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Full Length Research Paper

Gender determination using primary teeth: A polymerase chain reaction (PCR) study

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The aim of this study was to assess the effect of various environmental factors on the preservation of pulp tissue in primary teeth as a source of DNA and its use for sex determination using polymerase chain reaction (PCR). 120 samples were grouped into 4 groups of 30 teeth each. Group I samples were kept immersed in a bucket of drainage water, group II, III and IV samples were buried in sand taken from seashore, burial ground and desert, respectively for a period of 2 months. Pulp tissue was collected from each sample and DNA was isolated. PCR amplification was performed and sex determination was done by detection of X and Y chromosome-specific alphoid centromeric repeat sequences. In group I, 86% of the samples exhibited correct gender interpretation by PCR amplification. In groups II, III, and IV, all the samples showed correct results indicating a significant difference in scores between group I and the remaining three groups. Teeth stored in dry conditions can serve as a better source of DNA as compared to the teeth stored in moist conditions and co-amplification of both X and Y specific sequences by PCR is a fast, specific, sensitive and reliable method providing sex determination

Key words: DNA, gender, polymerase chain reaction (PCR), forensics

INTRODUCTION

The rights of children and their aspirations are of paramount importance in our demonstration towards an inclusive and equitable society. Investment in the well-being of children is an investment in the future of the country. Eradication of malnutrition and improving general and dental health is receiving topmost priority. At the same time issues relating to child protection are high on every government's agenda.

Child abuse is shrouded in secrecy and there is a conspiracy of silence around the entire subject. Certain kinds of traditional practices that are accepted across many countries, knowingly or unknowingly amount to child abuse. Existing socio-economic conditions also render some children vulnerable and more at risk to abuse, exploitation and neglect. It is time that this is recognized and appropriate remedial measures taken. Lack of empirical evidence and qualitative information on the dimensions of child abuse and neglect makes it difficult to address the issue in a comprehensive manner.

In a study conducted by Ministry of Women and Child Development, Government of India (2007), state-wise break up of overall incidence of physical abuse revealed that in all the 13 states covered under the study, the reported incidence of physical abuse was very high, in fact uniformly above 50%. Annually, large numbers of children go missing and there is little attempt to track them or trace them.

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The global scenario is also not much different as compared to the Indian situation, according to an overview given by the United Nation Secretary General's Study on violence against children, WHO estimated that almost 53,000 children that died in 2002 were due to child homicide (Ministry of Women and Child Development, Government of India, 2007).

In the past, there were many efforts made by numerous professionals to detect and document various cases of child abuse, but unfortunately most of them focused on the children who are living. But definitely there will be a group of children who could have lost their life due to the same barbaric act which went unnoticed, and these unfortunate children also deserve justice as much as the living children do. Determination of the victim's identity is the first step in any kind of forensic investigation and determination of gender is one of the important dimensions of this process. During the past few decades physical evidence has become increasingly important in criminal investigations. Courts often view eye witness accounts as unreliable and biased. Physical evidence such as DNA, fingerprint and trace evidence may independently and objectively link suspects to a crime, disprove an alibi, or develop important investigative leads. Gender identification can also provide valuable information about past human societies, culture and their life styles.

There are no reliable methods available for sex determination in primary teeth using morphometric analysis (Murakami et al., 2000). With the improvement of technology, increased speed, sensitivity and specificity, DNA analysis has revolutionized the field of forensics and PCR method is one of them. Bones and teeth are the only material available in markedly decayed/skeletonized bodies. The most important variables upon which identification and availability of DNA depend are time after death and type of soil (pH, humidity, temp, bacteria, etc) where in the bodies are buried (Dragan, Unpublished).

If found reliable in tissues exposed to such conditions, PCR method of sex determination can contribute immensely in gender identification in forensic as well as anthropological, archeological and paleontological researches.

The aim of this study was to assess the effect of various environmental factors on the primary teeth in the preservation of pulp tissue as a source of DNA and its use for sex determination using PCR.

