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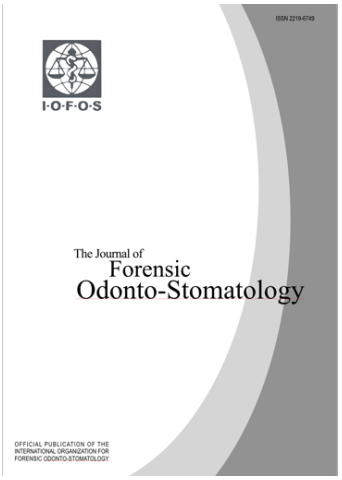

THE JOURNAL OF FORENSIC ODONTO-STOMATOLOGY

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CONTENTS

Application of an ancestrality estimate method through morphoscopic analysis of Brazilian skulls

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KEYWORDS

Forensic Sciences, Forensic Dentistry, Ancestry Estimation

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ABSTRACT

The objective of the investigation was to test a technique originating in the United States to verify the accuracy of the ancestry estimate in a sample of Brazilian skulls. The sample consisted of 120 skulls of Brazilian adults from the collection of the Institute of Education and Research in Forensic Sciences (IEPCF), Guarulhos-SP, Brazil, with ancestry determined by routine examination. In the skull, the following structures were analyzed: anterior nasal spine (ANS), inferior nasal aperture (INA), interorbital breadth (IOB) nasal aperture width (NAW), nasal bone structure (NBS), and post bregmatic depression (PBD). The Hefner and Ousley technique was applied to classify ancestry. Data were registered and correctness percentage was obtained. To improve ancestry prediction, Machine Learning techniques were used. Regarding the error of the studied method and the correctness of the complete sample, the study presented a percentage above 50%, with values such as 56.33% for whites and 53.07% for non-whites. The most frequent ANS parameter was marked (37.5%), INA straight (31.7%), IOB wide (38.3%), NAW medium (32.5%) NBS triangular (29.2%), and PBD present (78.3%). The application of the method in the sample of the study showed a limitation to estimate ancestry, suggesting a need for adaptation for its applicability in the Brazilian population.

INTRODUCTION

The existence of sexual, ancestral, and age dimorphism in human skeletons, and its importance in medical legal investigations is well established. According to contemporary literature, the biological profile is outlined through the analysis of bone structures, in turn, this analysis can be performed through numerous methods that are classified into two main groups: qualitative also known as morphological methodologies, and quantitative also known as morphometric methodologies. Qualitative or morphological methods have as main objective to obtain biological parameters through subjective analysis of anatomical characters. On the other hand, quantitative or morphometric methods recommend the use of linear measures, projections, angles, and indices to outline the demographic characteristics of the remains under analysis [1].

In human identification, one of the main characteristics in the design of the biological profile is the estimation of sex, age,

height, and ancestry. In the latter case, the geographic origin is of fundamental importance in establishing the identification of a forensic case.[2]

In addition, the determination of ancestry has been little explored, though skulls can provide substantial information in this regard. After the pelvis, the skull remains one of the parts of the human skeleton that has the most information on this subject.[3] These characters can be estimated through a visual comparative analysis by the correspondents of known origin called the qualitative method or through measurements of the cranial anatomical structures called the quantitative method. The quantitative methodology applied to the skull is called craniometry, which can be defined as being a technique, or conventional system, which determines the measurement of the skull in a universally systematized way, which allows the comparative evaluation between studies carried out by different researchers.[4]

Within forensic anthropology, there are several discussions regarding the terminologies used in the studies, such as the concept of ethnicity, ancestry, and race.[5] On one hand, the word ethnicity, derived from the Greek ethnos, has a slight differentiation with the term ancestry. Ethnicity means a multi-purpose concept, which builds the identity of an individual summarized in kinship, religion, language, shared territory, and nationality, in addition to physical appearance, while the term ancestry encompasses similar genetic characteristics within a given population, but distinct when comparing two groups. These genetic patterns have a great influence on physical traits and are constant throughout human life.[6] Considering the miscegenation processes of the human population of the 21st century, in the present study we adopted the term ancestry, considering the geographical origin [7] as a parameter that has the potential to assist in the identification of a skeleton in the area of forensic anthropology.

Klales & Kenyhercz[7] applied the Hefner technique [8] to an American collection of whites and blacks (Hamann-Todd Collection) and concluded that the technique could be applied among this population group, since the success rate found was high, and the agreement between the exams was adequate. Considering that techniques based on a reference population may not be suitable for application in other groups, the study's objective was to test a technique originating in the United States to verify the accuracy of the ancestry estimate in a sample of Brazilian skulls.

MATERIAL AND METHODS

This study followed the recommendations of the Declaration of Helsinki for research involving human subjects and was approved by the Ethics and Research Committee of the Faculty of Dentistry of the University of São Paulo (FOUSP) (reference number 71476817.4.0000.0075).

The sample consisted of 120 skulls of Brazilian adults (supplementary table) from the collection of the Institute of Education and Research in Forensic Sciences (IEPCF), Guarulhos-SP, Brazil, with ancestry determined by routine examination.

The Hefner and Ousley technique[9] was applied to classify ancestry. The result of the analysis was compared with the examination carried out by an identification specialist, who determined ancestry in the service routine. The rate of correct answers has been described.

In the skull, the following structures were analyzed [8]: anterior nasal spine (ANS), inferior nasal aperture (INA), interorbital breadth (IOB) nasal aperture width (NAW), nasal bone structure (NBS), and post bregmatic depression (PBD). These structures, analyzed according to the author, were then automatically reclassified according to the index called Optimized Summed Scoring Attributes (OSSA), which considers the 6 structures mentioned in a dichotomous way and integrates them in a decision tree, to estimate ancentrality [9] (Chart 1).

The classification of each skull was made according to the parameters described above, and the results were included in a program provided by the author of the technique, which automatically classifies ancestry. These results were compared to the routine examination (prior registration), given to each skull by traditional methods, by the Service's routine.

To carry out the research, an examiner (JCM) was properly trained by an experienced professional (TTLC), who was the gold standard for calibration. Both performed a re-analysis in 10% of the sample.

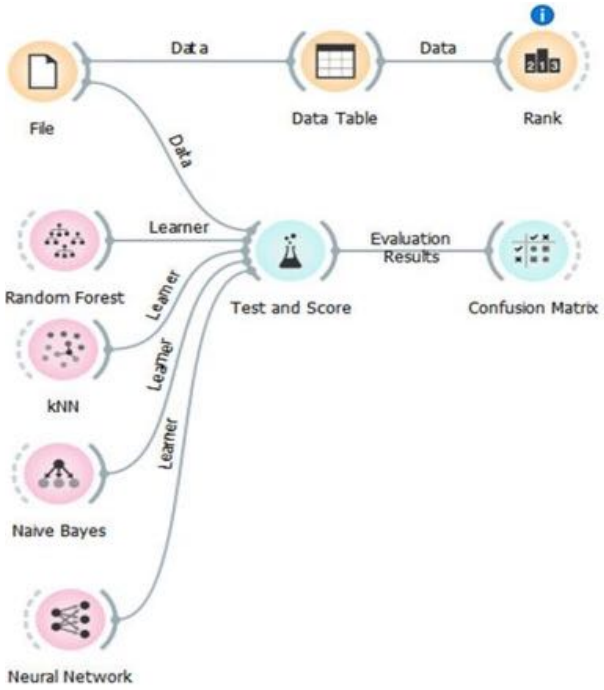
To improve ancestry prediction, Machine Learning techniques were used. Figure 1 shows the flowchart of the analysis of this technique. The data were worked in the R language and

Python. The steps were: check the best weight of the studied variables and check the accuracy of the algorithms.
All measurements were performed without any prior knowledge of the ancestors of the skulls by the examiners. The statistical test to be performed was the linear regression model. The STATA 13.0 program was used to enter and process the data.

Chart 1. Hefner's classification (8) dichotomized by Hefner & Ousley (9)

	Hefner's classif. (8)		Hefner & Ousley's classification (9)
ANS - Anterior nasal spine	1 2 3 Missing	Slight Intermediate Marked -	0 1 1 Missing
INA - Inferior nasal apertura	1 2 3 4 5 Missing	Pronounced slope Moderate slope Straight Partial Sill Sill -	0 0 0 1 1 Missing
IOB - Interorbital breadth	1 2 3 Missing	Narrow Intermediate Wide -	1 1 0 Missing
NAW - Nasal aperture width	1 2 3 Missing	Narrow Medium Broad -	1 1 0 Missing
NBS - Nasal bone structure	0 1 2 3 4 Missing	Low/Round Oval Marked Plateau Narrow PLateaw Triangular -	0 0 1 1 1 Missing
PBD - Postbregmatic depression	0 1 Missing	Absent Present -	1 0 Missing

Figure 1. Flowchart of the machine learning technique used to predict ancestry



RESULTS

To assess the reproducibility of the ancestry estimation method, the intra-examiner, and inter-examiner concordance tests were performed and the results indicate that the method is reproducible, as they presented an agreement that varied between moderate to perfect in both inter (0.467-1.0) and intra (0.529-1.0) analyses. (Table 1).

When checking the results, it was possible to understand that both in the inter-examiner and intra-examiner analysis, the variable that showed the best performance was the anterior nasal spine (ANS) showing 1.0 and 0.874, respectively, and the one that had the least agreement was the width of the nasal opening (NAW) showing 0.467 and 0.529.

Regarding the error of the studied method and the correctness of the complete sample, the study presented a percentage above 50%, with values such as 56.33% for whites and 53.07% for non-whites (Table 2).

Table 1. Results regarding intra-examiner and inter-examiner agreement for the ancestry estimation method applied in the study

Variable	N(10%)	*Intraobserver*		*Interobserver*	
		Kappa	p-valor	Kappa	p-value
ANS	12	0.874	<0.001	1.000	<0.001
INA	12	0.579	<0.001	0.657	<0.001
IOB	12	0.609	<0.002	0.625	<0.002
NAW	12	0.529	<0.002	0.467	<0.024
NBS	12	0.529	<0.002	0.750	<0.001
PBD	12	1.000	<0.001	0.833	<0.001
Ancestry	12	1.000	<0.001	1.000	<0.001

Hefner's method (2009) recommends the qualitative analysis of some structures of the skull that were analyzed and classified as the one proposed, thus generating a number called frequency that expresses the number of skulls that were classified within each specific characteristic. Since some skulls were not eligible for classification of some of the structures proposed by the technique, the method occurred in a small number of the sample without altering the continuity and proposals of the study (Table 3). The most frequent ANS parameter was marked (37.5%), INA straight (31.7%), IOB wide (38.3%), NAW medium (32.5%) NBS triangular (29.2%), and PBD present (78.3%).

Table 2. Results regarding intra-examiner and inter-examiner agreement for the ancestry estimation method applied in the study

	% correctness	**% error**
White	56.33	43.67
Black	53.07	46.93

Table 3. Results for each analyzed sample structure in frequency and percentage for the ancestry estimation method applied in the study

	N	**Freq**	**%**
ANS			
1	120	30	25.0
2	120	39	32.5
3	120	45	37.5
INA			
1	120	12	10
2	120	22	18.3
3	120	38	31.7
4	120	24	20.0
5	120	21	17.5
IOB			
1	120	32	26.7
2	120	41	34.2
3	120	46	38.3
NAW			
1	120	54	45.0
2	120	39	32.5
3	120	25	20.8
NBS			
0	120	13	10.8
1	120	14	11.7
2	120	14	11.7
3	120	33	27.5
4	120	35	29.2
PBD			
0	120	24	20.0
1	120	94	78.3

DISCUSSION

New methods of data analysis are emerging with the increasing complexity of statistical analysis. Techniques for estimating ancestry tend to perform better, when the subjects
under analysis comes from a population that already has a cataloged database.[10] In the case of the Brazilian sample, there is no international database that has samples of this population, so it is to be expected that the performance of techniques created in other groups will not be fully satisfactory.

Navega et al. [11] created a database (AncesTrees) where 23 craniometric variables can be inserted and compared with the original database, which has data from some population groups: African, Austro-Melanesian, East Asian, European, Native American, and Polynesian. Thus, when trying to solve a specific case, the expert can select some geographic origins that are more likely or not to select any, and let the program estimate the possibilities of geographic origin.[11]

One possibility to remedy this limitation could be to adopt parameters established for other population groups from Latin America. However, this strategy is not effective either, as it does not historically consider the cultural and historical processes of each group that inhabits the region.[12]

When using the support vector machine as a predictor model to differentiate American White, American Black, Guatemalan, and southwest Hispanic (Amerindian ancestry) skulls, Hefner et al.[13] observed that the rate of success was 72% for Guatemalans and 94% for American Black; when associating the Guatemalan and southwest Hispanic groups, an increase in this rate was observed, with results of 96% for American Black, 77% American White and 91% for Hispanics.[13] A similar algorithm correctly classified 60% of a Colombian sample.[14]

When analyzing skulls from the historical collection of Hispanics in the United States, and comparing them with a recent sample of migrants from Mexico, significant differences were found in the morphology of these skulls; thus, the authors state that if the term << Hispanic >> is used within a research context, it should be clearly defined regarding the origin and the time period of the group".[15]

It is known that there is human variation, and therefore, analyses of the skulls and jaws should be performed and the results presented in a probabilistic way. [9] Concerning machine learning, there are many possibilities for analysis [16]; thus, before the data analysis, we performed a model to verify the performance of the various possibilities, allowing an accuracy increase.[16]

In a study with a Brazilian sample using a semi-automatic tool, Jurda & Urbanová [17] analyzed sex and ancestry using mesh to mesh processing (distances of reference anatomical landmarks). In the skull, there is a need to manually establish the reference points, which could hinder the use of fully automated models.[17] Also, examiners must be thoroughly trained to perform the technique.

When using the 3D-ID software to verify the correct classification of sex and ancestry in a sample from Greece, it was observed that the program presented moderate reliability, with a correct classification at 70.9% when comparing individuals to the European population.[18] In a study to verify the intra-examiner error in the analysis of macromorphoscopic characteristics in the identification of skeletal remains, the observer's experience was analyzed, new technologies and inherent error of the method were also analyzed; it was concluded that the possible analysis errors can be predicted and that they do not limit the use of techniques in forensic anthropology.[19] Other studies have shown that, depending on the anatomical characteristic under analysis, there may be wide variations.[20]

CONCLUSIONS

The application of the method described in the sample of the present study showed a limitation to estimate ancestry, suggesting a need for adaptation for its applicability in the Brazilian population.

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Supplementary Table. Sample composition

Skull	Sex	Age	Ancestry
1	M	43	B
2	M	37	W
3	F	38	W
4	M	32	W
5	M	43	W
6	M	41	B
7	M	40	W
8	F	70	B
9	F	57	W
10	M	76	W
11	M	61	W
12	F	70	B
13	F	90	W
14	M	56	W
15	M	76	W
16	F	104	W
17	F	64	W
18	M	46	W
19	F	77	W
20	F	56	W
21	M	64	B
22	M	61	W
23	F	44	W
24	F	44	W
25	M	35	B
26	F	56	W
27	M	97	W
28	F	69	B
29	M	72	W

30	M	70	W
31	M	72	W
32	M	46	B
33	M	34	W
34	F	82	W
35	M	57	W
36	F	61	B
37	M	79	W
38	F	37	B
39	F	43	W
40	M	67	W
41	F	74	W
42	M	43	B
43	M	18	W
44	F	78	W
45	F	82	B
46	M	30	B
47	F	77	W
48	M	81	B
49	F	34	W
50	M	58	B
51	F	56	B
52	F	84	W
53	M	36	W
54	F	80	B
55	M	66	W
56	M	61	W
57	F	71	W
58	M	64	W
59	M	65	W
60	M	81	W
61	F	71	W
62	F	79	W
63	F	60	W
64	F	49	B
65	F	79	B
66	F	43	B
67	M	36	B
68	F	20	W
69	F	38	B
70	F	24	B
71	M	46	W
72	F	52	B
73	M	32	B
74	M	48	B
75	M	40	B
76	F	48	W
77	F	36	B
78	M	40	W
79	M	80	W
80	M	25	W
81	M	56	W
82	M	40	W
83	M	68	W
84	M	61	W
85	F	62	W
86	F	68	W
87	F	38	B
88	F	50	B
89	M	60	B
90	M	89	W
91	F	68	W
92	F	61	B
93	M	23	W
94	M	41	W
95	F	40	W
96	F	94	W
97	F	45	W
98	M	87	B
99	F	48	W
100	M	62	B
101	M	40	B
102	M	28	B
103	F	80	B
104	M	28	B
105	M	31	W
106	M	34	W
107	M	28	W
108	M	40	B
109	F	64	W
110	M	65	B
111	M	35	B
112	M	38	B
113	M	21	B
114	M	24	W
115	M	25	B
116	M	23	B
117	M	17	W
118	F	14	W
119	M	30	W
120	M	22	B

Dentinal translucency and width of cementum: predicting the age over 55 years in South Indian adults using extracted sectioned teeth

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KEYWORDS

Dental age estimation,
Root dentin translucency,
Width of cementum
Age threshold,
Probability

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ABSTRACT

The main intention of this article was to evaluate the reliability of root dentine translucency (RDT) and the width of cementum (CW) in indicating the age over 55 years in forensic and criminal investigations. 600 non-restored, single rooted teeth (300 males and 300 females) which were extracted for periodontal or orthodontic reasons were collected. Each tooth was sectioned longitudinally until the desired thickness (250 µm) was obtained. Both the length of the RDT and CW were measured from these unstained ground sections of teeth using ImageJ computer software. Pearson's correlation coefficient indicated a very strong and positive correlation for RDW and CW with age in both sexes. Cut-off values of RDT= 7.07 and CW= 52.06 were obtained using the maximum Youden's index. The value for the area under the curve (AUC) was 0.987 for RDT and 0.910 for CW was seen as indicating a very high discrimination. The performance of these cut-off values was tested in a separate sample of sectioned teeth (n= 300) and was analyzed using contingency tables in both sexes. The sensitivity was 88.2% and 92.3%, while specificity was 98.9% for RDT in males and females. For CW, the sensitivity was 96.1% and 90.3%, and specificity was 76.7% and 74.4% in males and females, respectively. Bayes post-test probability was 98.9% for RDT in both sexes, while 80.5% in males and 78% in females, for CW. Based on our study findings, it can be concluded that both variables have performed well in predicting the age over 55 years. Further research concerning the radiographic study of secondary dentine deposition to predict legal age thresholds would be a great benefit for living adults who require age estimation in civil proceedings.

