

# Forensic STR Identification of Human Teeth Samples Exposed to Various Acidic and Alkaline Chemical Conditions in the Iraqi Population

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## ABSTRACT

Forensic odontology has transformed human identification of forensic applications. This study evaluated the effect of various acidic and alkaline chemical solutions on dental DNA STR loci used in human identification. Teeth samples were divided into 3 groups of hydrochloric acid (HCl), nitric acid (HNO<sub>3</sub>) and sodium hydroxide (NaOH), in comparison to control teeth or blood DNA samples of the same individual. Following DNA extraction, a total of ten STR loci (CSF1PO, FGA, D13S317, D16S539, D21S11, D2S1338, TH01, D18S51, D5S818 and D8S1179) were amplified and sequenced. Results showed that DNA concentrations and purities decreased significantly in HCl and HNO<sub>3</sub> chemical conditions, but were less affected in NaOH whereas only HNO<sub>3</sub> showed DNA degradation. In addition, amplification of dental STR loci showed lower size products than their control blood counterparts. Moreover, the number of amplified STR loci in samples exposed to HNO<sub>3</sub> and HCl were fewer than those in NaOH. To evaluate STR loci, sequencing analysis was performed to compare STR motif frequency, DNA identity, and gaps between teeth and blood DNA. Results showed that the ratio of tooth/blood STR frequency was significantly different among HNO<sub>3</sub>,

HCl and NaOH (25%, 8.5% and 33.4%, respectively). Moreover, gap percentages were lower in samples exposed to NaOH and HNO<sub>3</sub> than HCl (9.8 and 9.3 vs 12.2%). Furthermore, NaOH alkaline solution had higher percent identities of STR loci, between teeth and blood DNA, than HNO<sub>3</sub> or HCl acidic conditions (58.7 versus 48.9 and 36.8%, respectively). The highest STR identity percentages was found for CSF, D18S5, and D2S1338 (64.7, 59.1, and 57.1%; respectively) whereas D5S818 was the lowest (16.9%). The most stable human identifier in the Iraqi population was the CSF locus whereas D5S818 was the least stable. In conclusion, dental DNA STR loci are more sensitive to HNO<sub>3</sub> than HCl or NaOH, which affected sequence STR frequency, identity and gap.

**Keywords:** human teeth; alkaline chemical conditions; STR identification

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## INTRODUCTION

Identification of human remains can be hindered by several factors like traumatic mutilation, carbonization or decomposition. Rapid and unambiguous identification is crucial, regardless of whether remains are found at a crime scene, following a mass disaster or under other circumstances (Zappa *et al.*, 2005). In some criminal cases, offenders may deliberately adopt various methods to prevent the victim's identification; for instance, the use of corrosive reagents to dissolve the body, as previously reported for mafia-related murders (Robino *et al.*, 2015). Destroying the body by immersing it in an acid, or some other caustic substances, is receiving considerable interest from forensic scientists (Gupta *et al.*, 2012; Raj *et al.*, 2013).

Dental identification has always been employed in natural and manmade catastrophes, particularly in mass casualties during aviation disasters (Jadhav *et al.*, 2009). Indeed, comparing post-mortem to ante-mortem dental records has been the most reliable method of identification. Compared to traditional methods, DNA analysis is a more reliable approach, with a higher degree of certainty. Teeth offer a good source of DNA within the mash, dentine, cementum and periodontal tendon filaments (Sweet *et al.*, 1999). DNA extracted from teeth could be preserved for a long time, even after rotting of remains (Garish *et al.*, 2010).

Human teeth could be exposed to corrosive chemicals in various contexts including: forensic cases, industrial chemical spills at work, and dentistry applications for engraving purposes. For instance, dental remains from the legendary Romanov case displayed convincing evidence

suggesting the exposure to a corrosive chemical (Maples and Browning, 1994; King and Wilson, 2003; Melia and Carr, 2005). It has been previously reported that teeth are totally disintegrated after 12–17 hours of submersion in hydrochloric acid, nitric corrosive or water regain, while sulfuric corrosive is inadequate even after 90 hours (Mazza *et al.*, 2005).

Short tandem repeats (STR) loci have been used since 1997 in the FBI Laboratory and represent the core of the Combined DNA Index System (CODIS). These STR loci dominate the area of genetic markers in humans (Butler, 2005). Since the human body can be dissolved partially or totally by immersing it in chemical corrosives, it is important for a forensic specialist to estimate the time needed for its complete destruction. The aim of this study is to determine the most stable STR loci for human identification, using teeth samples that were exposed to various chemical conditions such as hydrochloric acid (HCl), nitric acid (HNO<sub>3</sub>) or sodium hydroxide (NaOH), in comparison to control teeth.