MATERIALS AND METHODS

Sample selection

A total of 120 non carious primary canines extracted for serial extraction or various other purposes were used as the study samples. Written informed consent was taken from the parents of the children whose teeth were selected for the study (by the co investigator). The teeth showing apical resorption beyond apical 1/3 were not included in the study.

Study groups

The selected samples were grouped into 4 groups of 30 teeth each. Coding and decoding was done by a co-investigator. The samples in group I were kept immersed in a bucket of drainage water (collected from the main sewer channel of the town) for 2 months. The teeth samples of group II, III and IV were buried in sand taken from seashore, burial ground and desert, respectively at a depth of 30 cm for a period of 2 months (Figure 1).

Pulp sample collection

After 2 months, teeth were taken out and washed thoroughly using distilled water. The teeth were then sectioned along the long axis, through the pulp chamber using hard tissue microtome and pulp was recovered (Figure 2A and B). The recovered pulp samples were stored and carried in 100% ethanol in labeled Eppendorf tubes for DNA isolation.

Isolation of DNA from dental pulp

DNA was isolated from dental tissue obtained from the teeth by proteinase-k digestion and phenol chloroform extraction methods (Zeljka et al., 2000). Isolated DNA was then diluted with tris-ethylenediaminetetraacetic acid (TE) buffer. The isolated DNA was then utilized for PCR amplification in Amplitaq DNA polymerase buffer for various thermal cycles.

Electrophoresis and sex determination

The PCR products underwent electrophoresis in 1% agarose gel at 200 V for 1 h. Ethedium bromide staining was performed and amplified bands of X and Y sequences were examined under UV radiation (Figure 3). The sex of a subject was considered to be male when both X and Y specific sequences were detected, but female when only the X-specific sequence was detected.

Universal precautions were taken to prevent cross contamination while handling the samples which included personal protective equipments (gloves, mouthmasks, haedcaps) and PCR was done in specifically designed setup in human genetics department. Disposable devices were preferred wherever possible to rule out cross contamination either from operator DNA or amplicons from previous experiments.

Data analysis was done using Chi square test of independence. Statistical significance was set at 5% level of significance ($p<0.05$). Percentage distribution of positive and negative results was compared among the groups.

RESULTS

In group I (teeth stored in drainage water), 26 out of 30 teeth (86%) showed correct gender interpretation. In groups II, III, IV (teeth buried in sand taken from beach, mud taken from burial ground and sand taken from desert, respectively) all the samples (30 in each group) showed correct results indicating a significant difference in scores between group I and the remaining three groups ($p<0.01$, 99% significance) (Table 1).

Gender wise distribution showed up in group I out of 26 male samples, correct sex determination was possible in 22 samples (73.3%) and 4 samples gave wrong results

Table 1. Comparisons between positive and negative results in different test groups.

Result		Group I	Group II	Group III	Group IV	Total
Positive	Count	26	30	30	30	116
	Within group (%)	86.7	100	100	100	96.7
Negative	Count	4	0	0	0	4
	Within group (%)	13.3	0	0	0	3.3
Total	Count	30	30	30	30	120
	Within group (%)	100	100	100	100	100

Chi-square=12.414, $p<0.05$.

Table 2. Chi-square tests.

Test	Value	df	Asym p. Sig. (2- Sided)
Pearson Chi-square	12414 [a]	3	0.006
Likelyhood ratio	11.514	3	0.009
Linear-by-linear	7.386	1	0.007
Association	120	-	-
No. of valid cases	-	-	-

[a] 4cells (50.0%) have expected count less than 5. The minimum expected count is 1.00.

(13.3%). Sex was determined correctly in all 4 female samples (Table 2).

In group II, correct sex could be determined in all 23 (76.7%) male and 7 (23.3%) female samples, whereas in group III, positive sex determination was possible in all the 24 (80%) male and 6 (20%) female samples, group IV also showed correct sex determination in all 23 (76.7%) male and 7 (23.3%) female samples.

DISCUSSION

Children are the most vulnerable group in our society. Child fatalities due to maltreatment represent the worst-case scenario in attempts to protect children. Although the untimely deaths of children due to illness and accidents have been closely monitored, the same cannot be said of children who have died as the result of physical assault or mere neglect. Interventional strategies targeted at resolving this problem face complex challenges.