INTRODUCTION

Establishing identity to unknown bodies or skeletal remains is of paramount importance in the legal context of forensic and anthropological practice.[1] To this end, estimating the age of the individual can be considered as the prime factor and it represents a key feature leading to the identification of the unknown. Depending on the context of the investigation, the forensic experts use either osseous or dental based methods. Even though skeletal methods are indicated to be reliable for age estimation, their poor resistance to the taphonomic process and variability in their maturation under the influence of environmental factors made experts look for alternative parameters.[2] Some dental tissues are extremely resilient,

resisting mechanical, chemical, and thermal stimulation even in the most adverse situations.[3, 4] Following the completion of the developmental changes occurring in teeth, one must rely on the changes in dental hard tissues for estimation of age. A variety of age estimation approaches have been proposed by researchers in the past. In 1925, Bodecker initially observed a relationship between secondary dentine apposition and increasing age.[5] Later, in 1950, Gustafson studied the regressive alterations in teeth and it was the first scientific method for estimation of age in adults.[6] The numerous approaches used to estimate age in adults have arisen as a result. Among them, root dentine translucency (RDT) was found to be reliable by many authors.[7-10] Similarly, studies also found a positive correlation between the cementum thickness and age, and its role in age assessment.[11-13]

In medico-legal cases, age is a valuable criterion to determine essential services, systems and entitlements. The major issues in age estimation in the living concern illegal migration of refugees, adoption, imputability (14, 16, 18 and 21 years in different countries), and old age pension (50, 55, 60, 65 years in different countries).[14] More often forensic experts are called for evaluation of the true age of adults for old age pension, access to medical care or other civil purposes (retirement). Unlike children, there are no age thresholds in adults that had medico-legal importance. However, in some circumstances, the services of doctors/forensic experts were utilized for evaluation of the true age of adults for multiple purposes, for example if they have attained the age of 55 years.[14, 15]Similarly, in criminal investigations the police often consult forensic experts to determine the age of unidentified human remains. In such instances, it is the duty of the experts to provide an age range that helps the police narrow down the search. Therefore, it is important to investigate the regressive changes in teeth with specific emphasis on the different age thresholds in adults.

To date, and to the best of our knowledge, no study has evaluated the specificity and sensitivity indices, and the probability in evaluating the age group of 55 years. The present study aims at deriving cut-off values separately by means of RDT length and CW, and to verify the performance of newly determined cut-off values in age indication over 55 years in a new sample.

MATERIAL AND METHODS

Sample

600 freshly extracted single rooted permanent teeth from healthy volunteers (300 males & 300 females) aged between 15 and 75 years were collected from the department of Oral and Maxillofacial Surgery and from private clinical practitioners. The extracted teeth were divided into six equal age groups, with each age group ranging up to 10 years span and consisting of 50 teeth with an equal number of male and female samples. The age, sex, reason for extraction and from whom teeth were extracted were noted. Teeth extracted for orthodontic or prosthodontic reasons and those who complained of mobile teeth due to periodontitis were collected. Teeth with pathologies like caries, hypercementosis, trauma and teeth with loss of tooth substance due to tooth-to-tooth contact due to pathological causes were excluded. After obtaining institutional ethical committee clearance (SVSIDS/OP/4/2018) and informed consent from the patients, extracted teeth were collected.

Study sample distribution

The Study Group sample (n=600) was randomly split into two sub-samples. One of the sub-samples was referred to as the test sample, while the other was referred to as the validation sample. A cut-off value was calculated, which is known to result in the best overall performance of the discrimination test. Subsequently, the second sample was utilized to verify the feasibility of the cut-off values, and to see if the person had attained the age of 55. For the whole sample, age and sex distribution is shown in Table 1.

Preparation of tooth specimen

The teeth were preserved in 10% neutral buffered formalin immediately after extraction to prevent dehydration. Later, each tooth was sectioned longitudinally (in buccolingual direction) using a diamond disc followed by Arkansas stone until desired thickness (250 µm) was obtained. The slides were washed in xylene and then put in DPX to view under a light microscope. Cover slips were placed for viewing using a stereomicroscope.

Table 1. Age and gender distribution of the overall (test & validation) sample

Age groups	Test sample			Validation sample		
	Males	Females	Total	Males	Females	Total
15- 25	25	25	50	25	25	40
26- 35	25	25	50	25	25	40
36- 45	25	25	50	25	25	40
46- 55	25	25	50	25	25	40
56- 65	25	25	50	25	25	40
66- 75	25	25	50	25	25	40
Total	150	150	300	150	150	300

Measurements

Cementum from the mid-root section was observed under light microscope and digital images were captured using Olympus Preogress C3 camera. Teeth with indistinct, invisible cementum were eliminated. The width of the cementum (CW) was measured between DCJ (dentino-cementum junction) to the outer surface of the cementum (**Figure** 1). The width of the cementum (X) was measured between DCJ (dentino-cemental junction) to the outer surface of cementum. Subsequently the width between two adjacent incremental lines (Y) was measured and the number of incremental lines (N) in each section was calculated using the formula: N= X/Y. Finally, the estimated age of individual (E) was calculated by adding average eruption age (T) in years of each tooth with total number of incremental lines. E= N+T. To measure the length of the root dentinal translucency (RDT), sections were observed and photographed under stereomicroscope (**Figure** 2). Using the image analysis software the apical translucent dentine (T) length was measured. In every tooth section measured, the length of dentinal translucency was measured from the coronal most extent to apical most extent of the dentinal translucency. Modified Bang and Ramm's formula was applied to all measurements and the age of the individual was estimated.

T= apical translucent dentine length

Linear regression:

$T \geq 9$ mm

Age= 35.5619+ (3.4828 x T).

Quadratic regression:

If $T \leq 9$ mm

Age = 29.9074+ (7.4507 x T) + (-0.4369 x T^2). All linear measurements were carried out with the help of computerized image processing programme (ImageJ, U.S. National Institute of Health, Bethesda, Maryland).

All extracted tooth samples were assessed by an experienced examiner who was unaware of the subjects' details. To check for any intra-examiner and inter-examiner variations, 50 extracted teeth were randomly selected and were re-assessed after an interval of 2 weeks.

Data management and Statistical analysis

The age, sex, RDT, and CW values of each tooth specimen were noted in a Microsoft Excel 2016 spreadsheet (Microsoft Office 2003, Microsoft, Redmond, WA). To avoid any future misunderstanding, each tooth specimen was assigned an identifying number, and all of the specimens' information was recorded against that number. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) 20.0 version for Windows (SPSS Inc., Chicago, IL, USA) and the significance level was set at 5% (P0.05). The intra- and inter-examiner variations were determined using the intra-class correlation coefficient (ICC). A binary logistic, generalized linear regression model was used on the test sample to validate the relevance of the factors (RDT, CW, and sex) on the discriminating of participants over 55 years old from those under 55 years old. The efficacy of the RDT and CW values was tested using a receiver operating characteristic (ROC) curve (a graphical figure that shows the entire trade-off between sensitivity and specificity over a succession of cut-off points). The discrimination test's greatest performance was determined using a single statistic, Youden's index (J), which is a function of sensitivity and specificity. RDT and CW cut-

off values were determined using the largest Youden's index.[16]The performance of the generated RDT and CW cut-off values was assessed using a 2X2 contingency table, which shows the number of true positives, false positives, true negatives and false negatives. We calculated the percentage of correct categorization (Ac), sensitivity (Se), specificity (Sp), predictive values, and likelihood ratios. Pre- and post-test probability of being 55 years or older were computed to distinguish between people aged 55 years and those aged >55 years. The likelihood that there are people in a given group who are 55 years or older before a diagnostic test is performed is known as pre-test probability (p_o). Post-test probability (PTP), on the other hand, can be defined as the likelihood that a person is 55 years or older when the diagnostic test identifies the condition. The standard form of Bayes' theorem for a positive result should be written as:

Post - test probability= $p_o \times Se / (p_o \times Se) + (1- p_o) \times (1- Sp)$

p_o is the pre-test probability of being 55 or older in this equation. It was evaluated using data from the Census of India website: https://www.censusindia.gov.in/2011census/C-series/C-13.html. It was computed using demographic data from the 2011 census to determine the proportion of individuals aged 55 to 75 years old and those aged 15 to 75 years old. According to this, the proportions for males and females were determined as 50% for males and 50% for females, respectively.

Figure 1. Images of sound upper right lateral incisor showing the incremental cemental lines under microscopic magnification

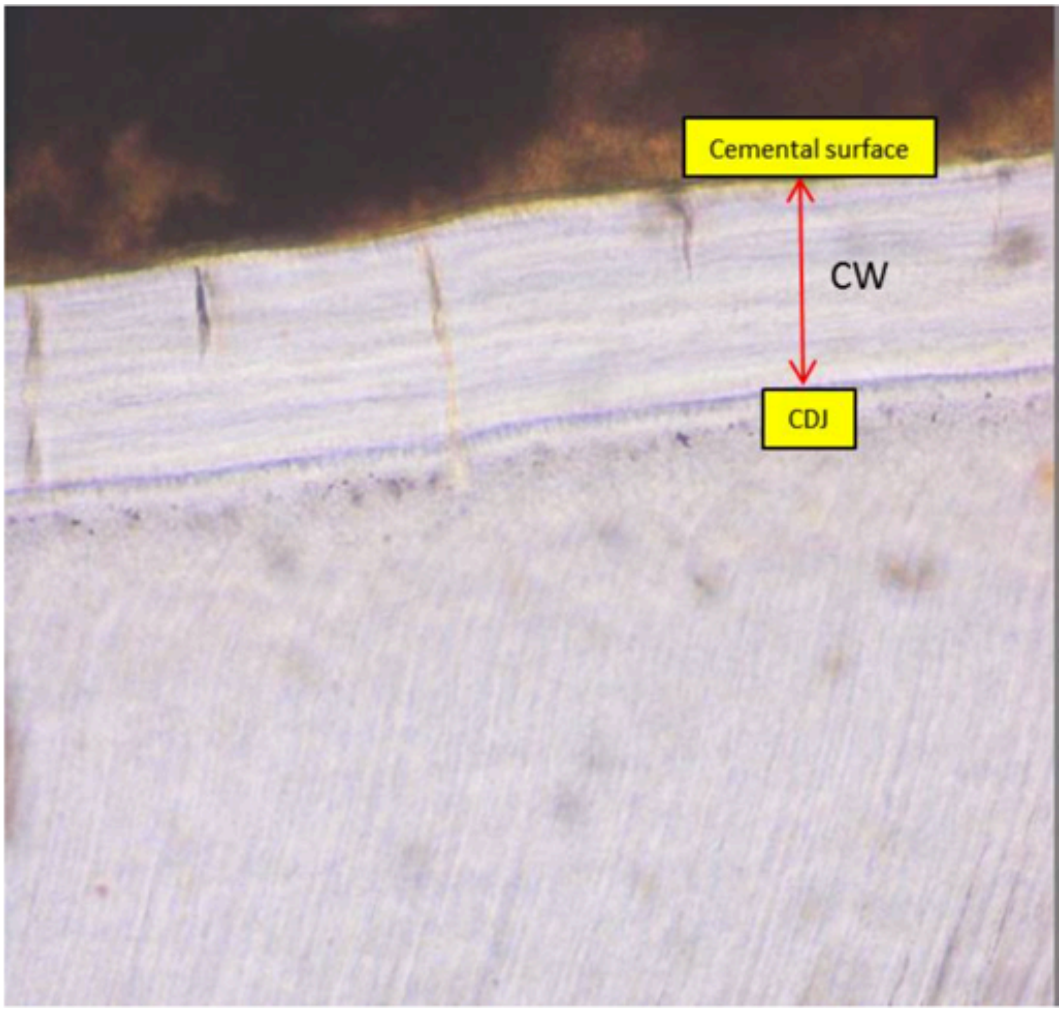
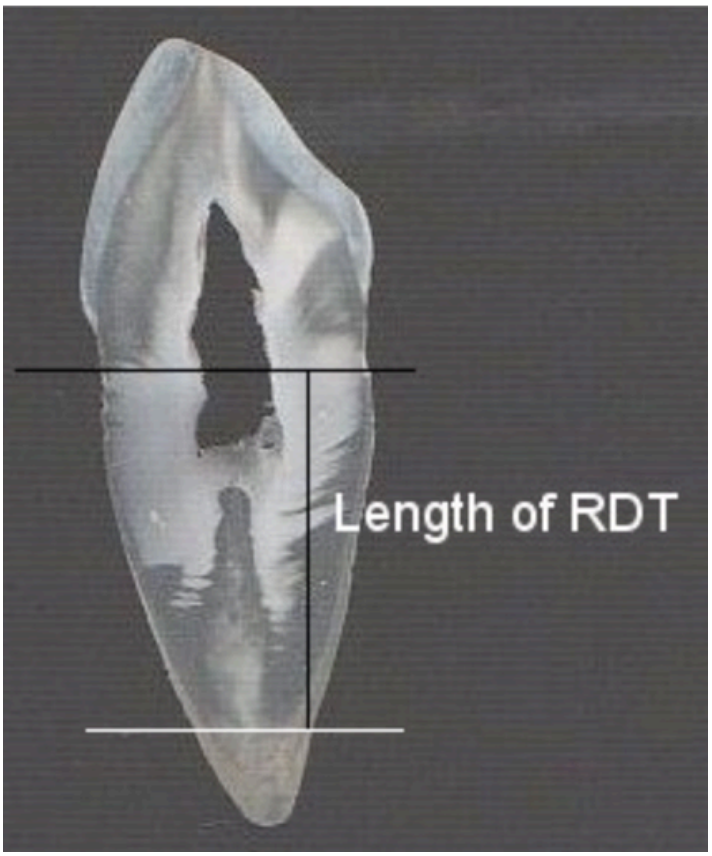


Figure 2. Measurement of the length of the root dentin translucency using ImageJ computer software



RESULTS

The total sample's average age was 44.78 + 17.4 years. Males and females, on the other hand, were 44.67 + 17.4 years and 44.89 + 17.5 years old, respectively. According to Pearson's correlation statistics, there was a significant and extremely strong relationship between age and RDT (R^2= 0.949), CW (R^2= 0.930). (Table 2 and Figures 3 & 4). The intra- and inter-examiner agreement ICC values for RDT were 0.917 and 0.891, respectively. For CW, the ICC for intra- and inter-examiner agreement was 0.905 and 0.872, respectively.

Except for sex (p=0.068), all of the predictor factors were statistically significant in the logistic regression model (Table 3). The overall model explained 0.644 (Cox & Snell R Square) to 0.890 (Nagelkerke R Square) of the variations in the ≥55 years and <55 categories.

Using dentine translucency, the area under the curve (AUC) was 0.987 (Figure 5). For the maximum value of the Youden index, which was 0.893 for the cut-off value of DT= 7.07, a cut-off value of dentine translucency was developed to differentiate that persons are ≥55 years or <55 years.

Using width of cementum, the area under the curve (AUC) was 0.910 (Figure 6). For the maximum value of the Youden index, which was 0.688 for the cut-off value of CW= 52.06, a cut-off value of cementum width was developed to differentiate whether persons are ≥55 years or <55 years.

Tables 4 and 5 provide the results of the contingency tables, which illustrate how the cut-off values performed in a different sample (n=300). For males, the Ac, Se, Sp, LR+, LR-, PPV, and NPV were 95.3%, 88.2%, 98.9%, 87.35%, 0.12%, 97.8%, and 94.2%, respectively, using the root dentine translucency (RDT>7.07). Females scored 96.6%, 92.3%, 98.9%, 90.46, 0.08, 97.9%, and 96%, respectively. In both males and females, the calculated Bayes post-test probability was 98.9% (Table 6).