## MATERIALS AND METHODS

### Teeth

A total of 15 molar teeth samples were collected according to the ethical approval of the ministry of health and ministry of higher education and scientific research in Iraq during the period from the 15<sup>th</sup> of January till the 15<sup>th</sup> of October 2017. The tooth surface was further brushed with a tooth brush then rinsed with distilled water (Ghareeb, 2010; Zagga *et al.*, 2014).

#### Teeth exposure to acids and alkaline chemicals:

A total of 15 teeth were kept in a dry environment at room temperature and were then divided into 3 groups. Concerning the experiments with acids, 10 teeth samples were divided into two groups. The first group included 5 teeth that were immersed in an aqueous solution of HNO<sub>3</sub> (total volume of 25ml: 16.25 ml of 65% HNO<sub>3</sub> + 8.75 ml of water) for 24h. The second group included 5 teeth that were immersed in an aqueous solution of HCl (total volume of 25ml: 14.6 ml of 37% HCl + 10.4 ml of water) for 24h. As for the experiments with alkaline solution, a total of 5 teeth samples were immersed in an aqueous solution of NaOH (2N) for 24hrs. Samples were photographed before and after placement in the various chemical solutions (Jadhav *et al.*, 2009).

#### Collection of blood samples

Blood samples of 2ml each were taken using disposable syringes from the same individuals (n=15) donating their teeth. Blood samples in EDTA tubes were then stored at -40°C (deep freeze) to be used later for DNA extraction.

#### Genomic DNA Extraction

Genomic DNA of whole blood was purified using the Genomic DNA Mini Kit according to the manufacturer's protocol (Geneaid, Taiwan). On the other hand, genomic DNA from teeth samples was extracted using Gene MATRIX Bone DNA Purification Kit according to the manufacturer's protocol (Roboklon, Germany).

#### Primers and PCR amplification

Ten STR loci were used in this study including CSF1PO, FGA, D13S317, D16S539, D21S11, D2S1338, TH01, D18S51, D5S818 and D8S1179. The sequences of the sets of PCR primers are given in Table 1. PCR was performed using the following conditions: denaturation at 95°C for 5 min then 35 cycles consisting of denaturation at 95°C for 30 sec, annealing at (59-60) °C for (18-30) sec, depending on the T<sub>m</sub> of the primer sets, and extension at 72°C for 30 sec, followed by a final extension step at 72°C for 10 min.

#### Sequencing and bioinformatics analysis

DNA sequencing for each sample was performed at Macrogen Company (Seoul, Korea). STR loci sequencing was performed using Genetic analyzer and the sequences were verified. Analysis of DNA sequences was also performed using online bioinformatics softwares at NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and EBI ([https://www.ebi.ac.uk/Tools/psa/emboss\\_needle/](https://www.ebi.ac.uk/Tools/psa/emboss_needle/)). Also, the SMS package and DNA pattern finder tools ([https://www.bioinformatics.org/sms2/dna\\_pattern.html](https://www.bioinformatics.org/sms2/dna_pattern.html)) were used to analyze DNA sequence identity, gaps, and STR motif frequency as well as teeth/blood STR frequency ratio.

#### Statistical analysis

Results are presented as the mean with error bars indicating the standard error of the mean (Mean ± SEM). Statistical analysis was performed using SPSS (Version 23) software. Significance is indicated by \*, p<0.05.

## RESULTS

#### DNA quality control for teeth immersed in various chemical conditions

The DNA samples showed variable concentrations and purities. Results revealed that concentrations decreased significantly in teeth exposed to all chemical solutions: HCl, HNO<sub>3</sub> and NaOH (9.3, 5.1, and 13.3ng/ µl; respectively), in comparison to controls (~26ng/l). As for the purities, a significant decrease was obtained for teeth DNA samples immersed in HCl or HNO<sub>3</sub> (1.4 versus 0.8), compared to controls (~1.8). However, non-significant variations were observed in DNA samples exposed to NaOH chemical solution (~1.5) (Table 2). On the other hand, blood samples showed concentrations of ~100 ng/µl with purities ranging between 1.6-2.3 (Data not shown).

