Human Sex determination from the dental pulp tissue using Polymerase Chain Reaction (PCR)

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KEYWORDS
Identification,
Odontology,
dental pulp,
PCR,
SRY gene.

ABSTRACT

Human identification means the determination of a person's individuality. Various methods are employed for sex determination analysis in forensic investigations including visual, clinical, microscopic, and advanced techniques. DNA is the molecule of choice for forensic analysis. Forensic odontology is a fast-growing and promising branch of forensic medicine, it is a vital and integral part of forensic science that is most widely utilized for solving many crimes. The current study aims to assess the reliability of using the teeth pulp tissue samples for sex determination regardless of the type of teeth (milk or permanent), using Multiplex PCR targeting the SRY protein and AR genes in male and female samples. Results: The PCR analysis on blood samples confirmed that these blood samples belonged to 42 males and 33 females. SRY gene and SRY protein on the Y chromosome was shown at 778bp and 254bp respectively in 42 dental pulp tissue samples, while the androgen receptor gene (AR) on the X chromosome (AR) was at 293bp in all cases. 100% of males showed negative Barr body with no false negative result, while 12.2% of females didn't show Barr body in dental pulp tissue samples and 87.8% showed positive Barr body in dental pulp tissue samples. sensitivity and specificity of the Barr body test on dental pulp tissue samples stained by H&E stain were 87.8% and 100% respectively. Conclusion: Dental pulp tissue is a significant source of sex determination.

Introduction

Human identification is the act of identifying a person. It occurs frequently in everyday life, in both civil and criminal instances (Kumar et al., 2012).

Sex determination is often considered one of the simplest tasks in the forensic investigation as the genitalia can directly suggest the sex of the individual. However, situations involving intersex, bodies in an advanced state of putrefaction, mutilated, fragmentary, and skeletonized remains can make the question of sex discrimination exceedingly difficult (Dey and Kapoor, 2015).

In forensic investigations, a variety of techniques are used to determine sex, including optical, clinical, microscopic, and other advanced methods. The strongest part of the human body is the teeth, which can resist powerful explosions and are unharmed by such incidents (Hinchliffe, 2011).

Forensic odontology is a fast-growing branch in forensic medicine, it is most widely utilized for sex identification and can aid in solving many crimes (Nambiar et al., 2014). In large-scale disasters, it is crucial for identifying people, especially when there are fires, explosions, decayed remains, or skeletonized bodies (Mayall et al., 2013).

Advanced approaches for analyzing sex determination have been shown to be more precise and produce reproducible results. DNA is the molecule of choice for forensic

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examination because of its sensitivity, genetic continuity, and discrimination (George et al., 2013). For instance, sex determination analysis uses DNA analysis for male- and female-specific sex-typing markers in X and Y chromosomes (Hemanth et al., 2008).

The SRY gene is widely used in the forensic examination based on the fact that it has been successfully identified in a variety of samples, including blood, saliva, teeth, and even epithelial cells attached to toothbrush bristles (Reddy et al., 2011).

Since the sex genome differs by the presence of the Y chromosome in males. Hence the sex- determining region Y (SRY) detection would distinguish a male DNA sample from a female one, as female lacks this SRY gene (Drobnic, 2006).

The aim of the present study is to assess the reliability of using the teeth pulp tissue samples for sex determination for forensic purposes.

Patients and Methods:

Patients:

The present study was a blinded study conducted on 75 cases of both sexes (42 males and 33 females), their age ranged from 5 to 68 years, they arrived at different dental clinics and hospitals complaining of dental problems. All patients were evaluated by dentists. All of the study participants gave written informed consent. It was approved by Ethical Committee (number: FORE 7-4).

Exclusion criteria:

- Patients on hormonal or immuno-suppressive therapy.
- Patients with severely decayed teeth, dental abscess, or necrotic teeth pulp.

Methods:

A sheet was created including personal history (name, age sex, etc.), medical history, dental history, history of other diseases (diabetes, blood disease, hypertension, etc.).

Two types of tissue from each participant were chosen in this study: dental pulp tissue, and blood. Two laboratory tests were chosen to evaluate their sensitivity in human sex determination. Polymerase Chain Reaction (PCR) was done on whole blood and dental pulp tissue samples (of molar and pre-molar teeth) of male and females. Barr body test on dental pulp tissue of both human sexes.