The Ac, Se, Sp, LR+, LR-, PPV, and NPV for males using the other predictor, CW > 52.06, were 83.3%, 96.1%, 76.7%, 4.14, 0.05, 68.1%, and 97.4%, respectively. Females scored 80%, 90.3%, 74.4%, 3.54, 0.13, 65.2%, and 93.5%, respectively. Males had an estimated Bayes post-test probability of 80.5%, whereas females had an estimated Bayes post-test probability of 78% (Table 6).

Table 2. Pearson's correlation between the age and the parameters tested (RDT & CW) in both males and females

Parameters	Sex (n)	Correlation	p- value
RDT	Males (150)	0.978	0.000*
	Females (150)	0.920	0.000*
CW	Males (150)	0.939	0.000*
	Females (150)	0.921	0.000*

*Statistically significant (p<0.05)

Figure 3. Scatter plot showing the relation between the age and the length of the root dentin translucency in males and females

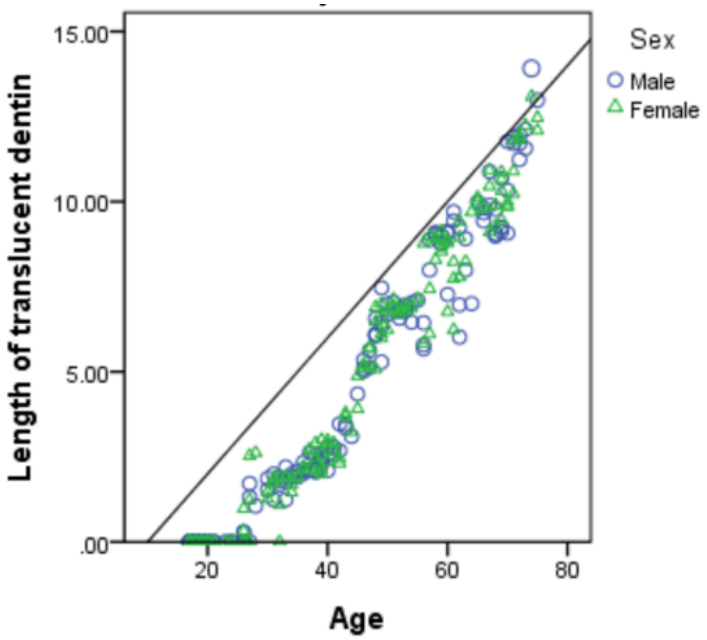


Figure 4. Scatter plot showing the relation between the age and width of the cementum in males and females

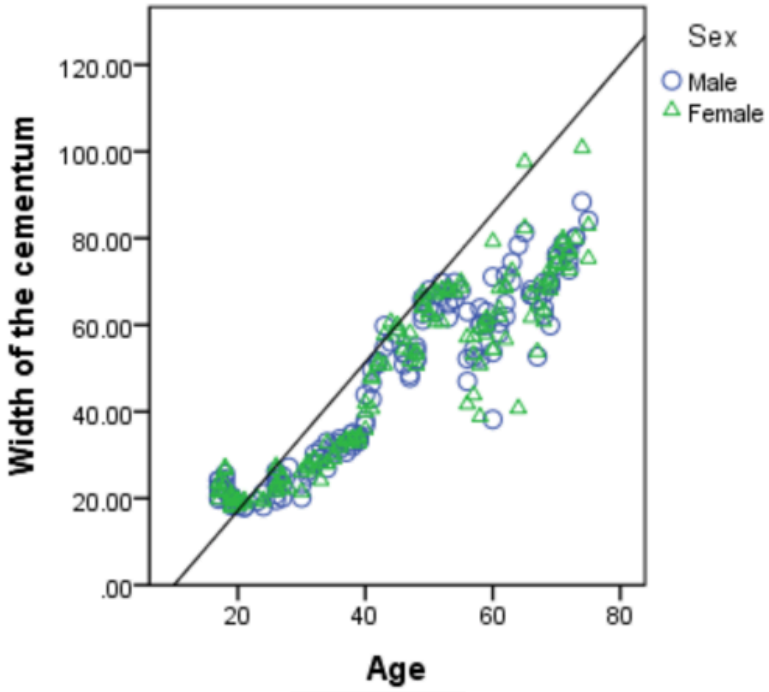


Table 3. Parameter estimates of the RDT and CW and sex as explanatory variables and ≥55 years and <55 years age as dichotomous dependent variable on Logistic Regression

	B	Std. Error	Wald	df	Sig.
Sex	-0.583	0.378	3.129	1	0.068
RDT	1.986	.367	29.232	1	0.000*
CW	-2.031	.440	31.578	1	0.000*
Constant	-12.062	3.102	15.125	1	0.000*

*Statistically significant (p<0.05)

Figure 5. The receiver operating characteristic curve for indicating the legal age of 55 years using length of the root dentin translucency

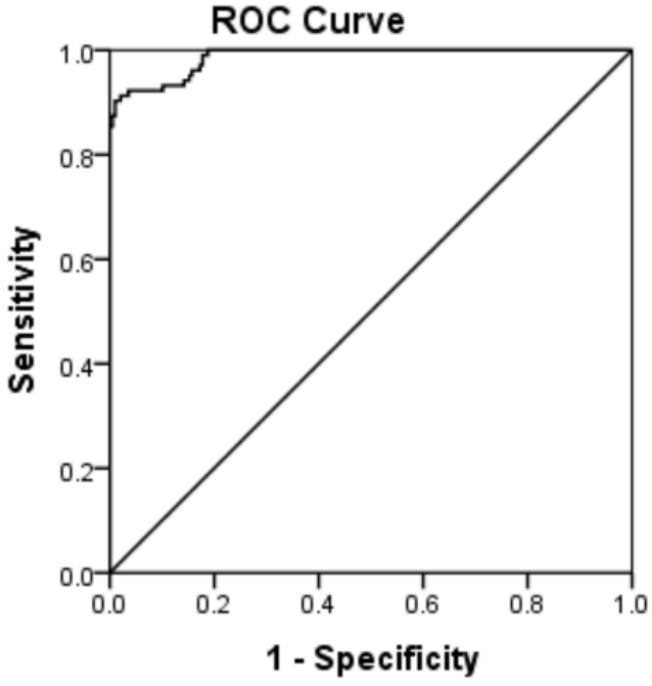


Figure 6. The receiver operating characteristic curve for indicating the legal age of 55 years using width of the cementum

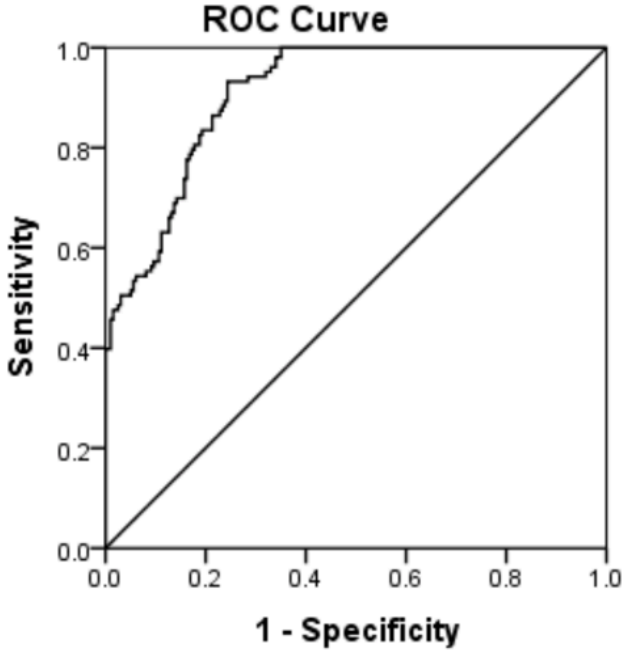


Table 4. Contingency table describing discrimination performance of the test (≥ 55 years and < 55 years) for RDT ≥ 7.07 in males and females

Test		Age Category		Total
		<55 years	≥55 years	
1.		**Males**		
DT≥7.07	**≥7.07**	1^{FP}	45^{TP}	46
	<7.07	98^{TN}	6^{FN}	104
Total		99	51	150
2.		**Females**		
DT≥7.07	**≥7.07**	1^{FP}	48^{TP}	49
	<7.07	97^{TN}	4^{FN}	101
Total		98	52	150

TP, true positive; FN, false negative, FP, false positive, TN, true negative.

Table 5. Contingency table describing discrimination performance of the test (≥ 55 years and < 55 years) for CW ≥ 52.06 in males and females

Test		Age Category		Total
		<55 years	≥55 years	
1.		**Males**		
CW≥52.06	**≥52.06**	23^{FP}	49^{TP}	72
	<52.06	76^{TN}	2^{FN}	78
Total		99	51	150
2.		**Females**		
CW≥52.06	**≥52.06**	25^{FP}	47^{TP}	72
	<52.06	73^{TN}	5^{FN}	78
Total		98	52	150

TP, true positive; FN, false negative, FP, false positive, TN, true negative.

Table 6. Parameter estimates of the RDT and CW and sex as explanatory variables and ≥55 years and <55 years age as dichotomous dependent variable on Logistic Regression

	Root Dentin Translucency		Width of the Cementum	
	Males	**Females**	**Males**	**Females**
Sensitivity	88.2 (76.1- 95.5)	92.3 (81.4- 97.8)	96.1 (86.5- 99.5)	90.3 (78.9- 96.8)
Specificity	98.9 (94.5- 99.9)	98.9 (94.4- 99.9)	76.7 (67.2- 84.6)	74.4 (64.6- 82.7)
Accuracy	95.3 (90.6- 98.1)	96.6 (92.3- 98.9)	83.3 (76.3- 88.9)	80 (72.7- 86.1)
LR+	87.35 (12.4- 615.6)	90.46 (12.85- 636.81)	4.14 (2.88- 5.94)	3.54 (2.5- 5.03)
LR-	0.12 (0.06- 0.25)	0.08 (0.03- 0.20)	0.05 (0.01- 0.20)	0.13 (0.06- 0.30)
PPV	97.8 (86.4- 99.6)	97.9 (87.2- 99.7)	68.1 (59.7- 75.3)	65.2 (56.9- 72.7)
NPV	94.2 (88.5- 97.1)	96 (90.4- 98.4)	97.4 (90.6- 99.3)	93.5 (86.2- 97.1)
Bayes PTP	98.9 (92.5- 99.8)	98.9 (92.8- 99.8)	80.5 (74.2- 85.6)	78 (71.4- 83.4)

LR+, positive likelihood ratio; LR-, negative likelihood ratio; PPV, Positive predictive value; NPV, Negative predictive value; Bayes PTP, Bayes post-test probability.

DISCUSSION

Root Dentine translucency

In the past, age estimation studies in adults focused on one of the regressive changes i.e. quantification of dentine translucency on extracted teeth.[10, 17] However, variations in the evaluation of this age-related change were observed in the literature with respect to their analysis (sectioned or unsectioned tooth),[18, 19] grading or the measurement of the variable,[20] measuring the length or area of the translucency,[21, 22] and the examination of the variable i.e. caliper based or the digital approach.[8, 23, 24] When dentine translucency was studied on the unsectioned extracted teeth, high variation was seen, especially the correlation between age and translucency (0.34 to 0.97).[18, 25]

Jigna S Shah et al. examined sectioned teeth for the clear appearance of dentine translucency on the tooth sections.[18, 26] Literature indicated a high correlation between the age and translucency, particularly in sectioned teeth.[8, 26, 27]On this point, our results also have shown very high correlation. On comparing results of this study with the other Indian studies alone,[27-29] the correlation values both in males and females were greater. Even though, Acharya and Vimi[27] showed a moderate correlation of translucency length with age, their findings were not truly reflected in our study. The possible difference between the results of our study to previous ones[27-29] is that; firstly, 300 sections used in the present study (relatively larger sample size) and secondly, coverage of wider range from younger age groups to the older with reasonably equal distribution of age groups and number of sections as per sex in each age group.

Width of the cementum

In addition to dentine, cementum is another calcified dental tissue. Unlike enamel, it is synthesized continuously and maintained throughout all stages of adult life.[30] Few have considered the analysis of cementum apposition and thickness as a better parameter for age estimation due to its distinctive location in the alveolar process.[31] Literature evidence has also suggested that the migration of cementum coronally in impacted teeth can also be helpful in age estimation.[32, 33] Studies in the literature have indicated that both the cemental thickness and cemental annulations are best correlated with age. When Kasetty et al.[13] studied both these variables in estimating age, they have reported a significant correlation. However, cemental thickness was statistically more significant than cemental annulations. Similar findings were also reported by Stein et al.[12] that quantitation of cemental annulations as a moderately reliable means of age estimation in adults. Additionally, Dias et al.[34] highlighted the difficulties that one can face while studying cemental annulations such as variability in their thickness, blurry or indistinct annulations, overlay of the single annulation at varying levels (could lead to misinterpretation of two lines) etc. These may result in bias while making the count and may account for moderate correlation of cemental annulations with age. Considering all these studies into account, the authors in this study examined the cemental thickness.

The present study also aimed to identify the relationship between the CW and age. Variables revealed a strong, positive and statistically significant correlation, which correlates with the findings of Priya Gupta et al.[11], Jigna et al.[10] and Kasetty et al.[13] Variations in the correlations between the age and cementum thickness have been reported in the literature based on the different areas (the apex or one third of the root length) and type of tooth (maxillary or mandibular) analyzed. Solheim[35] indicated that the cemental thickness analyzed at the one third of root length as most accurate predictor of the age. Similarly, when we analyzed the cemental thickness at middle third of the root, we observed a very high correlation, both in males and females. However, these findings were contrary to that of Jigna et al.[26] and Kasetty et al.[13] where they found apical thickness of cementum as a better predictor of age in adults.

Misclassifications and probability

When it comes to the prediction of attainment of age thresholds in medico-legal cases or in criminal proceedings, it is of supreme importance that age misclassifications should be avoided or kept to a minimum. In children and sub-adults, who were involved in criminal proceedings, only age estimation methods that have exhibited high specificity and very probability (>90%) must be used.36 However, under civil law, the context of specificity and probability will be different; a

probability of around 51 percent may be adequate for determining age in civil procedures.37 In the current study, when RDT was used to indicate age over 55 years, a probability of 98.9% was observed for both males and females, respectively. On the other hand, CW has produced lesser probability values which is 80.5% in males and 78% in females. However, in the context of civil proceedings, both these variables have performed well and therefore can be applied for estimation of age in adults, especially in evaluating the age threshold of 55 years in the population.

Limitations and recommendations

Each method of age estimation in practice has its own set of limitations and benefits. One of the main limitations of the present investigation is the use of destructive methods that involve the sectioning of the teeth. However, destruction of evidence may not be permitted by our legal system.[18] The findings in the present study can only be useful in the context of crime investigations, where determination of age was a key factor in the identification of the unknown remains. Further studies could be warranted to study the secondary changes i.e. secondary dentine deposition in the radiographs, dentine translucency in extracted and unsectioned teeth for predicting the attainment of age thresholds of importance in adults.

CONCLUSIONS

In criminal investigations, forensic experts are often requested by the police to provide the age range of the unknown human remains that may help them in proceeding with further investigation. In this study, two popularly used parameters in adult age estimation i.e, the RDT and CW for indicating the age over 55 years were investigated and it was found that there is significant influence of both parameters on the age of the subject. Their thickness increased with age that was statistically significant in both sexes. Both parameters also have exhibited very high probability (approximately 80%) in predicting the age over 55 years. However, factors like need for the extraction of the teeth, sectioning of the teeth and procedural difficulties make age assessment using these parameters less reliable. Moreover, they cannot be applied to living individuals who need age estimation in civil proceedings. Hence, there is a need to find alternate parameters such as study of secondary dentine deposition in radiographs for predicting age thresholds in adults.

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Applicability of the Demirjian, Willems and Haavikko methods in Croatian children

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KEYWORDS

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ABSTRACT

Age estimation is an inescapable part of every identification process. During growth and development, it is possible to estimate age based on the developmental stages of teeth. The aim of this study was to evaluate three frequently used methods for dental age estimation on a broad sample of Croatian children. The sample comprised 1996 digital, standardized orthopantomograms of children (1121 boys and 875 girls) aged 5 to 16, collected in four major Croatian cities. Age was estimated according to the Demirjian, Willems and Haavikko methods and the accuracy of the estimation was evaluated. The Kappa for intra-examiner agreement was 0.83 for the Haavikko stages and 0.92 for the Demirjian stages. Using the Demirjian method, the average overestimation of age was 0.80 years for boys and 0.84 years for girls. The Willems method overestimated the mean age by 0.41 years in boys and 0.22 years in girls. The Haavikko method underestimated the mean age by 0.60 years in boys and 0.80 years in girls. The Willems method proved to be the most accurate and can be used for dental age estimation among Croatian children. The Demirjian and Haavikko methods showed greater deviation between dental and chronological age and require adaptation when used in the Croatian population.

