.

The primers used are as mentioned below:

ALU-STYa

Forward Primer 5'- CAT GTA TTT GAT GGG GATAGAGG-3'

Reverse primer 5'- CCT TTT CAT CCA ACT ACC ACTGA-3'

ALU-STXa

Forward primer 5'- TGA AGA AAT TCA GTT CAT AGCTTG T-3'

Reverse primer 5'- CAG GAG ATC CTG AGA TTA TGT GG-3'

STATISTICAL ANALYSIS

The mean values, standard deviation, and ranges (maximum and minimum) were calculated for each variable. The resulting data were analysed using SPSS software version 21. Data were expressed as mean ± standard deviation. Differences between different variables were analysed using Mann–Whitney test. Pearson's correlation coefficient was carried out to determine the level of correlation between the findings of the two methods. A p value ≤ 0.05 was considered statistically significant.

RESULTS

The mean weight of tooth powder used in our study was 73.56 ± 2.28 mg in the RRCF method

and 170.40 ± 7.04 mg in the conventional method (Table 1). For all the samples, the mean DNA concentration was 155.64 ± 89.82 ng/ml in the RRCF method and 102.77 ± 55.0 ng/ml in the conventional method (Table 2).
The mean DNA concentration per mg of tooth powder was 2.21 ng/m/ in the RRCF method and 0.60 ng/ml in the conventional method. The mean DNA concentration was 1.68 ng/ml in the group of incinerated samples, 1.24 ng/mlin samples buried in soil, and 1.14 ng/ml in samples submerged in water. Twenty one samples were amplified in both the RRCF method and the conventional method.

Table 1. Comparison of powder weights by Mann–Whitney U test

METHOD	MEAN WEIGHT	MEAN RANK
RRCF (N=27)	73.56 ± 2.28 mg	14
CONVENTIONAL (N=27)	170.40 ± 7.04 mg	41
p value	0.0001 (significant)	

Table 2. Groupwise comparison of mean DNA concentrations (A)

METHOD	MEAN CONC. SOIL ng/ml	MEAN CONC. INCINERATION ng/ml	MEAN CONC. WATER ng/ml	MEAN CONCENTRATION OVERALL ng/ml
RRCF METHOD	145.57	191.83	129.52	155.64
CONVENTIONAL METHOD	93.47	127.15	87.69	102.77
DIFFERENCE	52.1	64.68	41.83	52.87

METHOD	MEAN RANK SOIL (N=9)	MEAN RANK INCINERATION (N=9)	MEAN RANK WATER (N=9)	MEAN RANK (OVERALL) (N=27)
RRCF	11.67	11.33	11.89	33.20
CONVENTIONAL	7.33	7.67	7.11	21.80
p value	0.085	0.145	0.058	0.008

(B) Mann-Whitney U test

Table 3. Comparison of mean DNA concentrations by weight in Mann–Whitney U test

METHOD	MEAN RANK SOIL (N=9)	MEAN RANK INCINERATION (N=9)	MEAN RANK WATER (N=9)	MEAN RANK OVERALL N=27
RRCF	13.56	13.78	13.67	40.07
CONVENTIONAL	5.44	5.22	5.33	14.93
p value	0.001	0.001	0.001	0.001

DISCUSSION

The RRCF method is a relatively new method of tooth sampling, with published data limited to only two studies, one by Cobb in 2002 and the other by Hughes-Stamm et al. in 2016. Cobb suggested the RRCF method for the first time with the view that it concentrates on the dentine-pulp rich area for obtaining powder of the tooth. This method approaches the tooth from the apical portion of the root, thereby preserving the tooth architecture and morphology with good amount of concentration of DNA per mg of tooth powder.[18]In the present study, we included three environmental parameters (soil, incineration, water) with the RRCF method to evaluate the efficacy and advantages of this method over the conventional method in terms of DNA yield.

The RRCF method used a lesser amount of dentine powder compared to the conventional method. The results were comparable to the study conducted by Hughes-Stamm et al., where the amount of tooth powder in the conventional method was significantly higher than in the RRCF method (Table 1).[19] This can be related to the powdering of only dentine near the pulpal region in the RRCF method in comparison to the complete grinding of the tooth in the conventional method. This implies that the RRCF method preserved tooth samples by utilizing only a fraction of the tooth specimen as compared to the conventional method, in which all the powder is used at once.

Our results indicated that the RRCF method yielded more DNA as compared to the conventional method (Table 2 & 3), and the difference was statistically significant. This was in accordance with the study by Cobb.[18] Hughes-Stamm et al. also observed that the DNA concentration was greater in the RRCF method as compared to the conventional method in most samples.[19] The first possible explanation of these results could be the selective processing of a DNA-rich zone of the dentine–pulp complex of the tooth in the RRCF method as compared to the conventional method processing the whole tooth, including the acellular cementum and part of the enamel. Secondly, the non-cellular part of the tooth could result in decreased yield by reducing the action of the reagents during the DNA extraction procedure. These results suggest the possible advantage of the RRCF method over the conventional method in terms of DNA quantification. The RRCF method reduces the chance of exogenous contamination by targeting a specific portion of the tooth. Moreover, it excludes the non-cellular component of the tooth, thereby decreasing the quantity of polymerase chain reaction (PCR) inhibitors and contamination.