Teeth sample preparation:

Sample preparation was done according to Kholief et al. method (Kholief et al., 2017). Pulp extirpation and storage were done according to Tilotta et al. method (Tilotta et al., 2010), and stained using hematoxylin and eosin stain according to Murugesan method (Murugesan et al., 2018).

DNA typing from blood and dental pulp tissue:

Genomic DNA from a total of 75 samples was isolated following standard protocols.

DNA extraction: using QI amp DNA Min Kit. Polymerase Chain Reaction (PCR): DNA Primer. Primers are used to amplify the SRY, SRY protein, and androgen receptor (AR) genes (Table 1).

The results were interpreted as follows:

SRY gene was detected by the presence of a 778bp fragment and AR gene was detected by the presence of 293 bp fragment, while 254 bp fragment was indicative of the human Y chromosome male-specific region (Y protein gene).

Table (1): Polymerase Chain Reaction (PCR): DNA Primers sequences.

Gene	Primer Sequence (5′→3′)	Bank accession number
SRY gene (Mohammed and Tayel, 2005)	Forward 5'-GGTGTTGAGGGCGGAGAAATGC-3' Reverse 5'-GTAGCCATTGTTACCCGATTGTC-3'	NC_000024.10
Androgen receptor gene (AR) (Mohammed and Tayel, 2005)	Forward 5'-CTCTGGGTTATTGGTAAACTTCC- 3' Reverse 5'-GTCCAGGAGCTGGCTTTTCCCTA- 3'	NC_000023.11
SRY protein gene (Skaletsky et al,2003) ;(Settin et al.,2008) ; (Osman et al.,201٤)	Forward 5'-CATGAACGCATTCATCGTGTGGTC 3' Reverse 5'-CTGCGGGAAGCAAACTGCAATTCTT 3'	NC_000024.10

Statistical Analysis:

The SPSS application version 22 for windows was used for the statistical analysis of the data (Kirkpatrick and Feeney, 2014). Data are shown as mean, range, frequency, and percentage. Sensitivity, accuracy, and specificity were tested on a VBV according to Baratloo et al. (2015). A probability of P less than 0.05 was considered statistically significant.

Results:

The 75 individuals in the current study were 42 males and 33 females, whose ages ranged from 5 to 68 years, and were seen at various dental clinics and hospitals. According to the type of teeth of the studies group, 9.3% of teeth samples were milk teeth, while 90.7% were permanent teeth (Figure 1).

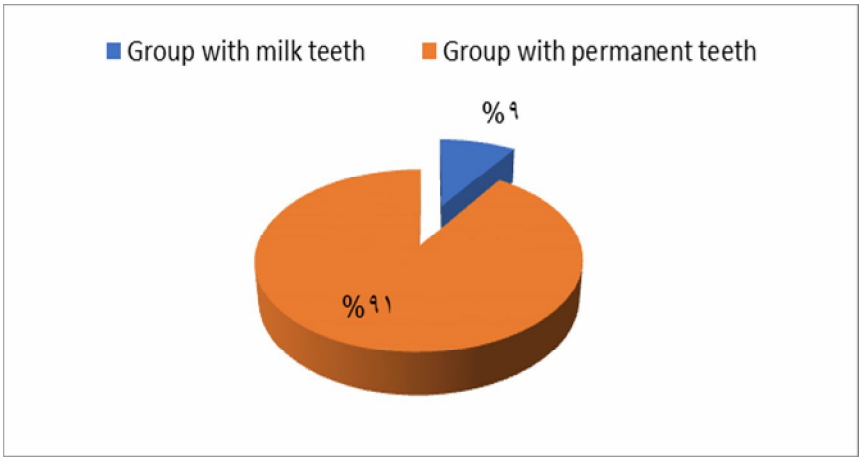

Fig. (1): The distribution of the studied cases according to the teeth type.

All 75 participants had blood sample drawn for PCR, according to the 100 base pair ladder gel electrophoresis, the SRY gene was detected at 778 base pairs, the SRY protein was detected at 254 base pairs (in all 42 male participants), and the AR gene was detected at 293 base pairs in both male and female cases. This exhibits a sex determination that is 100% accurate (Table 2 and Figures 2 & 3).