INTRODUCTION

Dental age estimation is a procedure used for clinical, forensic and archaeological purposes. During the period of growth and development, it is possible to estimate age by assessing tooth development. In their development, teeth follow a specific pattern that has been well documented since the discovery of the X-ray. It should be noted, however, that the first scientific record of teeth being used to estimate age dates back to 1837 when Saunders[1] presented a study that estimated age based on the eruption of permanent teeth. Although easily noticeable, tooth eruption is not considered a reliable age indicator as it is highly susceptible to the influence of extrinsic factors and varies significantly from child to child.[2]

The advancement of dental radiology facilitated the development of numerous methods for dental age estimation. Using a French Canadian population sample, in 1973 Demirjian developed a method that estimates age based on the developmental stages of seven permanent left mandibular teeth.[3] It has proven the most widely used and tested method

for dental age estimation. In 2001 Willems adapted the Demirjian method by simplifying the procedure and improving the accuracy of age estimation in a sample taken from the Belgian population.[4] The Haavikko method was developed using a Finnish population sample in 1974. The age estimation is based on the average chronological age for the developmental stages of a few selected teeth.[5,6]

The need for age estimation has increased over recent years due to migratory flow initiated by war, conflict and economic crises. The high rate of asylum procedures, missing children, human trafficking and cases related to legal consent and child abuse demands a reliable and accurate method for age estimation that serves to protect and ensure children's rights.

Dental development is mainly under the genetic influence, unlike skeletal development which is, apart from genes, greatly influenced by endocrinal disorders and nutrition.[7] Therefore, especially in legal processes, dental age estimation should never be omitted in age assessment.

Since every population has its singularities, scientists are encouraged to test the applicability of the existing methods and, if necessary, to adapt them for the specific population to ensure accuracy of age estimation.[8] The aforementioned methods have been tested in populations worldwide.[9–13]

The aim of this study is to evaluate the dental age estimation methods developed by Demirjian, Willems and Haavikko on a large sample of orthopantomograms of Croatian children and to provide guidelines for dental age estimation in the Croatian population.

MATERIAL AND METHODS

1996 digital, standardized orthopantomograms (OPGs) of children (1121 boys and 875 girls) aged 5 to 16 were collected in four major Croatian cities (Zagreb, Split, Osijek and Varaždin) (Table 1). All the individuals participating in this study were referred by their dentists for radiological diagnostics and no OPG was taken solely for the purpose of this investigation. Informed consent was obtained from parents or legal guardians to enable the data to be used for scientific purposes. The study was approved by the Ethics Committee of the School of Dental Medicine in Zagreb.

Table 1. Sample structure

Age (years)	Total sample			Sample for Demirjian and Willems method			Sample for Haavikko method		
	Total	Boys	Girls	Total	Boys	Girls	Total	Boys	Girls
5 - 5.99	9	4	5	9	4	5	9	4	5
6 - 6.99	43	31	12	43	31	12	43	31	12
7 - 7.99	137	84	53	129	78	51	135	82	53
8 - 8.99	209	124	85	202	121	81	206	121	85
9 - 9.99	227	122	105	215	116	99	226	121	105
10 - 10.99	229	135	94	213	130	83	228	135	93
11 - 11.99	253	126	127	237	120	117	253	126	127
12 - 12.99	213	112	101	202	108	94	212	112	100
13 - 13.99	242	133	109	224	123	101	236	129	107
14 - 14.99	231	142	89	211	128	83	229	141	88
15 - 15.99	203	108	95	183	100	83	200	107	93
Total	**1, 996**	**1, 121**	**875**	**1, 868**	**1, 059**	**809**	**1, 977**	**1, 109**	**868**

All OPGs were taken with the Cranex device (Soredex, Finland), thus ensuring a standardized procedure for digital OPGs. The OPGs were coded without information pertaining to name, sex, date of birth and date of record. Developmental stages of the permanent teeth were assessed by one investigator (IB) using the developmental scale introduced by Demirjian [3] (Figure 1) and Haavikko [5] (Figures 2 and 3). Age was estimated according to the Demirjian [3], Willems [4] and Haavikko [6] methods. Since Demirjian and Willems use the same developmental scale and assess seven left mandibular teeth in their analysis, all OPGs missing at least one of the required teeth (31-37) were excluded. Therefore, the sample for Demirjian and Willems method was 1868 (1059 boys and 809 girls). Haavikko uses a different process of tooth selection for dental age estimation – teeth 11, 43, 44 and 46 for children under 10 years of age and teeth 13, 43, 44 and 47 for children older than 10. Consequently, the sample for the Haavikko method was 1977 (1109 boys, and 868 girls) (Table 1).

After a period of two months, 100 randomly chosen OPGs were reassessed for testing intra-examiner reliability and kappa statistics were applied.

Dental and chronological age were compared in each method and the p value was calculated for all age groups using the Wilcoxon signed-rank test. For comparison of accuracy between the Demirjian, Willems and Haavikko methods, square deviations were used, thus enabling each deviation between dental and chronological age (positive and negative) to be noted. Overall accuracy of age estimation for each method was presented as a percentage of the correct estimation within intervals of ±0.5, ±1, ±1.5 and ±2 years.

Statistical analysis was carried out by SAS software (SAS Institute INC., Cary, NC).

Figure 1. Assessment of developmental stages introduced by Demirjian for age estimation using the Demirjian and Willems method

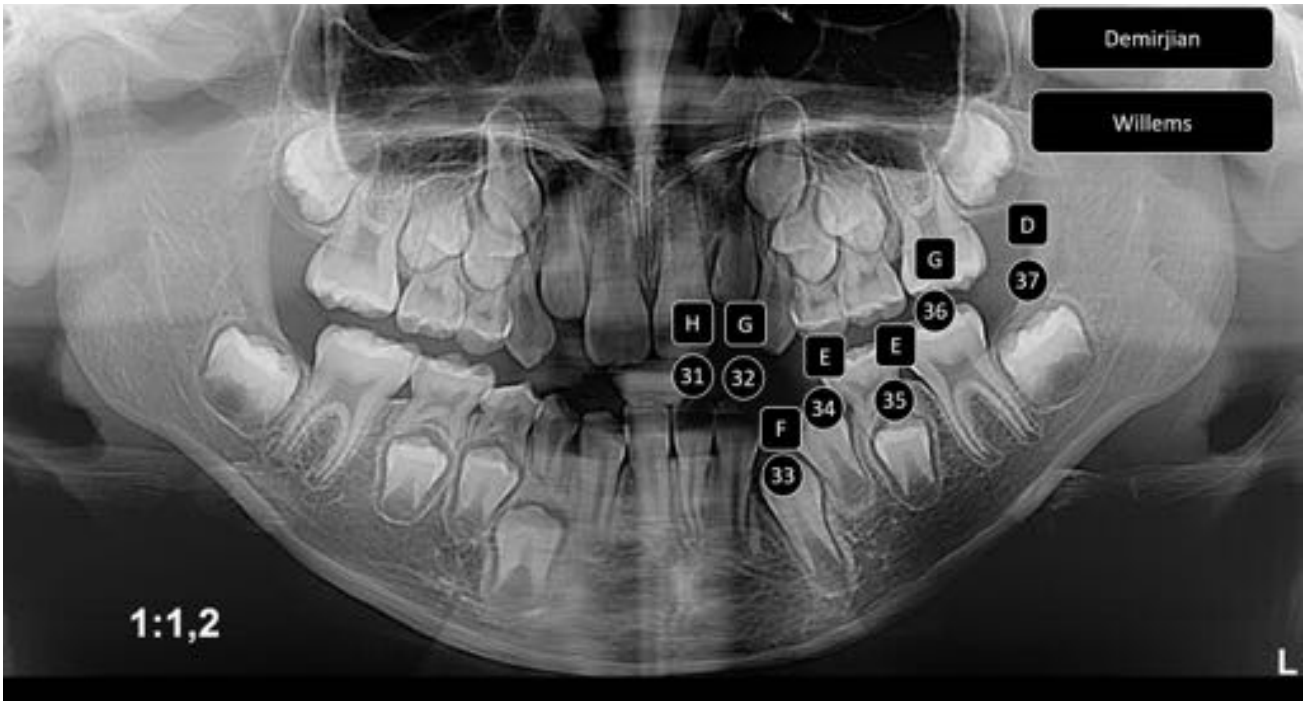


Figure 2. Assessment of developmental stages introduced by Haavikko for children younger than 10 years (teeth 11, 43, 44 and 46 are used)

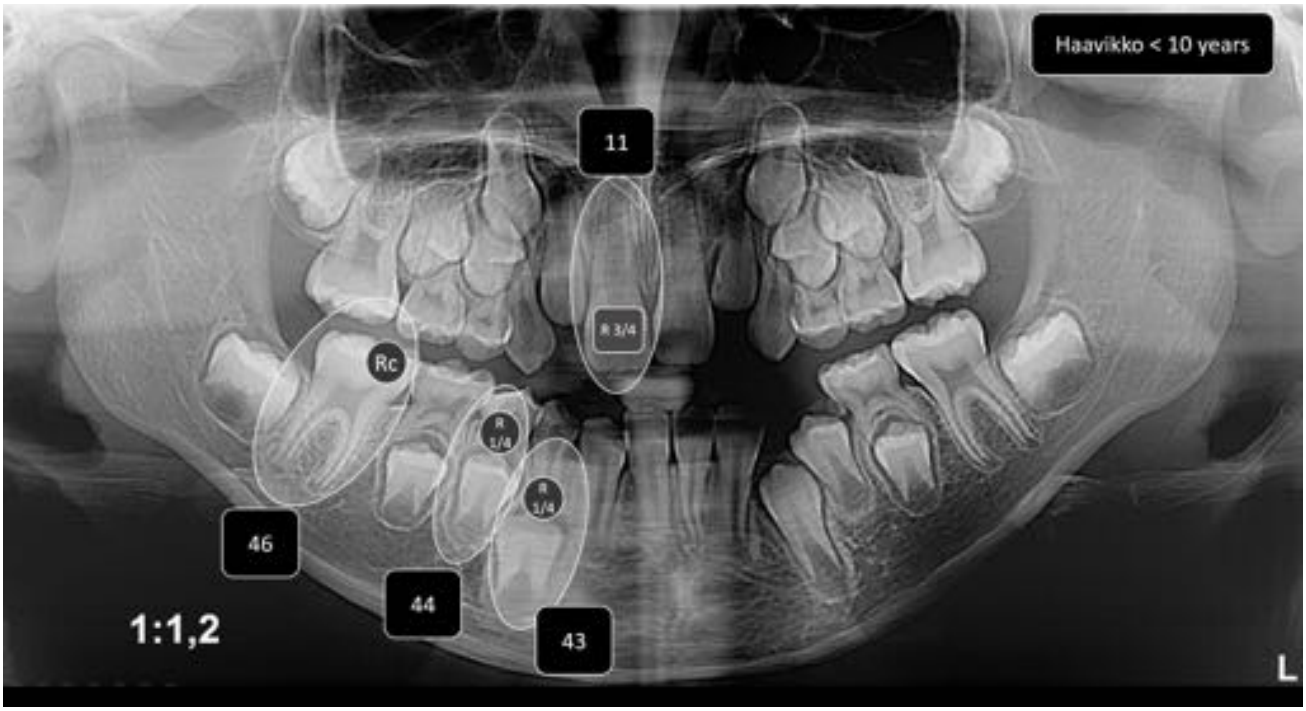
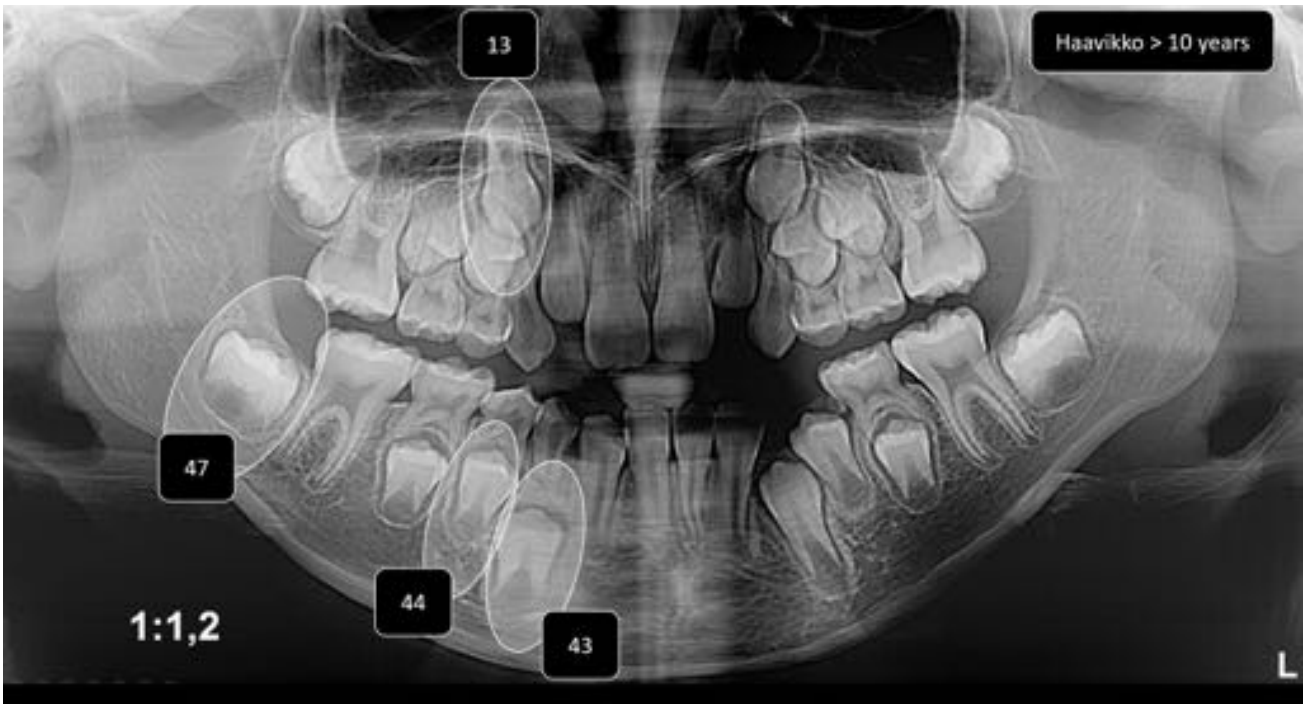


Figure 3. Assessment of developmental stages introduced by Haavikko for children older than 10 years (teeth 13, 43, 44 and 47 are used)



RESULTS

The Kappa value for intra-examiner agreement was 0.83 for the Haavikko stages and 0.92 for the Demirjian stages.

Using the Demirjian method, the average overestimation of age was 0.80 years for boys and 0.84 years for girls (Table 2). The average deviation between dental and chronological age was significant in all age groups ($p<0.001$) except the youngest. The Willems method overestimated the mean age by 0.41 years in boys and 0.22 years in girls (Table 3). The average deviation between dental and chronological age was significant in five out of eleven age groups. The Haavikko method underestimated the mean age by

0.60 years in boys and 0.80 years in girls (Table 4). The deviation was significant in seven out of eleven age groups.

In the comparison of the Demirjian, Willems and Haavikko methods, the differences between dental and chronological age (overestimations and underestimations) were presented as square deviations (Table 5). The accuracy of age estimation in the form of the percentage of correct estimations within the interval for all three methods is shown in Table 6.

Table 2. Demirjian method: comparison of chronological and dental age

Boys					Girls				
age group	Chronological age (years)	Dental age (years)	Deviation (years)	p*	age group	Chronological age (years)	Dental age (years)	Deviation (years)	p*
5 - 5,99	5,66	6,63	0,97	0,125	5 - 5,99	5,33	5,82	0,49	0,063
6 - 6,99	6,65	7,74	1,09	< 0.001	6 - 6,99	6,62	7,66	1,04	< 0.001
7 - 7,99	7,57	8,49	0,92	< 0.001	7 - 7,99	7,60	8,16	0,56	< 0.001
8 - 8,99	8,51	9,23	0,72	< 0.001	8 - 8,99	8,51	8,97	0,45	< 0.001
9 - 9,99	9,51	10,06	0,55	< 0.001	9 - 9,99	9,52	10,46	0,94	< 0.001
10 - 10,99	10,46	11,13	0,67	< 0.001	10 - 10,99	10,51	11,43	0,92	< 0.001
11 - 11,99	11,42	12,15	0,72	< 0.001	11 - 11,99	11,50	12,93	1,43	< 0.001
12 - 12,99	12,47	13,28	0,81	< 0.001	12 - 12,99	12,48	13,69	1,22	< 0.001
13 - 13,99	13,49	14,51	1,02	< 0.001	13 - 13,99	13,52	14,75	1,22	< 0.001
14 - 14,99	14,47	15,49	1,02	< 0.001	14 - 14,99	14,42	15,13	0,71	< 0.001
15 - 15,99	15,43	15,81	0,37	< 0.001	15 - 15,99	15,49	15,78	0,29	< 0.001
Total	10,51	11,32	0,80		Total	10,50	11,34	0,84	

* p value in Wilcoxon signed-rank-test

DISCUSSION

In this survey three widely used methods for dental age estimation, Demirjian, Willems and Haavikko, were evaluated for accuracy among Croatian children aged 5 to 16 years. The strength of this study lies in the large and representative sample drawn from different regions across Croatia. In addition, all the OPGs were standardized and taken with the same device, which provided images of the highest quality. To date, a sample of this size and quality for the specific purpose of dental age estimation has yet to be recorded in the Croatian population and represents a valuable addition to the scientific literature currently available.