#### Amplification of ten STR loci for teeth exposed to various chemical conditions

Following DNA extraction, PCR amplification was performed for a total of 10 STR loci including CSF1PO, FGA, D13S317, D16S539, D21S11, D2S1338, TH01, D18S51, D5S818 and D8S1179 (Table 1). Results on control blood samples showed successful DNA amplification for all STR loci (n=10) of all individuals included in the study, which constitute a positive control for data comparison with teeth samples (Figure 1A, Left Panel). Using blood samples, the obtained amplification bands for all STR loci were at the expected lengths, as summarized in Table 1.

#### Effect of chemical conditions on STR Frequency of teeth DNA

DNA extracted from teeth samples immersed in HNO<sub>3</sub>, HCl, or NaOH conditions showed single and clear amplification bands (8 out of 10, 8 out of 10, and 9 out of 10; respectively), therefore, the amplified samples were then sequenced. Products were analyzed to validate DNA sequences before estimating STR frequency, gap and identity. Results showed that the quality of DNA trace histograms were not affected by HNO<sub>3</sub> (Figure 2A). However, teeth immersed in HCl or NaOH were affected (Figure 2B and 2C, respectively). In the present study, STR frequencies, gaps and identities were analyzed between teeth and blood samples of the same individual, chosen randomly from the Iraqi population. First, the changes of STR frequencies in teeth samples exposed to different chemical solutions were estimated based on the ratio between teeth to blood (Teeth/Blood or T/B), the latter considered as control. For each locus, the ratio between the frequency of STR in teeth, relevant to the various chemical conditions, and their blood counterpart, was estimated (Figure 3 and Table 3). Data showed that the highest T/B STR frequency ratio was obtained for the NaOH alkaline condition (58.7%) followed by HNO<sub>3</sub> acidic solution (48.9%), whereas the HCl condition showed the lowest frequency (36.8%). All these variations in frequency ratios, for each chemical condition, were significant (p value < 0.05).

Immersion of teeth samples in HNO<sub>3</sub> acidic solution showed variable frequencies, in comparison to blood samples (Table

3). In fact, all samples revealed frequencies in 8 STR loci, except for D8S1179 and FGA, which were completely absent. The lowest frequency was for D13S317 locus whereas the highest was for D21S11. On the other hand, samples immersed in HCl acidic solution showed frequencies for 8 STR loci, except for D5S818 and D16S539. The lowest frequency was for FGA locus whereas the highest frequencies were within three loci; D18S5, D2S1338 and CSF. As for samples immersed in NaOH alkaline solution, frequencies were revealed in 9 out of 10 STR loci, except for D5S818. The lowest frequency was for D8S1179 locus whereas the highest was for CSF (Table 3). The ratio of T/B was also used to estimate the stability of each STR locus in these chemical conditions. Results showed that CSF locus was the most stable (~64.7%). On the other hand, D5S818 (16.9%) and D8S1179(31.3%) were the least stable.

#### Effect of chemical conditions on Gaps in the STRs of teeth DNA

Gaps in STR DNA sequences were analyzed and showed that the percentage of gaps was mostly affected by the HCl condition (~12.2%) followed by NaOH and HNO<sub>3</sub> (9.8% and 9.3%, respectively) (Figure 4 and Table 3). Significant differences (p value <0.05) were found for these conditions (Figure 4).

Teeth samples exposed to HNO<sub>3</sub> showed variable gap percentages, in comparison to blood samples counterparts. In fact, all samples showed gaps ranging between 4% and 19.1%, for all STR loci except for D8S1179 and FGA. Moreover, samples exposed to HCl showed even higher gap percentages, ranging between 9.1% to 22.4% except for D5S818 and D16S539. Furthermore, teeth samples exposed to NaOH showed gap percentages ranging between 7% to 17.1% (Table 3). All STR loci DNA sequences showed different percentages of gaps. Higher gap percentages were revealed for STR loci D2S1338 and THO which showed 14.9% and 14.1%; respectively. Low gap percentages were demonstrated for STR locus D5S818 (4.3%).

#### Effect of chemical conditions on the identity of STR sequences in teeth DNA

The highest effect on STR sites in teeth DNA was for the HCl condition (8.5%) followed HNO<sub>3</sub>(25.3%) whereas DNA immersed in NaOH was the least affected (33.5%). Significant differences in percentages of identity, between teeth DNA in comparison to blood controls, were observed for the ten STR loci for all these chemical conditions (HNO<sub>3</sub>, HCl and NaOH) (Figure5, p<0.05).