Table (2): Sex determination by PCR analysis on blood samples.

Gel electrophoreses	Blood PCR	
	Male (n=42)	Female (n=33)
SRY gene	Base pair (778)	None
SRY protein	Base pair (254)	None
AR gene	Base pair (293)	Base pair (293)

n: number), PCR: Polymerase Chain Reaction, AR: androgen receptor genes, SRY: sex determining region of the Y chromosome.

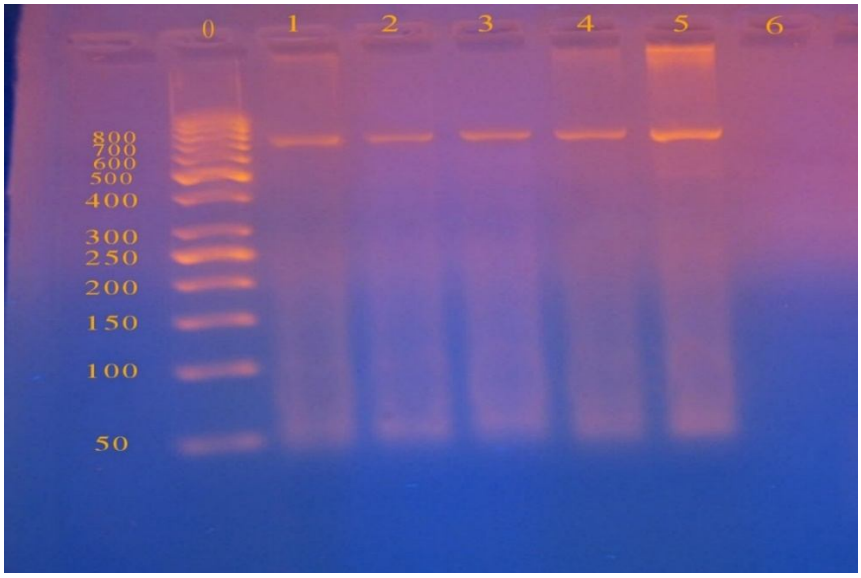

Fig. (2): shows the PCR products of amplification of the SRY gene of Y chromosome from genomic DNA extracted from fresh blood of male cases.

It reveals that:

Lane 0: DNA marker with 100 base-pair.

Lane 1, 2, 3, 4, 5: bands of PCR products of Y-chromosome in male samples. SRY gene shown at 778 base-pair (white arrow).

Lane 6: negative control sample (nuclease free water).

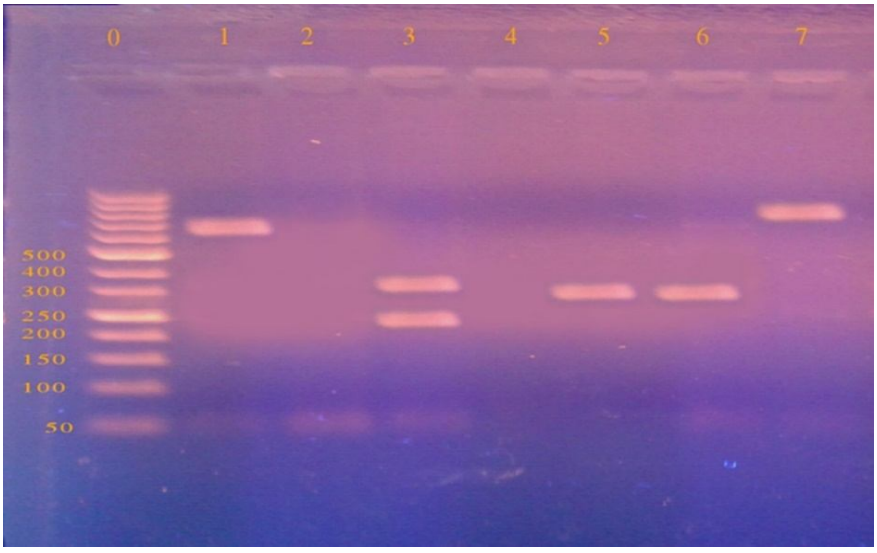

Fig. (3): shows electrophoresed PCR products of amplification of the SRY gene, SRY protein of Y chromosome and AR gene on X chromosome from genomic DNA extracted from fresh blood samples of male and female cases.