The Demirjian and Willems methods use a simple and well explained developmental scale which consists of eight stages for the seven mandibular teeth.[3] The simplicity of usage as well as good reproducibility favours their application in dental age estimation.[14] However, OPGs with mandibular hypodontia cannot be assessed as they do not meet the basic requirement of the methods, which is the presence of all seven left permanent mandibular teeth. The Haavikko method is based on a more complicated developmental scale which counts twelve different stages.[5] When compared to the Demirjian and Willems methods, the advantage of this approach is that the selection excludes the most frequently missing teeth.[6]

A potential weakness of the sample used in this study is the comparatively small number of OPGs in the two youngest age groups. Therefore, the results of age estimation for children younger than seven years should be read with caution. Nonetheless, the statistical methods used in the study ensure that the results in the other age groups are not compromised. The problem with the small number of OPGs in the youngest age groups is well documented in the literature as, for ethical reasons, there is no justification in taking OPGs exclusively for scientific purposes.[12]

Table 3. Willems method: comparison of chronological and dental age

Boys					Girls				
age group	Chronological age (years)	Dental age (years)	Deviation (years)	p*	age group	Chronological age (years)	Dental age (years)	Deviation (years)	p*
5 - 5,99	5,66	5,88	0,23	0,875	5 - 5,99	5,33	4,97	-0,36	0,313
6 - 6,99	6,65	7,43	0,77	< 0.001	6 - 6,99	6,62	7,15	0,53	0,042
7 - 7,99	7,57	8,42	0,85	< 0.001	7 - 7,99	7,60	7,71	0,11	0,097
8 - 8,99	8,51	9,01	0,50	< 0.001	8 - 8,99	8,51	8,27	-0,24	< 0.001
9 - 9,99	9,51	9,68	0,17	0,674	9 - 9,99	9,52	9,54	0,02	0,894
10 - 10,99	10,46	10,64	0,18	0,207	10 - 10,99	10,51	10,65	0,14	0,653
11 - 11,99	11,42	11,60	0,17	0,587	11 - 11,99	11,50	12,16	0,66	< 0.001
12 - 12,99	12,47	12,68	0,21	0,064	12 - 12,99	12,48	13,03	0,56	< 0.001
13 - 13,99	13,49	13,88	0,39	0,005	13 - 13,99	13,52	14,25	0,72	< 0.001
14 - 14,99	14,47	15,25	0,78	< 0.001	14 - 14,99	14,42	14,66	0,24	0,041
15 - 15,99	15,43	15,67	0,24	< 0.001	15 - 15,99	15,49	15,52	0,02	< 0.001
Total	10,51	10,92	0,41		Total	10,50	10,72	0,22	

* p value in Wilcoxon signed-rank-test

Table 4. Haavikko method: comparison of chronological and dental age

Boys					Girls				
age group	Chronological age (years)	Dental age (years)	Deviation (years)	p*	age group	Chronological age (years)	Dental age (years)	Deviation (years)	p*
5 - 5,99	5,66	4,94	-0,72	0,250	5 - 5,99	5,33	4,50	-0,84	0,063
6 - 6,99	6,65	6,09	-0,56	< 0.001	6 - 6,99	6,62	6,32	-0,30	0,519
7 - 7,99	7,57	6,97	-0,60	< 0.001	7 - 7,99	7,60	7,07	-0,52	< 0.001
8 - 8,99	8,51	7,85	-0,66	< 0.001	8 - 8,99	8,52	8,10	-0,42	< 0.001
9 - 9,99	9,51	8,87	-0,65	< 0.001	9 - 9,99	9,52	9,27	-0,25	< 0.001
10 - 10,99	10,46	10,14	-0,32	0,015	10 - 10,99	10,54	10,31	-0,23	0,120
11 - 11,99	11,42	11,25	-0,17	0,231	11 - 11,99	11,50	11,51	0,00	0,388
12 - 12,99	12,48	12,34	-0,14	0,743	12 - 12,99	12,48	11,96	-0,52	< 0.001
13 - 13,99	13,49	13,04	-0,45	< 0.001	13 - 13,99	13,50	12,35	-1,15	< 0.001
14 - 14,99	14,47	13,63	-0,84	< 0.001	14 - 14,99	14,42	12,47	-1,94	< 0.001
15 - 15,99	15,43	13,80	-1,63	< 0.001	15 - 15,99	15,48	12,66	-2,82	< 0.001
Total	10,51	9,90	-0,61		Total	10,50	9,68	-0,82	

* p value in Wilcoxon signed-rank-test

Table 5. Square deviations between dental and chronological age (years²) for the Demirjian, Willems and Haavikko methods (a lower value indicates more accurate age estimation)

Boys				Girls			
age group	**Demirjian**	**Willems**	**Haavikko**	**age group**	**Demirjian**	**Willems**	**Haavikko**
5 - 5.99	1.93	2.00	1.33	5 - 5.99	0.44	0.35	1.19
6 - 6.99	1.64	1.56	0.90	6 - 6.99	1.41	0.82	0.80
7 - 7.99	1.27	1.15	1.01	7 - 7.99	0.67	0.24	0.78
8 - 8.99	1.08	0.62	1.14	8 - 8.99	0.79	0.39	0.70
9 - 9.99	1.63	1.14	1.38	9 - 9.99	1.67	0.80	0.39
10 - 10.99	2.02	1.64	2.13	10 - 10.99	2.50	2.00	1.10
11 - 11.99	3.20	2.46	2.01	11 - 11.99	3.58	2.12	0.59
12 - 12.99	2.50	1.68	1.06	12 - 12.99	2.99	2.06	0.55
13 - 13.99	2.78	2.08	0.82	13 - 13.99	2.62	2.09	1.45
14 - 14.99	1.92	1.87	0.86	14 - 14.99	1.66	1.90	4.02
15 - 15.99	0.52	0.82	2.78	15 - 15.99	0.49	0.63	8.01
Total	20.48	17.02	15.42	Total	18.81	13.40	19.59

Table 6. Accuracy of age estimation for the Demirjian, Willems and Haavikko methods (percentage of correct estimations within interval)

Precision (%)	**Demirjian**	**Willems**	**Haavikko**
Boys			
± 0.5 years	31.6	34.1	28.8
±1 year	56.4	62.2	58.3
± 1.5 years	73.7	80.5	79.1
± 2 years	86.4	91.0	92.0
Girls			
± 0.5 years	27.2	40.4	34.2
±1 year	53.9	64.3	58.0
± 1.5 years	70.3	78.6	73.9
± 2 years	83.1	90.4	83.1

On average, the Demirjian and Willems methods overestimated age, while the Haavikko method underestimated age in boys and girls alike. Among the evaluated methods, the Willems method proved to be the most accurate, followed by the Haavikko and Demirjian methods. The mean deviation between dental and chronological age is similar to the survey conducted in the Croatian population by Čuković-Bagić et al.,[15] who report an average overestimation of 0.92 years in boys and 1.00 years in girls when using the Demirjian method, and an average

underestimation of 0.5 years in boys and 1.00 years in girls using the Haavikko method. While testing the Willems method among Croatian children, Galić [16] found that it overestimates the mean age by 0.58 years in boys and 0.32 years in girls. In another survey among Bosnian-Herzegovinian children,[17] the Willems method showed an overestimation of 0.42 years in boys and 0.24 years in girls, while the Haavikko method underestimated age by 0.09 years in boys and 0.29 years in girls. Contrary to the aforementioned study by Galić et al.[17], the Haavikko method showed greater deviations underestimating age in the Brazilian [18], Turkish [19] and Malaysian[20] population.

According to Esan et al.,[21] the Demirjian method significantly overestimates age in most populations, while the majority of the studies analysed here do not report significant overestimation using the Willlems method. Another meta-analysis by Wang et al.[12] reports that in polled data the Willems method shows a slight overestimation of age but also observes a significant difference for different ethnicities. For example, in Kosovar population, Kelmendi et al.[22] found Willems method underestimates age on average by 0.14 years in boys and 0.24 years in girls.

Apart from ethnic specificities, the positive secular trend represents another challenge for accurate age estimation using the known methods. Earlier dental development has been observed in today's children compared to their peers from a few decades ago.[23,24] In a survey among Dutch children, Vucic et al.[24] stress "the necessity of taking the year of birth into account when assessing dental development within a population with a wider time span". Moreover, from the documented secular trend there derives an obvious need to test previously conducted studies and the developed methods based on shifting observations and requirements over a period of time. Testing both the known and developing new methods represents a continuous challenge in forensic odontology.

Recently, Bedek et al. have developed new models for dental age estimation that surpass the accuracy of the Willems method.[25] In addition to a higher level of accuracy, the advantage of the new models is the possibility of their application in cases with incomplete dentition (hypodontia and incomplete human remains), which was not possible using the previously existing methods. The potential of these models has been recognized by Sheriff et al.[26] who tested them among the South Indian children. They proved to be accurate and suitable for dental age estimation.

Information pertaining to dental age estimation can be presented in a variety of ways. In this investigation, by way of comparison with other studies, the average deviation between dental and chronological age was used as it is the most common form of presenting results. However, we believe that a the more precise expression of the accuracy of a certain method is the percentage of correct estimations within intervals of ± 0.5, ± 1, ± 1.5 and ± 2 years. Using the average error as the main reference in age estimation might mislead the user in an individual case. Liversidge[9,27] claims that each average deviation between dental and chronological age, no matter how large, is always smaller than the possible and existing difference between individuals in the same population. Consequently, it is possible to conclude that a small average deviation could be the result of a sample comprising an equal number of individuals whose development is faster or slower than average. In legal terms in particular, the result of the individual age estimation should be presented as the probability within the age interval.

Pruvost et al.[28] highlighted the problem in age estimation provided by forensic physicians. In a sample of 498 files regarding age assessment they found that 71% of estimations were incompatible with the age claimed by adolescent. The percentage of incompatible estimations dropped to 3% when age was estimated from population specific data presented in published studies including those regarding dental age estimation. These results clearly indicate lack of research experience and up to date information which lead to negligence of professional standards in age estimation.

With regard to legal processes, an age estimation of the victim or the accused person may be required, especially in cases that involve trafficking, asylum seekers, child labourers and sex workers with a missing or doubtful birth certificate.[29] Knowledge and experience in dental age estimation as well as an awareness of its limitations are the desirable characteristics of an experienced clinician and should not be a matter of concern solely for forensic physicians and odontologists.

In the period between 2010 to 2020, emigration from the Republic of Croatia increased from 10000 to 40000, encompassing approximately 6500 children. The results of the present study could be applied in the aforementioned cases both for the children living abroad and those living in Croatia.[30]

CONCLUSIONS

Considering the average overestimation and accuracy within the age interval, the Willems method can be used for dental age estimation in Croatian children. Due to the average deviation between dental and chronological age as well as the low percentage of correct estimations within the age interval, the Demirjian and Haavikko methods were not sufficiently accurate for Croatian children.

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Identifying a victim of alligator attack and scavenger fish in the Brazilian Amazon rainforest using smile photographs: a case report

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ABSTRACT

Reports of deaths caused by alligators or crocodiles are rare in the literature. These deaths may be related to sharp force trauma caused by the teeth of these animals, with or without mutilation, or even drowning after seizure and submersion of the victim. It is difficult to forensically identify bodies in cases of mutilation of the upper limbs during the attack or when the corpse is in an advanced stage of skeletonization. Smile photographs are an important source of *ante-mortem* references for comparison. We report a human identification based on a photograph of a victim, with the absence of limbs caused by an alligator attack and the advanced skeletonization stage due to scavenger fish action in the Amazon within only 36 hours after his disappearance. The description of alligator attacks and the marks observed on the victim's body are essential to help medical and forensic professionals diagnose the injuries found and, consequently, define the cause of death

INTRODUCTION

Human deaths caused by wild animal attacks are common across the world. However, deaths caused by alligators or crocodiles are rare, even though they can cause serious injuries.[1–4] Such deaths normally result from haemorrhage or drowning and are related to sharp force trauma caused by these animals' teeth, with or without mutilation of limbs. Publications describing these injuries are few,[4–6] despite their importance in the diagnosis of these injuries in forensic practice and for a better comprehension of the risks to which riverside populations are exposed.

In forensic practice, it is difficult to identify the victim when one or more limbs of the corpse are missing, especially if the body is in an advanced skeletonization stage. In this context, dentistry is of great value in human identification. Dental information present in *ante-mortem* documentation of the alleged victim, such as dental records, x-rays, and study models, can be compared with dental data found in the corpse for identification.[7,8] However, this process is hampered when the alleged victim has never undergone any dental procedure or when the family is unable to present dental records. In these cases, casual photographs can be used to compare with dental evidence to identify the victim.[7,9,10]

Thus, this work aimed to report a case of forensic human identification comparing *ante-mortem* photographs of a missing person with the dental characteristics found in a corpse of a possible victim of an alligator attack, who had his body skeletonized by scavenger fish from the Brazilian Amazon.

CASE REPORT

The victim and three other individuals (two children and an adult) were on the banks of the Madeira River, in the Brazilian Amazon region. The adults were on the beach repairing a small wooden boat and the children playing in the river. A witness reported that one of the children began to drown and the two adults entered the river to rescue them. During the return to the beach, the victim screamed, submerged, and disappeared in the river, as if something had pulled him into the water.

Authorities then began searches and rescued the remains of the corpse 36 hours after the accident in a shallow area 80 metres away from the disappearance site and close to the riverbank. The rescue group reported the presence of a black cayman of medium size, about 3 metres long, close to the victim's bones. They also reported the presence of several fish around the corpse, which were feeding on the remains.

The necropsy examination found an incomplete corpse, almost completely skeletonized, consisting of a cranium, three ribs, left upper limb, pelvic girdle, and right lower limb (Fig. 1). Anthropological examination showed that it was a single individual with male characteristics in late adulthood.

Figure 1. Human skeleton composed of the cranium, three ribs, left upper limb, pelvic girdle, and right lower limb

The examination verified sharp force injuries in the pelvic girdle, measuring from about 0.7 to 1.9 cm in diameter, representing small, depressed bone fractures in a circular shape. Many injuries were arranged in the form of two symmetrical lines converging with each other, the largest one measuring about 16 cm in length, which together formed a "V" (Fig. 2).

Some cartilages and the gingival mucosa were preserved, and there were shallow circular puncture wounds on the bone surfaces (Fig. 3), compatible with the action of scavenger fish from Amazonian rivers, such as candiru and piracatinga fish.[11–13]

Figure 2. Posterior view of the right iliac bone Sharp force injuries, several of them in a row forming two converging lines, shaped like a "V", compatible with an alligator bite

Figure 3. Top view of the cranial vault. The arrows point to multiple circular puncture wounds compatible with the action of scavenger fish, such as candiru and piracatinga

In the face of the impossibility of performing fingerprint analysis due to the skeletonization stage of the body, and the high cost and long waiting time for results of DNA analysis at the local institution, searches started for the victim's dental documentation to enable identification. In the absence of medical records and traditional dental documentation, the family provided a photograph of the alleged victim's smile for comparison with the dental data of the skeletonized remains.

Two forensic techniques were employed for the dental analysis of the case: direct comparison and computerized delineation of the incisal edges of the teeth.

For direct comparison, *ante-mortem* and *post-mortem* images were paired (Figs. 4 and 5), after obtaining them at approximate and stagger angles of incidence, allowing comparison between the dental characteristics found (Table 1).

To analyze the morphology of the smile line, experts drew a line corresponding to the incisal contours of the upper anterior teeth (Fig. 6) in both photographs using the Power Point® software (Microsoft®, Redmond, USA), enabling the comparison between the outlined incisal edges.

The analyzed points were compatible, both in the direct comparison and in the analysis of the computerized delineation of the incisal edges of the upper anterior teeth. These two dento-legal analyses enabled the identification of the victim.