Dental evidences have continued to provoke controversy within the field of forensic dentistry. The differing views surrounding interpretation, methodologies and admissi-bility are cornerstones of the arguments (McNamee and Sweet, 2003).

Sex identification is the first step in personal identifi-cation in forensic medicine. In general, the sex of an unidentified body can be determined based on anatomical characteristics of the external genitalia or whether the gonads are ovaries or testis. However, bones and teeth are the only available material for sex determination in markedly decayed and skeletonized bodies.

Sex differences in dental morphometric values are not distinct except in the permanent canine teeth, and determination of the sex from a random single tooth is extremely difficult. There has been no method to distinguish the sex particularly based on milk teeth (Murakami et al., 2000). Sex determination from pulp material can be done by different methods like fluorescence Y chromosome test, Southern blot test, etc. PCR stands above all mentioned methods since the high rate of sensitivity and specificity have been noted in previous experiments (Kumar and Hegde, 2005).

Teeth are considered as a good source to obtain genetic material. This is true mainly because of their great tissue resistance against external influences. Malaver and Yunis (2003) extracted DNA obtained from dentin and cementum of 20 corpses that had been buried for at least 5 years. Pulp tissue is a loose connective tissue and it degrades easily when compared with other dental tissues. Pfeiffer et al. (1999) studied the influence of the environment on DNA degradation in teeth that were kept underneath the soil. They observed that a tooth with opened pulp exposed to external agents showed a significant degradation. Lessig and Edelmann (1995) showed that pulp can be source of DNA in teeth that had been kept or obtained under different conditions such as teeth that had been extracted when the person was alive

T ab l e 3 . Comparisons of positive and negative results among different sexes in test groups (Chi square test was not performed because of lack of frequency distribution).

Group			Positive	Negative	Total
Group I	Male	Count	22	4	26
		% Total	73.3	13.3	86.7
	Female	Count	4	0	4
		% Total	13.3	.0	13.3
	Total	Count	26	4	30
		% Total	86.7	13.3	100
Group II	Male	Count	23	-	23
		% Total	76.7	-	76.7
	Female	Count	7	-	7
		% Total	23.3	-	23.3
	Total	Count	30	-	30
		% Total	100	-	100
Group III	Male	Count	24	-	24
		% Total	80	-	80
	Female	Count	6	-	6
		% Total	20	-	20
	Total	Count	30	-	30
		% Total	100	-	100
Group IV	Male	Count	23	-	23
		% Total	76.7	-	76.7
	Female	Count	7	-	7
		% Total	23.3	-	23.3
	Total	Count	30	-	30
		% Total	100	-	100

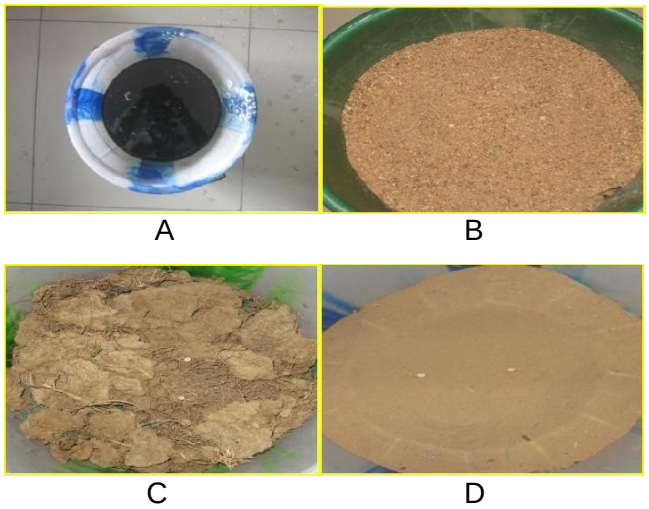

Figure 1. Photographs showing study groups (A) group I (Drainage water), (B) group II (seashore sand), (C) burial ground mud, and (D) desert sand.