Lane 0: showing 100 bp DNA ladder.

Lane 1,7: shows SRY gene (778bp) on Y chromosome indicating male sex.

Lane 2,4: negative control sample (nuclease free water).

Lane 5, 6: showing amplified AR gene on chromosome (293bp) indicating female sex.

Lane 3 showing both SRY gene (254 bp) and AR gene (293) on X chromosome, indicating male sex.

By analysis of the dental pulp tissue of all participants and on visualization on 100 bp (base pair) DNA ladder, the SRY gene was detected at 778 base pairs, the SRY protein was detected at 254 base pairs in all 42 male participants, while the AR gene (on x chromosome) was detected at 293 base pairs in both male and female cases (Table 3 and Figures 4 & 5).

Table (3): Sex determination from dental pulp tissue using the PCR.

Gel electrophoreses	Dental pulp tissue	
	Male (n=42)	Female (n=33)
SRY gene	Base pair (778)	None
SRY protein	Base pair (254)	None
AR gene	Base pair (293)	Base pair (293)

n: number, PCR: Polymerase Chain Reaction, AR: androgen receptor genes, SRY: sex determining region of the Y chromosome.

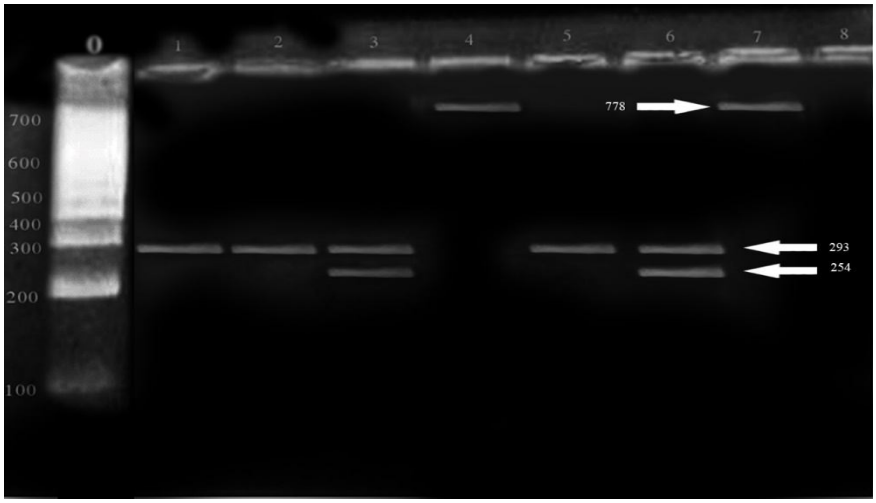

Fig. (4): shows the PCR products of amplification of the SRY gene of Y chromosome from genomic DNA extracted from dental pulp tissue samples of male and female cases (2% agarose gel electrophoresis stained with ethidium bromide):

Lane 0: showing 100 bp DNA ladder.
Lane 1, 2, 5: showing amplified AR gene on chromosome (293bp) indicating female sex.
Lane 3 and 6: showing both SRY gene (254 bp) and AR gene (293) on X chromosome, indicating male sex.
Lane 4, 7: shows Sex SRY gene (778bp) on Y chromosome indicating male sex.
Lane 8: negative control sample.