Figure 4. Photograph of the smile presented by the family of the possible victim The arrows show characteristics described in Table 1

Figure 5. Photograph of the corpse at the same angle as the photograph of the alleged victim. The arrows show the same characteristics found in *ante-mortem* photography, described in Table 1

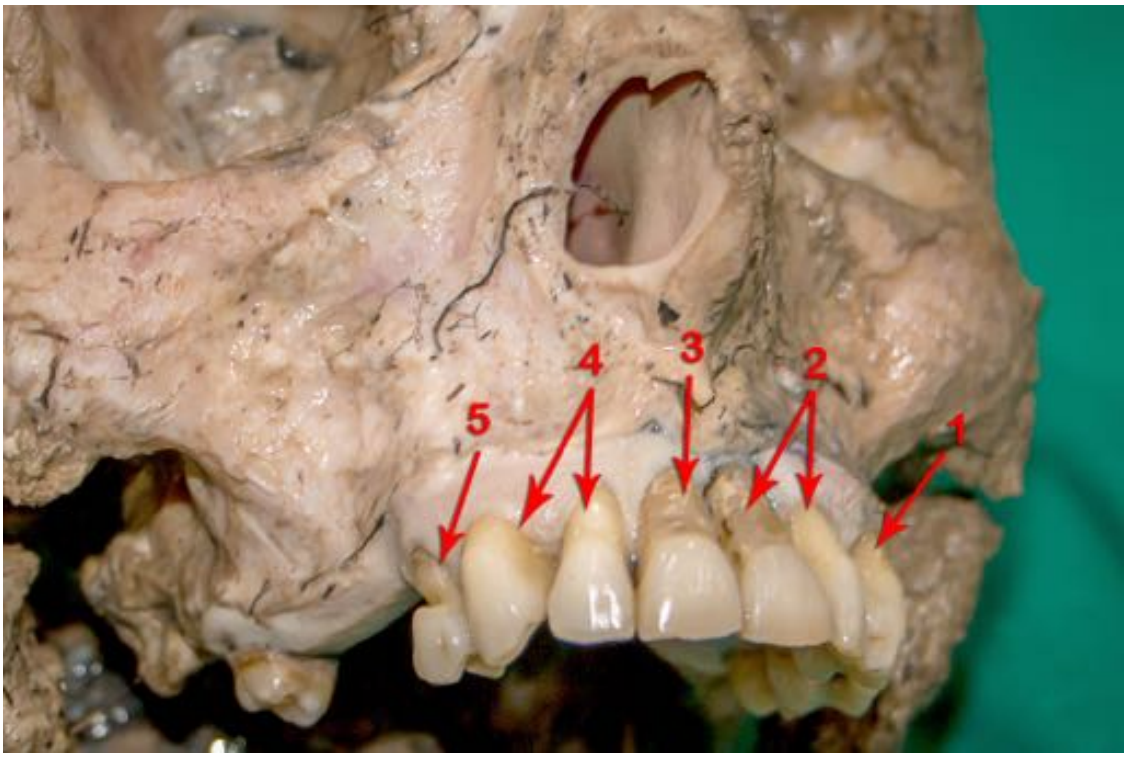
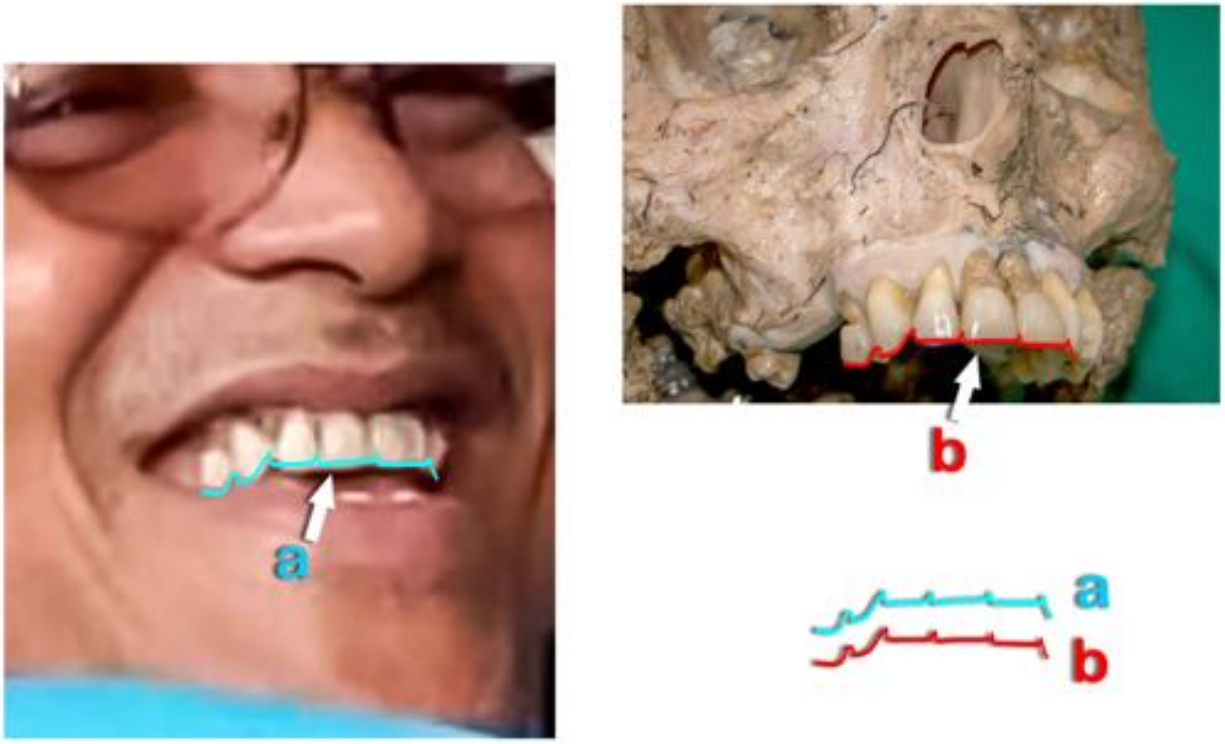


Table 1. Direct comparison between the dental characteristics observed in the photograph of the alleged victim (Fig. 4) and the dental arch of the corpse (Fig. 5)

ANTE-MORTEM	POST-MORTEM
Tooth 23 (upper left canine), proclined (fig. 4, arrow 1);	Tooth 23 (upper left canine), proclined (fig. 5, arrow 1);
Tooth 22 (upper left lateral incisor) slightly longer than tooth 21 (upper left central incisor) (fig. 4, arrow 2)	Tooth 22 (upper left lateral incisor) slightly longer than tooth 21 (upper left central incisor) (fig. 5, arrow 2)
Tooth 11 (upper right central incisor) with quadrangular shape, with greater wear in the mesial third of the incisal edge (fig. 4, arrow 3)	Tooth 11 (upper right central incisor) with quadrangular shape, with greater wear in the mesial third of the incisal edge (fig. 5, arrow 3)
Teeth 12 (upper right lateral incisor) and 13 (upper right canine) slightly diverged in the root apex direction (fig. 4, arrow 4)	Teeth 12 (upper right lateral incisor) and 13 (upper right canine) slightly diverged in the root apex direction (fig. 5, arrow 4)
Tooth 14 (upper right first premolar) with signs of cervical injury compatible with abfraction (fig. 4, arrow 5).	Tooth 14 (upper right first premolar) with an abfraction-type cervical lesion (fig. 5, arrow 5).

Figure 6. Comparison of computerized delineation of incisal contours between *ante-mortem* (a) and *post-mortem* (b) dental images



DISCUSSION

There are two alligator species (Alligatoridae family) with a history of attacking humans in the rivers of the Amazon basin: the jacaretinga (Caiman crocodilus), which can reach up to three metres in length, and the black cayman (Melanosuchus niger), which can reach up to six metres in length. The latter is known by natives of the Amazon region as “the devourer of men”, given its bite strength and extreme agility in the water environment. The black cayman has about 70 to 80 teeth arranged in a V-shape, which, combined with the strength of its jaw, can hold the prey without allowing any reaction, making its attacks mostly fatal. Smaller crocodiles usually only take a single bite. However, up to a third of attacks involve repeated bites.1,6

The state of Rondônia, in the Brazilian Amazon region, has registered a notorious presence of these alligators in recent years, especially in the Cuniã Lake region, on the banks of the Madeira River. The absence of natural predators and the abundance of food, such as fish and small animals, led to a disorderly growth in the alligator population in the region from 2000 onwards, which increased attacks on humans, especially children and fishermen.[6,14]

In the present case, the wounds found in the iliac bone during the autopsy were compatible with those caused by multiple black cayman bites.[1]They were sharp force injuries represented by depressed small bone fractures in a circular shape, mostly arranged in two symmetrical lines, forming the letter "V", compatible with the distribution of the alligator's teeth.

In this context, careful examination of bite marks is essential to exclude other possible forms of injury and to reach a conclusive result. This type of circular wound in the bone caused by an animal bite, when observed in isolation, and the absence of circumstantial evidence may lead to misinterpretations of penetration of foreign bodies, such as projectiles and other sharp objects, or can be mistaken by *post-mortem* damage.[2,3]

The statements of the witnesses, who saw the victim sink and disappear into the river, suggest that the *cause of death* was mechanical asphyxia by drowning, a common cause among victims of alligators in the Amazon, which usually kill by seizure and drowning, followed by disarticulation of limbs and ingestion.[6]However, it was not possible to prove the *cause of death* during the autopsy due to the absence of internal

organs and parts of the body that could present other fatal injuries.

The shallow circular wounds found in the cranial vault, in well-delineated shape, are compatible with the action of scavenger fish common in Amazon rivers, represented by candiru fish (Cetopsis candiru), baby whale catfish (Cetopsis coecutiens), and vulture catfish (Calophysus macropterus).[11–13]This explains how the body was found almost totally skeletonized only 36 hours after the victim's disappearance.

Due to the challenges in the use of fingerprint identification techniques, associated with the high cost and long waiting time for results of DNA analysis at the local institution, forensic dentistry analysis was performed.

In the face of the compatibilities found in the anthropological analysis between the body and the disappeared man, and the circumstantial similarities between the corpse and the report of the witnesses of the disappearance, dental technique of analysis and comparison of photographs of the victim's smile was used to identify the body. This is a practical, fast, and reliable method for scientific standards.[8,10,15,16]Such images are usually available as they are in the possession of family members and/or available on social networks. They may show teeth shape, size, and proportions, biometric characteristics of oral anatomical structures, gingival contours, positioning of long axes of the teeth, contact points, and shape of the incisor teeth of the supposed victim's smile.[10,15,17]Care must be taken in obtaining as clear images as possible and allowances should be made for different photographic lenses. All these elements allow the identification of a corpse through comparison between *ante-mortem* and *post-mortem* images.[18]Caution should be taken when image quality is compromised, particularly when it becomes blurry when expanded. In these cases, caution should be taken in relying on photographic evidence only and it is prudent that further evidence including circumstantial evidence is utilized to confirm identity.

In these cases, the expert must record images of the corpse at the same angle of incidence as the photographs of the alleged victim so that the comparison is as accurate as possible. The more teeth visible in the *ante-mortem* photograph, the more detail can be seen and the greater the probability of identification. The use of easily accessible software, such as Power Point® (Microsoft®, Redmond, USA), makes it possible to draw lines that demarcate the teeth limits and the incisal line, adding visual characters to the analysis and facilitating the visualization for lay people of the comparison of characters used to identify the victim.

CONCLUSIONS

The description of alligator attacks and the signs observed on the victim's body are essential to help medical and forensic professionals in diagnosing the injuries found and, consequently, in determining the cause of death. This paper contributes to a better understanding of the risks for people that use water environments for work and leisure, in addition to directing policies for isolation of areas and environmental education in great risk regions.

In identification, the absence of dental records, smile photographs are an alternative tool for obtaining ante-mortem dental information to be compared with post-mortem data. Such photographs can indicate an individual through morphometric characteristics, representing an efficient way to assist victim identification.

PERMISSION TO USE IMAGES

Permission to use images was granted by the victim's relatives through a specific Informed Consent Form.

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The CSI effect in Forensic Odontology. A systematic review

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ABSTRACT

The popularity of forensic science in recent decades is substantially related to the high rate of watching television programs dealing with the investigation of criminal cases, such as "CSI: Crime Scene Investigation" (CSI: Crime Scene Investigation, 2000) and the two sequels, "CSI: NY" (CSI: NY, 2004) and "CSI: Miami" (CSI: Miami, 2002). These medical-based TV series portrayed forensic science in a favorable way, encouraging viewers to experience differently this scientific field. Although it is considered a minor social phenomenon, the reality seems to diverge. The aim of this study is to systematically review the existing literature on the impact of the "CSI effect" on crime scene management, analysis, and interpretation of evidence on forensic odontology cases. Electronic research was attempted among four (4) different electronic databases from January 2005 to October 2021. After removing articles according to inclusion-exclusion criteria, the final selection resulted in 5 articles. The results indicated that forensic-based TV series provided a sense of plausibility not dependent on factual accuracy. In addition, an increasing pressure on law enforcement personnel and investigators to collect DNA at crime scenes, regardless of whether it was relevant to the case was also observed. The popularity of these TV shows has contributed to growing public interest in forensic science programs and hence the "CSI effect" had a greater impact on individuals who systematically watched such television series.

INTRODUCTION

As it is well known, television has a catalytic effect on shaping public opinion on various issues. So, in the 1990s, the popularity of medical-based television dramas enhanced public perception of health issues [1]. During the last decade, several TV series of crime or/ and legal dramas have been very popular worldwide [2,3]. Kim et al. (2009) referred to 33 U.S. television programs featuring forensic investigations and judicial proceedings commenting that, the abundance of information had almost become "part of the (popular) culture" [4].

Due to the growing dissemination of information concerning forensic science and criminal justice through crime television programs, there is a general perception that these series have dramatically influenced public beliefs about crime scene management and evidence's analysis and interpretation. In

particular, the public has raised high expectations and, in some cases, has been misleading concerning the management of real criminal cases [4-6].

The impact of crime television shows on the public perceptions of collection, management, analysis, and interpretation of forensic evidence has been dubbed the "CSI effect (Crime Scene Investigation effect)", a term that began to appear in the mainstream media as early as 2004. Although it is considered a minor social phenomenon, the reality seems to be different [5]. The research question posed was "Does the SCI effect has an impact on forensic odontology cases?" This study aims to systematically review the existing literature on the impact of the "CSI effect" on crime scene management, analysis, and interpretation of evidence in forensic odontology cases.

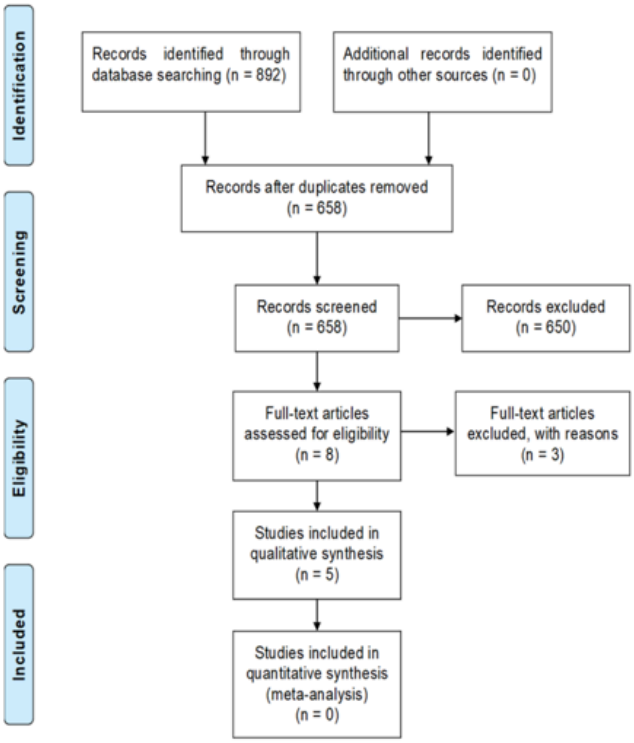
MATERIAL AND METHODS

A systematic review of literature on the influence of the "CSI effect" and its utilization in forensic dental evidence was conducted. The search strategy and inclusion of the studied articles was based on the PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analyses) statement published in 2020 [7]. Electronic research was attempted of the 'SCOPUS', 'COCHRANE Library', 'PUBMED' and 'Web of Science' databases from January 2005 to October 2021 using the search terms,''CSI Effect and impact'' OR ''CSI Effect and impact and forensic Odontology'' OR ''CSI Effect and impact and forensic medicine" OR "CSI Effect and impact and forensic science. Abstracts were examined for relevance to the defined review question. Full texts of case reports, technical notes, in vitro and experimental studies on humans in English were included. Newsgroup articles, systematic reviews, letters to the editor, animal experimental studies in a language other than English were excluded. Two researchers independently reviewed each abstract and title for potential relevance to the research question. Articles included by either researcher were subjected to full-text screening. At the full-text screening stage, the researchers reviewed the full text of each article for inclusion, and disagreements were resolved by discussion between them.

RESULTS

The database search resulted in 892 articles. After removing duplicate records (n=234), as well as full texts that were not available or did not agree with the inclusion criteria, the selection finally resulted in 5 articles as presented in Fig.1. Of the five articles identified, three reported data from adult population, jury-eligible participants [8-9], one reported data from volunteers aged from 13-43 years old [11],

Figure 1. PRISMA Flow Diagram



one reported data derived from the transcripts' content analysis of the first six seasons of CSI [1]. Only two out of five articles reported participants' very high degree of confidence in DNA testing [1,9]. Of the five articles identified, two articles provided the view that watching CSI series and other forensic crime shows usually contains exaggeration and conveys a sense of plausibility that did not depend on the accuracy/absence of facts[8,11]. No systematic reviews or high-level evidence studies were identified in this review. The articles finally included in this review were published between 2012 and 2019, as shown in Table 1.