Figure 2. (A) Primary canine after longitudinal sectioning and (B) pulp excavation.

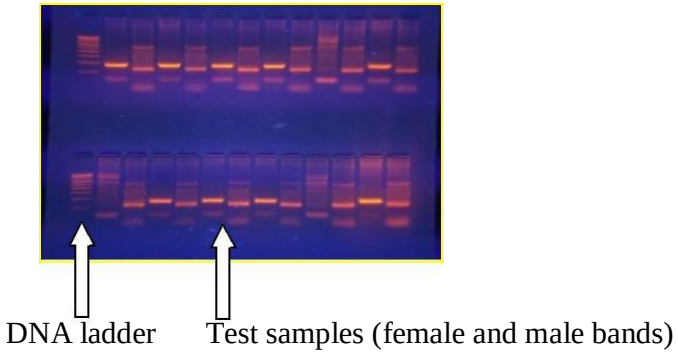

Figure 3. Gel electrophoretic picture showing X and Y specific amplifications.

and after death and kept in room temperature for 12 and 6 months, respectively (Da Silva et al., 2007).

In the present study, primary canine teeth were selected as the samples as these teeth are comparatively more protected teeth in the dental arch both from traumatic injuries and dental decay and compared to other primary teeth, initiation of resorption is late in primary canines. The teeth showing resorption beyond apical third were not included in the study.

It appears that the most important variables upon which identification by DNA depends are, the extent of time after death, the type of the soil in which the bodies were buried (temperature, bacterial composition, pH, etc), and the method of DNA extraction (Dragan, Unpublished).

DNA analysis of teeth for sex determination must be able to be carried out in bodies which are markedly decayed or skeletonized bodies that provide no other materials. In such bodies, dental DNA is considered to be considerably more decomposed and fragmented. Therefore, in this study highly sensitive method was followed as adopted by Murakami et al. (2000).

The Y and X chromosome-specific alphoid repeat sequences which were examined in this study were repeated several thousand times per genome, respectively and can be amplified by PCR if part of these copies remains intact (Murakami et al., 2000). Therefore, this method is more advantageous with regard to sensitivity than methods using a single copy base sequence.

In group I, sex was determined accurately in 26 of 30 teeth (86%). Among the remaining 4 teeth, no amplified band of Y chromosome specific sequence was observed in 3 teeth, although the samples were obtained from males and in 1 male sample, neither X nor Y specific amplification bands appeared. In these teeth therefore, decay due to bacteria was considered to have progressed markedly by infiltration of drainage water in to the pulp cavity and DNA of pulp tissue was considered to have been degraded to such a degree that amplification of the Y chromosome specific sequence by PCR became impossible. In these three samples, the amplified band of the X chromosome specific sequence was detected although that of the Y chromosome specific sequence was not, possibly because the amplified fragment of the X chromosome specific sequence, which is shorter than that of Y chromosome-specific sequence, was less liable to be effected by DNA degradation due to decay and because the number of repetitions of the X chromosome specific sequence in a single genome is several tens of times greater than that of the Y chromosome specific sequence (Murakami et al., 2000). In the remaining one tooth, it was considered that autolysis and DNA fragmentation reached such an extent that even detection of X chromosome specific sequence became impossible. Chances of PCR inhibition by substances in drainage water also was not ruled out.

The findings of our study were in accordance with the findings of Murakami et al. (2000) who mentioned that 'in wet state, pulp tissue gradually lyses due to autolysis and decay and its sampling becomes difficult'. This can be further accelerated in drainage water which is rich in various types of bacteria and chemical substances making DNA sampling difficult to near impossible.

The results of the present study indicate that sex determination of teeth by means of PCR is considered to be extremely useful for identification of markedly decayed or skeletonized bodies, which has been difficult using the conventional morphological methods contributing in identification of victims of various incidents and disasters including child abuse.

Sex determination is an important step in personal identification in forensic sciences. Surrounding environment has a definite role in preservation of pulp tissue; primary teeth can be regarded as an effective tool in preservation of pulp tissue which can provide DNA for sex determination and other DNA analysis procedures.

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