The environmental conditions were defined in a way that they included the maximum conditions for the forensic testing like accidental fire, drowning, or soil burial. The negative effect on DNA yield from teeth buried in soil for six months was shown by Schwartz et al.[15] Samsuwan et al. also showed that one year of tooth burial in soil is better in comparison to six months of burial. They hypothesised that in one year the specimen becomes dry and it is easy to extract the DNA.[20]The results of our study were partly not in accordance with the previous literature, wherein the soil-buried samples showed better results as compared to incinerated samples.[21] Garcia et al. found that samples exposed to soil yielded the maximum quantity, followed by incinerated samples, while samples submerged in water gave the minimum yield.[21] Chowdhury et al. and Kumar et al. also found similar results to those of Garcia et al. [22, 23]

Teeth immersed in water for more than one month have shown detrimental effects for DNA extraction.[19]The low value of mean DNA concentration in samples kept in water and soil might be related to the DNA degradation due to exposure to microorganisms. The results could not reach significant values, probably due to the small number of samples in each group.[24]

In samples buried in soil, the degradation of DNA depends upon the accessibility of the apical foramen. The apical foramen is not protected by alveolar bone and is a gateway for bacterial entry and exit to the pulpal region. Secondly, microorganisms from soil can act as a source of exogenous contamination. The exogenous contamination reduces the DNA yield, thereby posing difficulty in isolation of endogenous DNA. Thirdly, chemicals like fulvic acid and humic acid that are indigenous to the soil increase the DNA degradation by facilitating the growth of microorganisms and may act as a PCR inhibitory factor. [7, 22, 24]

Samples submerged in water showed the poorest result due to the dilution effect. Because of the presence of water, the rate of DNA hydrolysis

would increase, and the degradation in water would be more than in any other condition like soil burial or incineration. [22]
Incineration at a temperature of 200° C was performed to simulate fire incidents.[10] Total genomic DNA is comprising of nuclear (nDNA) and mitochondrial DNA (mt DNA).
It was interesting to note that the group of incinerated samples showed the best results, followed by those buried in soil and the samples submerged in water, showing the lowest concentration by both methods (Table 2). The possible reason for the better yield of incinerated samples could be the ability of enamel and cementum to withstand a higher temperature (>200° C for four minutes) for degradation and protecting the dentinal and pulpal portion of the tooth.[25]
The best possible explanation for the difference in results was the low incineration temperature. Garriga et al. showed that physical degradation of enamel and dentine was seen at a temperature of 200° C for ten minutes, along with a decrease in the quantity of DNA yielded. The fragmentation of the crown and fracture of the root occurred at a temperature greater than 300 °C for four minutes or more. Once the encapsulation of enamel and dentine was lost, there was tremendous decrease in the DNA yield.[16] In our study, the incineration temperature was 200° C for four minutes, and in all samples the macroscopic examination of the specimen showed no evidence of fracture or fragmentation. Thus, at this temperature the encapsulation of dental hard tissue was intact, and therefore the DNA-rich zone was preserved.
This inter-group comparison implies that for a lower temperature of incineration (<200°C), the degradation effects were lower as compared to the degradation in soil and water. Moreover, the samples immersed in water have the lowest DNA quantity compared to any other environmental exposures.
DNA quantification was also analysed in terms of DNA yield per mg of tooth powder of dentine. In all samples, the mean DNA yield per mg of dentine powder was significantly higher in the RRCF method as compared to the conventional method. It has been hypothesised that dentine and pulp have more cells with mtDNA and nDNA, whereas enamel is devoid of nuclear content.
Hughes-Stamm et al. found that the DNA yield per mg of tooth powder in the RRCF method was high in all samples.[19] It was hypothesised that the efficiency of DNA yield was due to the lower amount of powder utilised for DNA extraction in the RRCF method. Also, the powder generated in the conventional method was considered suboptimal due to the possibility of contamination from pulverisation of the complete sample. The utilisation of a relatively lower amount of powder for DNA isolation highlights the efficiency of the RRCF method.
The mean concentration per mg of DNA yield was analysed in three environmental conditions (Table 3). The incineration group showed the best yield per mg of dentine powder, followed by samples buried in soil and samples immersed in water. The efficiency in terms of concentration per mg of dentine powder was significantly higher in the RRCF method as compared to the conventional method in all three groups. The mean DNA concentration obtained in the incineration group was highest, followed by the soil group and the samples immersed in water. The reason for this high efficiency can be attributed to the utilisation of a low amount of powder in the RRCF method.
Hughes-Stamm et al. compared DNA quality by measuring the average number of alleles recovered. Overall, they recorded a higher amplification in the RRCF method.[18] It was postulated that the contamination due to suboptimal powder and delay in PCR cycles in the conventional method accounted for the difference in amplification.
The DNA amplification of the water-immersed group showed a positive result in comparison to the conventional group. DNA amplification depends upon many factors, such as purity, concentration of DNA, and presence of inhibitors. The grinding of the whole tooth has many inhibiting factors, which do not allow downstream amplification. [19]
Apart from better efficiency, the RRCF method has an added advantage of preservation of tooth samples by utilizing only an internal part of the tooth. Thus, if needed, samples could be further analysed, as required many times by forensic experts. In addition, due to the approach of tooth samples from the apical portion of the root, the crown portion remains intact. Thus, the morphology and anatomy of the tooth are also preserved. The major disadvantage of the RRCF

method is the laborious and time-consuming filing of tooth samples with H files.

CONCLUSIONS

A higher mean DNA concentration by the RRCF method in all three environmental conditions was indicative of better DNA yield in comparison to the conventional method. The RRCF method yielded better results in terms of DNA concentration per mg of tooth powder. Amongst different environmental conditions, the incinerated samples yielded the best results. However, the water-immersed samples showed comparable results in both methods. Further research with broader criteria of environmental exposures coupled with a larger sample size is required.

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