Table 1. Articles included in this systematic review

Year	Authors	Title	Country	Material	Journal
2012	Ley et al	Investigating CSI: Portrayals of DNA testing on a forensic crime show and their potential effects	Unite Kingdom	Transcripts of first six (6) of CSI	Public Understand. Sci.
2019	Ribeiro et al	Beliefs about error rates and human judgment in forensic science	Australia	On-line questionnaire	Forensic Sci. Int
2013	Chan	An investigation into the CSI effect on the Malaysian population	Malaysia	Self-administered form (on-line)	Australian Journal of Forensic Sciences
2019	Slak et al	Do Fictional Forensic and Criminal Investigation Television Shows Influence Students' Enrollment Decisions?	Slovenia	On-line questionnaire	Journal of Criminal Justice Education
2012	Smith & Bull	Identifying and measuring juror pre-trial bias for forensic evidence: development and validation of the Forensic Evidence Evaluation Bias Scale	United Kingdom	10-item scale questionnaire (on-line)	Psychology, Crime & Law

DISCUSSION

In this review, an extensive survey of previous literature on forensic evidence and the "CSI effect" among four different electronic databases was attempted. The "CSI effect" involves the growing expectation that crime scenes will reveal forensic evidence that can be scientifically analyzed through forensic science and technology expertise, such as DNA testing, fingerprint or bitemarks analysis, and which could be supported in court[12]. The collection and use of forensic data are very significant for criminal investigations and

prosecutions[13] because these procedures contribute to identifying the essential elements of a crime, to determining the guilt or innocence of the persons involved at the scene of the crime[14]. Of the five articles identified, three articles suggested that watching crime series (such as CSI: New York - CSI: Miami – NCIS – BONES - Forensic Heroes 3 (FH3)) forensic techniques (DNA testing and genetics in general) sometimes portrayed in more equivocal or complex ways [1,8,11]. Especially, Ribeiro et al. (2019) assessed their sample's responses about forensic techniques and concluded that people did not blindly believe that these scientific procedures were highly accurate[8]. Their sample consisted of 101 Australian adults (age range 20 to 70 years) recruited by an Australian market research company (December 2015) and remunerated $5.95 AUD for their involvement. Individuals completed an online questionnaire (on their own computers or electronic devices) in which they were required to rate their general knowledge of forensic procedures and their expectations of the accuracy of each stage on a percentage scale. Their responses were processed with the Qualtrics survey software and correlated each other using a) single sampled t-test, b) Pearson's correlations (r). The respondents had varying convictions about the accuracy of different forensic techniques and believed that forensic science's process involved a significant amount of human judgment and was relatively prone to errors. Although the authors' article revealed very limited support for the CSI effect, however, participants considered forensic dentistry as the procedure with the highest accuracy (89,26%) with almost similar results to DNA analysis (89,95%) [8].

Chan (2013) investigated Malaysian viewers' expectations of forensic science who were influenced by the knowledge obtained from watching a particular forensic-themed drama series [Forensic Heroes 3 (FH3)] [11]. Their sample consisted of 131 participants (age range 13 to 43 years) who submitted an online form filled with their responses and sent it back either by e-mail or via the Facebook electronic platform. The questionnaire form comprised general questions for volunteers and thirty-four (34) items that could be graded on a scale of 1 to 5 (1= strongly disagree, 5 = strongly agree), covering five (5) main conduct; s categories: general belief, forensic scientists, and the profession, conduct, and ethics, forensic laboratory, forensic evidence, and investigation. The participants were divided into three groups: cohort 1 (n=65), who were definitely affected by the particular TV forensic–
based show (FH3), cohort 2 (n=47) who had not watched the selected TV program, and cohort 3 (n=19) consisting of forensic science professionals who would discern the outcome's impact. Statistical processing was performed using Minitab 15 (software program) and the statistically significant difference between the selected groups was assessed using the Mann-Whitney U test. The results demonstrated that CSI effect's influence between the three (3) cohorts was insignificant. The belief of solving all cases was more pronounced in the first cohort and viewing specific forensic-themed programs presented a false reality characterized by hyperbole [11].

In the study by Ley et al. (2012), it was found that CSI forensic-based series portrayed a sense of plausibility not dependent on factual accuracy or their absence. The authors evaluated scripts of 51 randomly selected episodes of the first six seasons of CSI. They focused on the different stages of DNA collection, analysis and utilization. Each examiner/coder determined on a percentage scale whether the CSI investigator: a) searched for DNA in unknown sources at the crime scene (66%), b) collected or stored at least one DNA sample from a known individual or animal (52%), c) compared at least one DNA sample with possible samples from a federal DNA database (29%), d) solved the case (88%). Although, there are a few limitations of this study's results. The subcategories' grading is subjective and based solely on coders' judgment, whose level of experience did not mentioned in the article. Additionally, they reported an extremely high rate of case resolution when adopting DNA analysis, and this has resulted in increased pressure on law enforcement officials and investigators to collect DNA at crime scenes, regardless of whether it is relevant to the case [1].

Slak et al. (2019) aimed to examine whether viewing's frequency of forensic and criminal investigative TV series had any impact on students' enrolment at the Faculty of Criminal Justice and Security (FCJS) at the University of Maribor [9]. Their sample consisted of 151 first-year students of FCJS who answered an online questionnaire. The survey's sample had a higher percentage of females than the Slovenian

population and was not selected randomly. The online form included questions about their initial source of knowledge regarding investigative work if they watched specific series that influenced their final choice to enroll in FMJS school, how the police were portrayed in specific series and basic demographic information. Their results revealed the complexity of viewing specific forensic and investigative TV programs as influential behavioral factors. All statistical assessment methods (Kruskal-Wallis H/ Kolmogorov-Smirnov/Shapiro-Wilk tests) demonstrated that viewing TV forensic-based series and films did not overly strong motivators for the first-year student. Specific statistical tests (Kolmogorov-Smirnov and Shapiro-Wilk) verified that participants' responses were highly dispersed and did not follow a normal distribution. Additionally, the authors considered the "CSI effect" as a useful educational factor, although further future research is needed to examine the effect where many questions remained unsettled [9].

Smith and Bull (2012) aimed to develop a more precise predictor of pre-trial jury bias that focuses on the interpretation of forensic evidence [10]. In the initial stage of their survey, participants [jury-eligible psychology postgraduate students, (N=219)] were requested to grade thirty-one items from an initial pool, according to some basic jury eligible criteria described by the Criminal Justice System for England and Wales (www.cjsonline.gov.uk). Participation in the research was advertised through various websites and via the authors' institution press office. Initially, the questionnaire's results were tabulated in Excel data and exported to SPSS (statistical package) for analysis. Items with correlations less than 0,3 (n=21) were excluded. The final version of the scale consisted of 10 items with sufficiently high inter-item and item-total correlation scores (greater than 0.3). At the second stage of the survey, 159 jury-eligible undergraduate psychology students at the researchers' university participated in exchange for partial course credit. The final sample consisted entirely of undergraduate students while the sample's majority (88%) was female population. Individuals rated the 10-item Forensic Evidence Evaluation Bias Scale (FEEBS) using a 5-point Likert scale (coded as 1-5). They also studied a fictional murder trial presented by the authors and were asked to evaluate the defendant's likelihood of being guilty of the crime (expressed as a percentage) and a final verdict (guilty/innocent). Their results showed that the DNA evidence was rated significantly stronger than all other evidence types and overall 46% of the final sample (73 participants) voted guilty while 86 individuals voted not guilty (54%). There are a few limitations considering the results of this article[10].

Of the five articles identified, three articles supported the opinion of watching forensic-based TV shows provided a sense of plausibility [8,9,11]. Their results are consistent with Nisbet et al.'s claim (2002), who demonstrated that such viewing was negatively related to the self-perceived understanding of DNA evidence and these learning opportunities can be displaced [15]. Slak et al. (2019) mentioned the CSI effect's usefulness as an educational tool, however watching systematically these TV series was not a determining factor in enrolment's final choice [9].

The most affected population group was the viewers, whose expectations were clearly higher than those of professionals and non-viewers, who considered that at least one error occurred at each stage of the forensic science process. Due to the topic's specificity, the articles' total number for assessing was low while existed a high degree of heterogeneity in terms of the results and in sample collection methods. More future research is needed to examine the "CSI effect" on forensic odontology cases applying more relevant evaluation and sample collection methods.

The studies included in this systematic review did not referred exclusively to dental data and presented heterogeneity in their sample and data collection and evaluation. Smith and Bull (2012) assessed the responses of their sample, which consisted of undergraduate students at the researchers' university whose participation was rewarded in exchange for partial course credit, influencing their judgment. Chan (2013) included underaged participants in his survey [11]. The sample of Slak et al. (2019) survey was not randomly selected and not representative of the Slovenian population as it focused exclusively on first-year students, while the "CSI effect" may have a greater impact on older age groups [9]. Ley et al. (2012) assessed DNA testing's usefulness as a stage of forensic science and not the "CSI effect" overall. Furthermore, they did not report their examiners' level of experience.

Due to the aforementioned heterogeneity and the lack of a sufficient number of decent quality primary studies, a meta-analysis was impossible to performed.

CONCLUSIONS

It seems that the "CSI effect" had a greater impact on individuals who systematically watched such television series and created unrealistic expectations about the cases-solving procedure. Non-viewers of TV forensic-based series and forensic scientists were not affected to the same extent. All participants evaluated DNA evidence as to the most significantly stronger than other types of evidence in forensic science. Therefore, an increasing pressure on law enforcement personnel and investigators to collect DNA at crime scenes, regardless of whether it was relevant to the case was also observed. Further research concerning the CSI effect is needed, in order to identify and reduce the impact of unscientific parameters on the management, evaluation and judicial use of forensic evidence.

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Forensic microbiology and bite marks: a systematic review

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ABSTRACT

The forensic role of microbiology in bite mark analysis as evidence in a court of law has not yet been explored, as the analysis of bite marks is mostly morphology-based. The aim of this systematic review is to investigate if the analysis of the oral microbiota may be helpful as a complementary forensic tool. Articles were searched on the PubMed database, using predefined data fields and keywords. The final selection included a total of 6 papers (out of 42). Our results indicated that the *Streptococcus* genus is a key player in the analysis of bite mark microbiology from a forensic perspective and its genomic analysis may facilitate the association of a bite mark to the perpetrator. However, much more research is still needed before this forensic strategy can be applied in real scenarios. There is a need to optimize and standardize the methods of microbiome analysis and to determine several factors that may influence the results, such as the frequency of bacterial genotypes in the human population and the temporal stability of the oral microbiome on human skin.

INTRODUCTION

A bite mark can be described as a physical alteration on the skin or other materials caused by teeth pressure.[1] As physical evidence, bite marks are analysed using morphological aspects, and a comparison between the suspect's dentition and the mark inflicted on the victim's skin is performed.[1-6] The analysis of the bite mark as biological evidence may explore the oral DNA left by the biter.[7] This DNA may originate from host cells (human DNA) or from the oral microbiome. Despite being very useful for human identification, human DNA bite mark analysis can be extremely difficult due to DNA's rapid degradation by nucleases present in the saliva or on the skin.[1,2,5,6] In comparison to human DNA, microbiome analysis can offer several advantages, namely due to microbial DNA ubiquity and diversity,[8] greater resistance to degradation (due to their cell wall and biofilm), and to the potential to distinguish monozygotic twins.[9] For these reasons, there is a growing interest in microbiology in forensic science, particularly in human identification. Specifically, oral microbiome may have a great potential in forensic investigation since it presents high diversity and quantity of organisms, high inter-personal variability and intra-personal stability, and also because saliva is an easily accessible biological fluid. Oral microbiota includes more than 700 species of

microorganisms, where the *Streptococcus* is the most prevalent genus, found in saliva and soft tissues.[10] The species *Streptococcus mitis*, *Streptococcus sanguinis,* and *Streptococcus oralis* are the most common initial colonizers of the teeth biofilm.[1]

Forensic microbiology uses microbiological methods in criminal and medico-legal investigations by analysing and interpretating microbial evidence.[1,11] In bite mark microbiology, it is intended to collect and amplify microbial evidence on the victim's skin to associate the biter to the bite mark, and, perhaps, use it as additional evidence in a court of law. To evaluate the viability of the use of microbial DNA as forensic evidence in crimes involving bite marks, we have reviewed experimental trials that addressed the collection of microbial DNA from human bite marks.

This systematic review aims to provide an up-to-date clear and objective assessment of how microbiology can assist the criminal investigation into the perpetuator's identification when a victim presents with a bite mark.

MATERIAL AND METHODS

This review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol[12] and was registered on the PROSPERO (International Prospective Register of Systematic Review - Centre for Reviews and Dissemination University of York) website with the registration number 2022 CRD42022292232.

The scientific articles chosen for this review were selected from PubMed database between October and December 2021, with its query ((forensic or forensics) AND ("microbiology" or "microbiome" or "*Streptococcus*" or "microorganism" or "microbes" or "microflora" or "microbial" or "bacteria" or "fungi" or "yeast" or "*Candida*") AND ("bites" or bitemark or "bite mark"). This query intended to respond to the following PICO question: In human victims presenting human bite marks, how can microbiology assist forensic science through the analysis of the transmission of microorganisms between the bite mark and the oral cavity of the aggressor as a tool for the identification of the biter.

First, articles that corresponded to reviews, systematic reviews, and meta-analyses were excluded. The selection of articles was made progressively, starting by reading the title, then the abstract, and, finally, by reading the full article. The eligibility assessment of each article was made independently by the three authors and disagreements were resolved by consensus, excluding all those who did not meet the established inclusion criteria.

Data were extracted from each primary study by the review authors and organised into a table, including title, authors, and year of publication, with the variants defining the population (number and type of participants), the type of study, the main objective, the intervention (microbial group assessed and method of analysis), and the outcome (major findings and quantitative results).

For risk of bias analysis in the individual studies, the Joanna Briggs Institute-Faculty of Health and Medical Sciences at the University of Adelaide protocol was followed.[13] This analysis was conducted by the authors separately, and articles were classified as to whether the risk of bias is "no", "yes" or "unclear" for each question present in the protocol, in all included articles. For each yes, a point was given, and articles scoring 6 or over were selected for this review.

RESULTS AND DISCUSSION

A total of 42 articles was obtained with the database search but only 6 were included for analysis and data extraction (Fig. 1). All articles presented a low risk of bias analysis and, therefore, were included in the review (Supplementary Table 1). The 6 articles selected were experimental studies published in English, 4 papers used volunteers performing self-inflicted bite marks[1,2,5,6] and 2 papers used only saliva samples.[3,4] The ability to recover and amplify microbial DNA and, subsequently, its reliability to distinguish between individuals and match each sample to the perpetuator was evaluated in the 6 studies, in which the genus *Streptococcus* was the selected microbial group. However, the methods of analysis vary between articles and each study presented extra specific objectives (Table 1).

In the study by Kennedy et al.[1] bite marks and teeth (upper and lower anterior teeth) were swabbed from 16 volunteers who self-inflicted bites on their upper arms. DNA was directly extracted, purified, amplified, and pyro-sequenced for 16S rRNA gene; 16S–23S rRNA intergenic spacer region (ITS); endoribonuclease P (rnpB); and RNA polymerase betasubunit (rpoβ) loci. The results demonstrated that the

analysis of the rpoβ is more likely to correctly distinguish samples than the pyro-sequencing of streptococcal 16S ribosomal RNA (16S rRNA) or the 16S-23S intergenic spacer (ITS). The 3 streptococcal DNA regions analysis to distinguish the participants showed that the probability of matching correctly between the bite marks and the teeth was 92% for ITS, 99% for 16S rRNA, and 100% for rpoβ, with a confidence interval of 95%. The species identified in bite mark and teeth samples and in all the 3 loci were *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus cristatus*. The species *S. mitis* was the most prevalent on the teeth surface, being responsible for the difference in values obtained, since the ropβ primers are specific to this bacterium while the 16S rRNA and ITS primers are comprehensive to other species. That said, the robustness of ropβ is due to the ability to distinguish participants by the exclusive analysis of a species with profound genotypic diversity, presenting a specificity of 100%.