Immersion of teeth samples in HNO<sub>3</sub>acidic solution showed variable percentages of identity, ranging from ~50% up till ~79% for 8 STR loci, except for D8S1179 and FGA, in comparison to blood samples. The lowest identical locus was D13S317 (~50%) whereas the highest was D21S11 (~78%). On the other hand, samples immersed in HCl acidic solution showed percentages of identity ranging from ~37% to ~53%for 8 STR loci, except for D5S818 and D16S539. The lowest identical locus was FGA (~37%) whereas the highest percentages of identity were within three loci; D18S5 (~53%), D2S1338 (~52%) and CSF (~50%). As for samples

immersed in NaOH alkaline solution, percent of identity ranged between ~50% and ~78% in 9 out of 10 STR loci, except for D5S818. The lowest identical locus was D8S1179 (~50%) whereas the highest was CSF (~78%)(Table 3). The stability of STR loci showed that the identity of STR's exposed to the various chemical solutions were different. In fact, CSF and D13S317 were the most stable loci (~39.6% and ~32.0%) whereas D5S818 locus was the least stable (~10.3%).

## DISCUSSION

Our data showed different percentages of identity, STR frequency and gaps. Previous investigators observed that morphological changes and STR loci may be varied when acid concentrations used are those that are most commonly available commercially (Mazza et al., 2005; Raj et al., 2013). In the cases of destruction of the body by acids, soft tissues are destroyed first, followed by bones and teeth. However, the identification by morphological assessment of teeth can be hampered by their dissolution. Use of advanced techniques like DNA analysis in such scenario has rarely been studied. In fact, samples containing degraded or trace amounts of DNA prevent resolving forensic cases and offer a limited opportunity for meaningful analysis or conclusion (Leticia et al., 2009). Hence, this study was undertaken to assess the effect of acid dissolution on DNA extraction. Pulp and hard tissues (dentin and cementum) are the sources of DNA from teeth. The corrosive action of inorganic acids affects the macromolecules of the teeth, including DNA. The low quantity or impure DNA in forensic samples often results in partial or unsuccessful STR profiles (Jamilly et al., 2009).

The degradation or an insignificant amount of biological material in a DNA extraction may result in the absence of target sequence, not allowing DNA amplification by PCR (Mukherjee and Biswas, 2005). Singh et al. (2013) showed that there was a significant decrease in both DNA and RNA yield and integrity with strong acids (HCl, HNO<sub>3</sub>). Thus, it suggested that decalcification of bone biopsies with EDTA or formic acid agents was associated with a significant improvement in the quantity and quality of recovered nucleic acid, compared to other agents such as nitric acid used in the present study. Nitric acid required at least 4 hours to penetrate the core of small experimental meat specimens (600–800 g) and completely degrade DNA. Therefore, it can be expected that the physical protection exerted by various tissues in whole body parts will further delay the effects of acids on cells surrounded by, or embedded in, highly resistant mineralized matrices such as those found in cortical bones and teeth (Robino et al., 2015). Recent studies used experimental models to mimic several factors which may affect forensic evidence tests (Nicole et al., 2017). The latter showed that exposing teeth samples to formaldehyde or sodium hypochlorite did not interfere in the extraction of DNA and the subsequent amplification process.

Sodium hydroxide (NaOH) is a commonly used reagent to denature the DNA by increasing the pH (Poltronieri et al., 2008; Chen et al., 2009). At an alkaline pH, OH<sup>-</sup> groups are predominant. They remove the protons in guanine and

thymine, thus breaking the hydrogen bonds between the two oligonucleotides (Shin and Day, 1995). The mechanism by which NaOH can help amplification was previously investigated, it has been proposed that denaturing conditions would release intercalated inhibitors and that denaturing washes would allow for their removal (Bourke *et al.*, 1999). Furthermore, it is also possible that alkaline (or denaturing) conditions alone could inactivate the inhibitors, thus removing the necessity for NaOH washes and potentially increasing the quantity/quality of DNA recovered.

However, it appears that the identification of skeletal remains by STR analysis is sufficient in the large percent of analyzed cases (Antonio *et al.*, 2001). The STR examination is a scientific investigation method that assesses precise STR loci found on DNA. The variable (polymorphic) nature of STR areas highlights the segregation between one DNA profile and another (National Commission, 2006). The success of a CODIS STR profile depends, not only on the total quantity of human genomic DNA employed in the multiplex PCR reaction, but also on the relative proportions of intact versus degraded template DNA (Swango *et al.*, 2006). The loci utilized as a proof in human identification are situated on non-coding portions of DNA, STR DNA loci represent the most conventional procedure as a proof to distinguish, differentiate and discriminate between human remains (Alonso *et al.*, 2005; Zietkiewicz *et al.*, 2012).