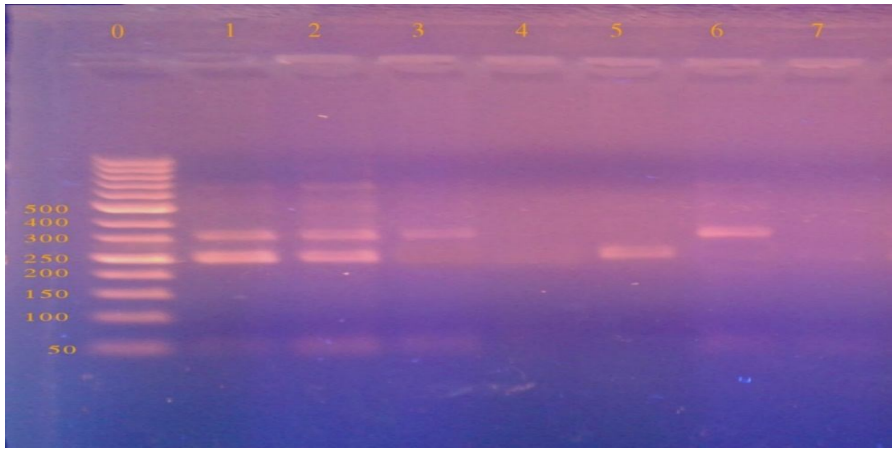

Fig. (5): shows the PCR products of amplification of the SRY gene, SRY protein of Y chromosome, AR gene on X chromosome from genomic DNA extracted from dental pulp tissue of male and female cases.

Lane 0: shows 100 bp DNA ladder.
Lane 1, 2: shows both SRY protein on Y chromosome (254 bp) and AR gene on X chromosome (293bp) indicating male sex.
Lane 3: showing X chromosome (293 bp) indicating female sex (white arrow).
Lane: 4 and 7: show of negative control.
Lane 5: Y chromosome (254 bp) (yellow arrow).
Lane 6: shows X chromosome (293bp).

On Sex determination using the Barr body test on the dental pulp tissue stained by H&E, there was no Barr body was shown in all males' samples (100%) with no false negative results, while Barr body was shown in 87.8% of females' dental pulp tissue samples. There is a highly statistically significant relationship between sex and Barr body on dental pulp tissue samples ($p<0.01$) (Table 4 and Figure 6).

Table (4): Barr body test on dental pulp tissue stained by hematoxylin and eosin.

Sex	Barr body in the dental pulp tissue		Total
	Absent	Present	
Male (n= 42)	42 (100%)	0 (0%)	42
Female (n=33)	4 (12.2%)	29 (87.8%)	33
Total (n=75)	46 (61.3%)	29 (38.6%)	75

n: number

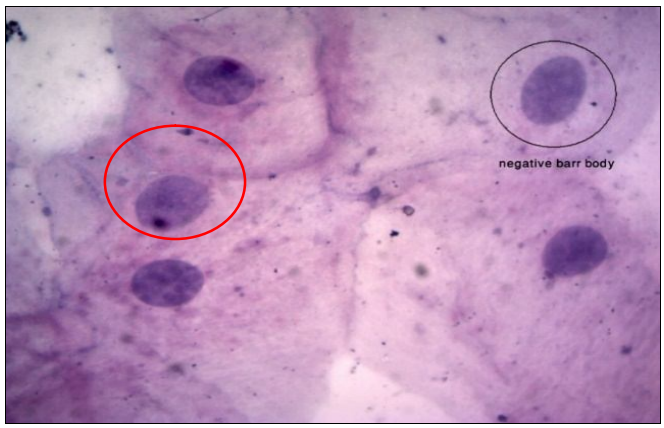

Fig. (6): Cytological smear of female dental pulp tissue stained with H&E showed fibroblast with condensed Barr chromatin in the periphery of the nucleus (red circle) and one cell show negative Barr body (100X, oil immersion) (black circle).

There are no positive Barr body cells in slides of male cases. The mean number of Barr body positive cells stained by H&E on dental pulp tissue samples in female cases was 30.4 and ranged from 3-62 /100 cells (Table 5).

Table (5): Mean number of Barr body positive cells on dental pulp tissue samples stained by hematoxylin and eosin.

	Mean	Minimum	Maximum	Total number
Male	0	0.0	0	42
Female	30.4	3	62	33
Total	13.4	0	62	75

Studying the association between tooth type (milk or permanent) and the presence of Barr body, demonstrates that there is no statistically significant relationship between Barr body on dental pulp tissue and the type of teeth (milk or permanent) (p>0. 05) (Table 6).

Table (6): Barr body on dental pulp tissue according to the type of teeth (milk or permanent).