Figure 1. Flow diagram leading to selection of the articles

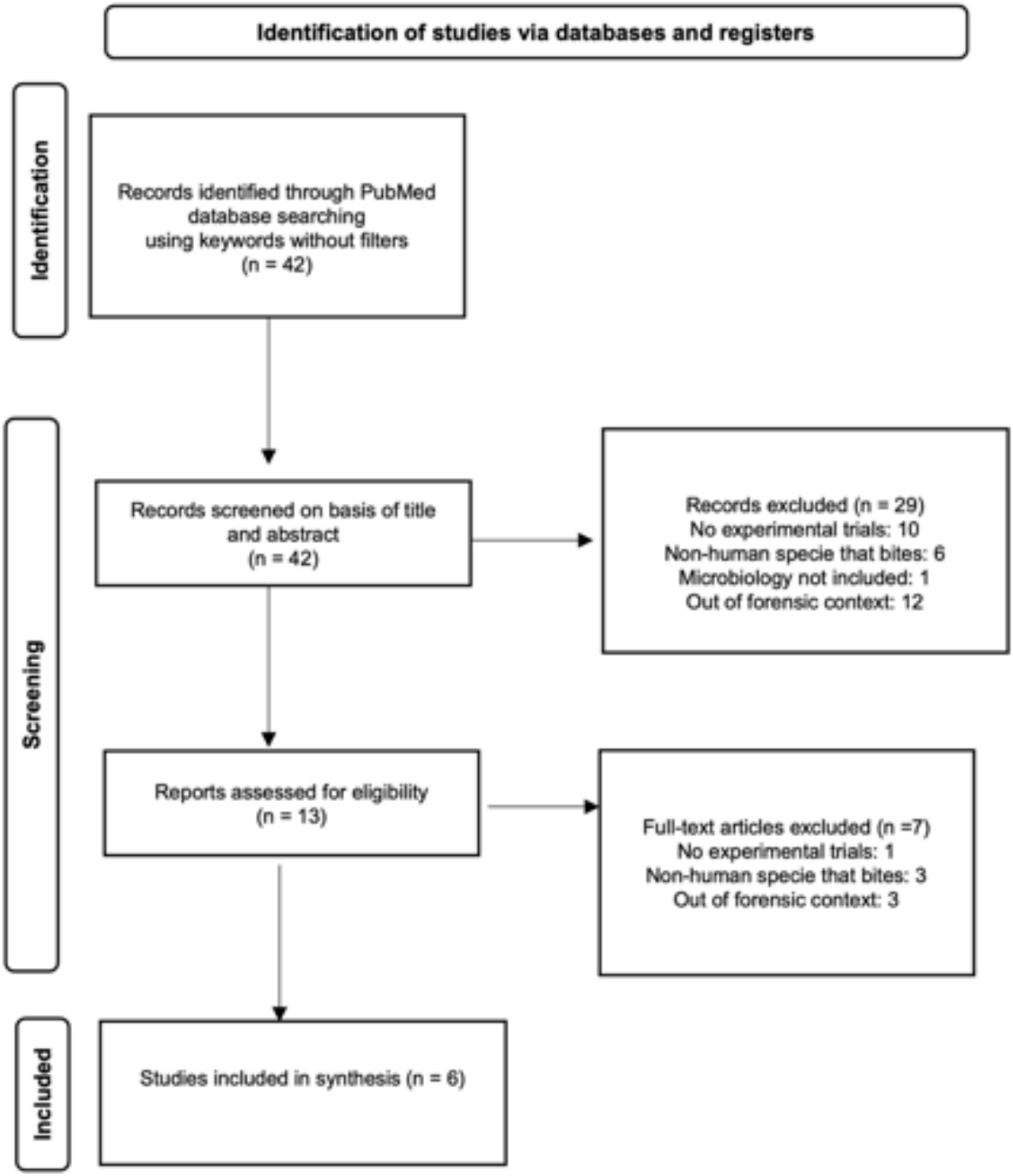

Table 1. Summarisation of the information obtained from the articles under analysis

Article	Participants	Species that bites	Main goal of study	Microbial group evaluated	Method of microbial assessment	Major findings	Identification
Kennedy et al.[1]	16 adults	Human	Capability of 3 genomic regions of streptococcal DNA to discriminate between participant samples.	*Streptococcus*	Pyro-sequencing of streptococcal 16S ribosomal RNA (16S rRNA) gene, 16S–23S intergenic spacer (ITS) and RNA polymerase beta subunit (rpoB)	Streptococcal DNA is capable of matching a bite mark to the teeth responsible. The probabilities of correctly distinguishing matching and non-matching teeth samples were 0.92 for ITS, 0.99 for 16S rRNA and 1.0 for rpoB. None of the skin control samples obtained prior to biting generated detectable amplicons using the streptococcus-specific fusion primers. Identified oral streptococci (*S. mitis, S. oralis and S. cristatus*).	Yes
Rahimi et al.[6]	8 volunteers	Human	Matching oral streptococci recovered from human bite marks with those from teeth.	*Streptococcus*	AP-PCR	400 colonies were analysed to yield a total of 106 genotypically distinguishable streptococcal strains. Between 8 and 23 genotypes recovered from each participant. The 2 most dominant genotypes from each individual composed more than 35% of all isolates from that site. Between 20% and 78% of bacterial isolates recovered at the start of the study were genotypically matched with isolates recovered 12 months.	Yes
Hsu et al.[5]	24 adults	Human	Explore the feasibility of directly amplifying bacterial DNA from bite marks from comparison with that from teeth.	*Streptococcus*	16S rDNA PCR	Streptococcal DNA can be amplified directly from bite marks. 8 of 15 bite mark amplicon patterns were matched to the corresponding incisor samples by correlation coefficients greater than 0,70 with one pairing scoring 1.0. The highest correlation between incisor amplicon profiles as 0,57, giving an indication of the level of co-incidental similarity between unrelated profiles.	Yes
Borgula et al.[2]	8 volunteers	Human	Matching *Streptococcus* isolates recovered from bite marks with the incisor teeth.	*Streptococcus*	DNA fingerprinting	Bacteria can be recovered from bite mark impress on human skin and could be matched exclusively to the teeth perpetrator. Oral streptococci recoverable decreased 30.8%/h over the first 3 hours and 5-6%/h between 6 and 24h. Manual rubbing decreased -80%; moderate physical exertion for 10 minutes decreased more than 95%. Streptococci were not recovered from control fabric squares that had not been bitten. 58 genotypes isolated from the teeth and 54 from bite marks. 60 distinct patterns were identified. Between 2 and 8 genotypically distinguishable strains were isolated from each tooth and bite mark.	Yes
Elliot et al.[4]	Saliva (2 donors)	Human	Distinguishing between oral isolates of S. *salivarius* from 2 persons.	*Streptococcus salivarius*	Pyrolysis mass spectrometry (PY-MS)	Differentiation of *S. salivarius* at strain level according to the origin of the isolate when we have different individuals. 78 spectra were generated. Major cluster which includes the three reference strains of *S. salivarius*	Yes
Brown et al.[3]	Saliva (1 donor)	Human	Determining if is possible to use "fingerprint" identification of oral bacteria.	*Streptococcus*	M-S agar plate	Suitable "fingerprint" typing scheme for oral bacteria may provide evidence relating to the identity of a suspect in such cases. Total counts decreasing at a rate of 44.8%/h and for *S. salivarius* 43.9%/h. Recoverable streptococci after 6 hours are still large	Yes

The study by Rahimi et al.[6] intended to evaluate the efficiency of AP-PCR (Arbitrarily primed polymerase chain reaction) to identify the biter, to assess the natural distribution of oral *Streptococcus* genotypes, and to examine their recoverability after 12 months. In this study, one of the 8 volunteers, whose identity was withheld from the laboratory investigator, and one extraneous individual, firmly bit their own upper arms with sufficient force to produce indentations that lasted for at least 10 minutes. The bite marks were covered with loose clothing for 6 hours and the area impacted by the mandibular incisors was swabbed with a moistened cotton-tipped applicator for further DNA analysis. Bacterial DNA samples from the 8 volunteers were obtained by swabbing the lower incisors incisal surface. All samples were cultured, and the Streptococcal genotypes were obtained from 50 randomly selected bacterial colonies from each sample. The analysis of the streptococcal DNA allowed to distinguish 106 genotypes, and, in each individual, 8 to 23 distinct strains were found. The bacteria were unambiguously matched to the biter by comparing the amplicon profiles with those from the 8 participants. In contrast, bacteria from an additional bite mark (extraneous individual) could not be matched to any of the 8 participants. The temporal stability of the *Streptococcus* genotypes was also evaluated, and an additional sample of the incisors from each participant was collected for analysis after 12 months; results showed that 20% to 78% of the catalogued bacterial genotypes were recovered after this period. Moreover, throughout the study period, none of the bacterial genotypes was shared between participants. This study demonstrated that the AP-PCR method facilitates a faster analysis of a large number of bacteria with no evident loss of resolution presenting discriminating power to be used in a forensic context. This approach to bite mark analysis may have an immediate application for individual identification, linking a suspect to the crime, within a limited number of individuals.

The study by Hsu et al.[5] intended to explore the consistency of the direct amplification of bacterial DNA recovered from the bite mark for comparison with oral samples. The streptococcal DNA was obtained from self-inflicted lesions by each of the 24 participants (after 3 hours and from the lower incisors), from unbitten control sites, adjacent to the bite marks, and from the lingual surfaces and lower incisors incisal surfaces of the biters. The Streptococcal DNA was amplified by PCR using primers specific for streptococcal 16S rDNA. The comparison of amplicon profiles was done by denaturing gradient gel electrophoresis (DGGE). Amplicon patterns generated from bite marks with 6 or more bands of DNA were compared with the incisors, where 8 (out of 15) coincided with the corresponding incisor, with a correlation coefficient greater than 0.70. This study provides support for a microbiologically based approach to the analysis of bite marks, using streptococcal DNA amplified directly from the bite mark.

The work of Borgula et al.[2] aimed to evaluate the feasibility of recovering oral *Streptococcus* from bite marks on human skin and clothing and the reliability in the correspondence to the *Streptococcus* collected from the incisors responsible for the bite, using genomic comparison. The samples were collected from self-inflicted lesions in the arms of the 8 volunteers, from various fabrics, and from the biter's lower incisors, cultured and the genomic profiles of the recovered bacteria analysed by DNA "fingerprints". After their analysis, it was concluded that recoverable oral streptococci of the skin decreased exponentially over time, namely by 30.8% per hour in the first 3 hours and 5% - 6% per hour between 6 and 24 hours after the bite. Of the 8 volunteers, 58 *Streptococcus* genotypes isolated from the teeth and 54 *Streptococcus* genotypes from the bite mark were compared, and, from each individual, it was possible to distinguish between 2 to 8 chains of each bite mark and dental source. It also demonstrates oral bacteria can be recoverable from the bite marks imprinted on the skin for up to 24 hours. Due to the extreme genotypic diversity of oral streptococci, the microorganisms recovered from the bite marks can correspond exclusively to the responsible tooth in each of the 8 samples, indicating that this approach can support the identification of the suspect of a crime, involving a bite mark.

In the study by Brown et al.,[3] the possibility of recovering up to 6.25h *Streptococcus salivarius* from ten-microlitre aliquots of whole saliva applied to human skin was evaluated. The saliva was plated in a selective medium, Mitis-Salivarius agar, to identify these bacteria and, subsequently, establish the time that *S. salivarius* can be recovered on human skin. The results of these

experiments showed a decrease of 44.8% per hour in the total number of *Streptococcus* and 43.9% per hour in *S. salivarius*; however, after 6 hours there is still a large amount of recoverable microbial material. Thus, the establishment of a suitable fingerprint typing scheme for oral bacteria may provide evidence relating to the identity of a suspect in such cases.

The study by Elliot et al.[4] had as its main objective to evaluate the applicability of Pyrolysis mass spectrometry (Py-MS) to distinguish samples of isolated *Streptococcus salivarius*, obtained from saliva samples of 2 different individuals. After collecting the saliva samples, the bacteria were cultured and isolated and submitted to Py-MS, where 78 spectra were obtained. This allowed authors to conclude that the analysis of the samples of *S. salivarius* by this method was able to distinguish between 2 different individuals.

Overall, all selected studies have shown that the analysis of the oral microbiome, particularly the genus *Streptococcus*, has the potential to be used in a forensic investigation, since, in theory, it is possible to match the bacterial profile from the bite mark found on human skin with that obtained from the teeth, and, therefore, connect an injury to the aggressor. In addition, it was possible to estimate the decrease in the recovery of the oral *Streptococcus* in a given period of time, and the rate of decay can exceed 30% in the first 6 hours,[2,3] demonstrating the importance of a rapid collection and storage. Moreover, it was also shown that, if the samples were collected and packaged correctly, the genotypes can be analysed after 12 months, demonstrating temporal stability of the oral streptococci.[6] Notwithstanding, further research should be done with a significantly higher number of participants.

The genus *Streptococcus* belongs to the most predominant classes in the oral microbiota, with a presence of about 20% in the salivary microbiota and 15% in plaque, and presenting a high genetic variability.[14] These reasons justify the focus of the studies analysed in this review on *Streptococcus* genus with interesting positive results. However, this may constitute also a limitation since the oral microbiota includes several other genus and species that could be explored as forensic tools as well.

Regarding the methods, 2 older papers only cultured samples in Mitis-Salivarius agar,[3,4] 2 papers compared the genotype profiles of isolated strains obtained after culturing samples in Mitis-Salivarius agar,[2,6] and the other 2 papers extracted streptococcal DNA directly from samples without culturing.[1,5] Previous studies have stated that culture-independent molecular methods show more promising results, better demonstrating the composition and variety of the oral microbiota,[15] since bacterial diversity in most environments is severely underestimated in surveys with culture-based techniques.[16] In many natural environments, less than 1% of organisms are culturable.[17] Due to the significant effort put into culturing oral bacteria, it is thought that about 50% of oral bacteria have been cultivated.[18] Therefore, in the studies where culture-dependent methods of analysis were used,[2-4,6] the results may not fully identify and characterise all the microbiota present in the samples, representing a limitation of these studies.

The different studies used different methods for the genotyping analysis of *Streptococcus*, namely amplification of specific genes (including 16S rRNA) and direct pyro-sequencing,[1] DGGE of 16S rRNA,[5] AP-PCR,[6] or whole genomic "fingerprinting" using several restriction endonucleases.[2] In a forensic context, most of samples have relatively low bacterial levels and require highly sensitive methods,[19] so it is, therefore, necessary to adopt standardised lab protocols (collection and analysis of samples)[20,21] and bioinformatics work,[22] enabling the removal of the biases associated with different extraction protocols, PCR reactions, and sequencing platforms[19] and to integrate the information and allow easy communication across studies.[23] Interesting to note, the study by Kennedy et al.,[1] showed that, in comparison with 16S rRNA and ITS, ropβ analysis appear to be the most robust method with the highest specificity. So, future studies should further explore these genetic markers as a possible relevant tool in bite marks analysis.

The studies focusing only on the saliva analysis[3,4] also demonstrated that saliva *Streptococcus* presents a relevant inter-individual variability regarding the streptococci profile, important for the forensic application of this microbial oral group. However, these older studies presented a significant lack of sensibility in species discrimination. Notwithstanding, the more recent studies using more discriminatory genetic tools also demonstrated a high discriminatory rate in what concerns perpetrator identification,

despite the reduced scope of participants evaluated in each study. Therefore, future studies with a larger number of individuals are necessary to validate oral *Streptococcus* as a relevant forensic tool.

Despite the positive results obtained, the studies also share several limitations that may not reflect the real conditions in which this analysis may be used, since there are factors, such as body lotions, antibacterial mouth washes, or body secretions, that can affect human microbiota, both on skin and oral cavity.[2] Also, in crime situations, some common behaviours associated with the victims, such as rubbing or washing the skin,[24] may negatively influence the survival of the bacterial community in the injuries that are inflicted on them during biting.[2]

There were some limitations of this study, namely in what performing a metanalyses is concerned. In fact, as the 6 selected studies had different goals and different methodologies, this kind of statistical approach was not an option.

CONCLUSIONS

This systematic review highlights that the microbiological evidence taken from bite marks may provide important information in the forensic investigation due to its ability to match the bacteria in the oral cavity, in particular from *Streptococcus* genus, with the bacteria recovered from the biter's mouth. However, much more research is still needed before this forensic strategy can be applied in real scenarios. There is still a need to optimize and standardize the methods of analysis and to determine several factors that may influence the results, such as the frequency of bacterial genotypes in the human population and the temporal stability of the oral microbiome in human skin.

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Supplementary Table 1. Risk of bias analysis

	1. Were the criteria for inclusion in the sample clearly defined?	2. Were the study subjects and the setting described in detail?	3. Was the exposure measured in a valid and reliable way?	4. Were objective, standard criteria used for measurement of the condition?	5. Were confounding factors identified?	6. Were strategies to deal with confounding factors stated?	7. Were the outcomes measured in a valid and reliable way?	8. Was appropriate statistical analysis used?	Overall appraisal
(Kennedy et al., 2012)	Yes	Yes	Yes	Yes	No	Not applicable	Yes	Yes	Include
(Rahimi et al., 2005)	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Include
(Hsu et al., 2012)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Include
(Borgula et al., 2003)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Include
(Brown et al., 1984)	Yes	Yes	Yes	Yes	No	Not applicable	Yes	Yes	Include
(Elliot et al., 1984)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Include

Note: Articles were classified as to whether the risk of bias is "no", "yes" or "unclear" for each question present in the protocol, in all included articles. For each yes, a point was given, and articles scoring six or over were included in this review.