Teeth type	Barr body on dental pulp tissue		Total	Chi square	p value
	Negative	Positive			
Milk teeth	4 (57.1%)	3(42.8%)	7	0.057	p >0.05 (NS)
Permanent teeth	42(61.7%)	26(38.2%)	68		
Total	46(61.3%)	29(38.6%)	75		

*P value.0.05 : non-signifiance (NS)

A discriminant functional analysis of Barr body test on dental pulp tissue samples stained by H&E stain reveals that 94.6% of cases have been correctly identified. Out of 33 females, 87.9% identified by the presence of Barr body on their dental pulp tissue samples, and 100% of males have been correctly identified with the absence of Barr body on their dental pulp tissue samples (Table 7).

Table (7): Discriminant analysis of Barr body test on dental pulp tissue samples.

Sex	Classification results				Total
	Predicted group membership				
	Count	%	Count	%	
Male	42	100%	4	12.1%	46
Female	0	0%	29	87.9%	29
Total	42	56%	33	44%	75
94.6% correctly identified					

Based on the cut-off point, represents the classification of people into males and females (male< 4, female ≥ 4) of Barr body count on 100 cells of dental pulp tissue. 100% of males below the cut-off point regarding the H&E-stained dental pulp tissue, while 87.8% of females above the cut-off point (Table 8).

Table (8): Sex Categorization based on the cut-off point.

Cut- off point	Dental pulp tissue stained by H&E	
	Male n (%)	Female n (%)
< 4	42 (100%)	4 (12.2%)
≥ 4	0 (0%)	29 (87.8%)

n (number), H&E: hematoxylin and eosin

Discussion:

In the process of personal identification by a forensic investigator in the case of explosions, natural disasters, and crime investigations, sex determination becomes the priority (Nagare et al., 2018). Dental pulp tissue is considered to withstand environmental conditions and save its viability for a long time by the protection of dental hard tissue (Hemanth et al., 2008).

Three genes were chosen in this study for PCR - dependent sex determination from blood and dental pulp DNA, sex- determining region of Y chromosome (SRY gene), SRY protein gene and AR gene.

The present study showed that the process of sex determination from blood samples by PCR amplification of Y-chromosome-specific regions: (SRY) gene, SRY protein gene and AR gene is completely successful. The PCR analysis done on blood and dental pulp tissue samples confirmed that these blood samples belonged to 42 males and 33 females.

This is almost agreed with Amornpan et al. (2017) who studied sex determination from bloodstains by PCR analysis and approved that SRY primer showed 100% accuracy on male identification. Also, Naik et al. (2012) determined sex by detecting SRY gene in 20 dental pulp tissue samples with 100% accuracy. Bharath et al. (2019) also studied SRY gene as a male specific marker and 100% accuracy was obtained. The SRY gene did not amplify in any of the female samples in his study.

Gender identification by amplification of SRY gene examined also by Reddy et al. (2011) who studied isolated epithelial cells from toothbrush and revealed 100% sensitivity and 73.3% specificity by amplification of the sex-determining region on the Y chromosome (SRY) gene. Zapico and Ubelaker (2013) reported that sex was determined successfully via PCR from incisors and molar teeth.

Osman et al. (2014) studied the validation of SRY gene marker (254 bp) for use in gender determination, as the presence of SRY gene-specific bands allowed male samples to be differentiated from female samples.

The present study reported that all males (100%) showed negative Barr body with no false negative result, while 87.8 % of females showed positive Barr body in their dental pulp tissue samples stained by H&E. Also, the study revealed that there is a highly statistically significant relationship between sex and Barr body test on dental pulp tissue samples stained by H&E stain. The mean number of Barr body positive cells on dental pulp tissue samples stained by H & E in female cases was 30.4 and ranged from 3-62

/100 cells. There was no statistically significant relationship between Barr body expressed in dental pulp tissue samples (stained by H&E) and the type of teeth (milk, permanent).

These results were like these of Khanna, (2015) who evaluated the relation between the presence of Barr body on dental pulp tissue by light and fluorescence microscope and gender determination, observed that the Barr bodies were most easily recognized after 15 days using the H & E stain (51.4% cells positive).

In contrast, Basnaker and Moolrajani (2016) study found that the prevalence of Barr body in the dental pulp tissue of males ranged from 0-2% whereas it ranged from 19 to 48% in the sample of females.

In accordance with the present study, Galdames et al. (2010) declared that male had no Barr body positive cells. While Das et al. (2004) observed that 24.92% of female pulp cells had positive Barr body.

The present study showed that the sensitivity and specificity of Barr body test on dental pulp tissue samples stained by H&E stain were 87.8% and 100% respectively. False negative results were seen in 21.2% of cases while there are no false positive results. The sensitivity and specificity of the Basnaker and Moolrajani (2016) study were reported to be 96% and 87%, respectively, which is less than this study.

Conclusion

- The tooth pulp tissue is a significant source in sex determination, as it has high reliability, accuracy and, sensitivity in DNA- dependent forensic sex determination. Sex chromosomes can be amplified from dental pulp tissue regardless of the type of teeth (milk or permanent). Using Multiplex PCR targeting the SRY protein and AR genes proved to be efficient in the sex determination of male and female samples.
- Barr body showed high specificity for forensic sex determination from both buccal mucosal smear and dental pulp tissue samples. Although the Barr body test has not shown a high sensitivity and accuracy compared to PCR -dependent sex determination, it can be used as a good positive test for assuring the female sex origin and excluding the male sex. Barr body test is an easy method, low cost, can be done on many tissues, and simple staining technique.

Recommendations:

Further research is recommended: on larger sample size and under different environmental conditions (different temperatures, humidity, pH, and storage media).

Conflict of interest:

The authors declare that they have no conflicts of interest in this research**.**

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تحديد الجنس البشري من أنسجة لب الأسنان عن طريق تفاعل البوليميراز المتسلسل

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المقدمه:

الاستعراف و تحديد الهوية ذو أهمية قصوى للمجتمع ويعني الاستعراف في الطب الشرعي تحديد الهوية الفردية للشخص و يتم استخدام طرق مختلفة لتحديد الجنس في تحقيقات الطب الشرعي . ويعتبر الحمض النووي هو الجزيء المفضل لتحليل الطب الشرعي بسبب تمييزه واستمراريته الجينية وحساسيته. و يستخدم طب الأسنان الشرعي على نطاق واسع لتحديد الجنس ويمكن أن يساعد في حل العديد من الجرائم.ويهدف هذا البحث إلى: تقييم استخدام عينات الدم وأنسجة لب الأسنان لتحديد الجنس لأغراض الطب الشرعي.

وقد طبق هذا البحث علي ٧٥ حاله من المترددين علي عيادات و مستشفيات الاسنان (٤٢ من الذكور و ٣٣ من الأناث) تم فحصهم بواسطه طبيب الاسنان وتم أخذ الموافقة المستنيره منهم. وتم استخدام العينات في تحليل تفاعل البلمرة المتسلسل(PCR) لأكتشاف الجين (SRY) علي كروسوم الذكري Y وأكتشاف الجين علي كروموسوم الأنثوي X . كما تم اخذ عينات من عصب الأسنان: لتحليل جسم بار(Barr body) -تحليل تفاعل البلمرة المتسلسل(PCR) لأكتشاف الجين (SRY) علي كروسوم الذكري Yوأكتشاف الجين ARعلي كروموسوم الأنثوي X.

و قد اظهرت النتائج في نهاية تحليل الحمض النووي ، وبعد مطابقة النتائج مع الجنس الأصلي الأصلية لعينات الدم ولب الأسنان نجاحًا بنسبة ١٠٠٪ في عملية تحديد الجنس باستخدام جين SRY وجين مستقبلات الاندروجين على (X Chromosome (AR وجين بروتين SRY مع عدم وجود نتائج سلبية كاذبة أو نتائج إيجابية كاذبة .

لم تؤثرنوع السن، سواءً كانت موقت أو دائم ، فى استخراج الحمض النووي منها بنجاح كما اتضح من خلال تضخيم جين مستقبلات الأندروجين في جميع العينات محل الدراسة .

أظهر ١٠٠٪ من الذكور جسم بار سلبيا بدون نتيجة سلبية خاطئة ، بينما لم تظهر ١٢,٢٪ من الإناث جسم بار في عينات أنسجة لب الأسنان و ٨٧,٨٪ أظهرن جسم بار إيجابيا في عينات أنسجة لب الأسنان. كانت حساسية وخصوصية اختبار جسم بار على عينات أنسجة لب الأسنان المصبوغه بصبغة H&E ٨٧,٨٪ و ١٠٠٪ على التوالي.