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Table 1: Sequences of primers that were used for 10 STR Loci.

STR loci	Loci	Frequency	Normal allele	Primer design	TM C°	Product size/pb
CSF1PO	5q33.1	TAGA	6-15	F: ACCTGCTAGTCCTTGGCAGA R: AAGGTGTCCTGGGAACACTG	60	443 bp
FGA	4q31.3	CTTT	15.2-17	F: GGGACCACAGCCACATACTT R: GCGAGTCTAGGGAGTCTCA	59	790 bp
D13S317	13q31.1	TATC	8-15	F: GGGTTGCTGGACATGGTATC R: TCCGAGGTTCTCTTCCTGTG	60	603 bp
D16S539	16q24.1	GATA	5-15	F: CCTAAGAATTTCGCTCCACA R: CGGATTCTGATTCTGCAGGT	60	695 bp
D21S11	21q21.1	TCTA	24-38	F: CCAGCTTCCCTGATTCTTCA R: CGAGGACTCACACAGAGTGG	60	596 bp
D2S1338	2q35	TGCC	15-28	F: TCAGGGTTTTGCTGTCACTG R: TCCCTCCTCTCCCTTCTCTC	60	326 bp
TH01	11p15.5	TCAT	4-13.3	F: CACAGGGAACACAGACTCCA R: CCTCTTAGGCCGACCTCTCT	59	407 bp
D18S51	18q21.33	AGAA	7-27	F: GCCACTGCACTTCACTCTGA R: CTGGATTGGAATCGCAGTTT	60	693 bp
D5S818	5q23.2	AGAT	7-16	F: TTCAAAATGAGGGGGTAAGC R: CATTCAATCAGGAGCATGTCA	60	709 bp
D8S1179	GRCh38_p7	TCTA	8-19	F: CCTTTGCCTGAGTTTTGCTC R: CTTTTGGCAGAAGGAACTGG	59	787 bp

Table 2: Concentration and purity of teeth DNA after exposure to acidic and alkaline conditions.

	Control	Chemical factors			P value
		HCl	HNO <sub>3</sub>	NaOH (2N)	
Concentration (ng/μl)	26±6.1 a	9.3±7.8 b	5.1±7.8 c	13.3±7.8 b	0.001
	1.8±0.1 a	1.4±0.1 b	0.8±0.2 b	1.5±0.1 a	0.01

Purity					
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Results are given as Mean  $\pm$  SD. Significance is shown by a, b and c, as given by Duncan's Multiple Range Test. Significant differences ( $p \leq 0.05$ ), in comparison with control are demonstrated by comparing a with b, a with c, and c with b. Similar letters (a) show no statistical significance.

Table 3: Effect of chemical factors on STR motif frequency (Fr), gaps and identity

STR	HNO <sub>3</sub> (65%)				HCl (37%)				NaOH (2N)			
	Blood STR Freq.	Teeth STR Freq.	Gap	Identity	Blood STR Freq.	Teeth STR Freq.	Gap	Identity	Blood STR Freq.	Teeth STR Freq.	Gap	Identity
D8S1179	15	0	0	0	9	1	13.3	44.3	16	6	15	49.5
D5S818	13	4	12.9	50.7	13	0	0	0	10	0	0	0
D16S539	11	5	11	68	12	0	0	0	10	4	9	66.3
D13S317	16	5	13.1	50.2	13	1	19.2	44.1	14	8	7	73.4
D21S11	29	10	4	79.1	27	4	15.2	41.2	24	9	10.2	50.1
D2S1338	25	4	19.1	59.1	24	2	13.5	52.2	26	5	12.2	60
CSF	13	6	10.1	66	14	2	9.1	50.1	12	7	9.8	77.9
FGA	16	0	0	0	15	1	22.4	36.9	15	7	8.7	76.4
D18S5	23	5	11.1	54.7	22	3	16.3	53	25	5	9.4	69.7
THO	11	3	11.9	61.6	12	1	13.2	46.1	11	2	17.1	63.4

Assessment was performed on individual teeth after alignment and comparison with their blood counterparts. Gaps represent deletions or insertions in teeth DNA, as calculated by the software.

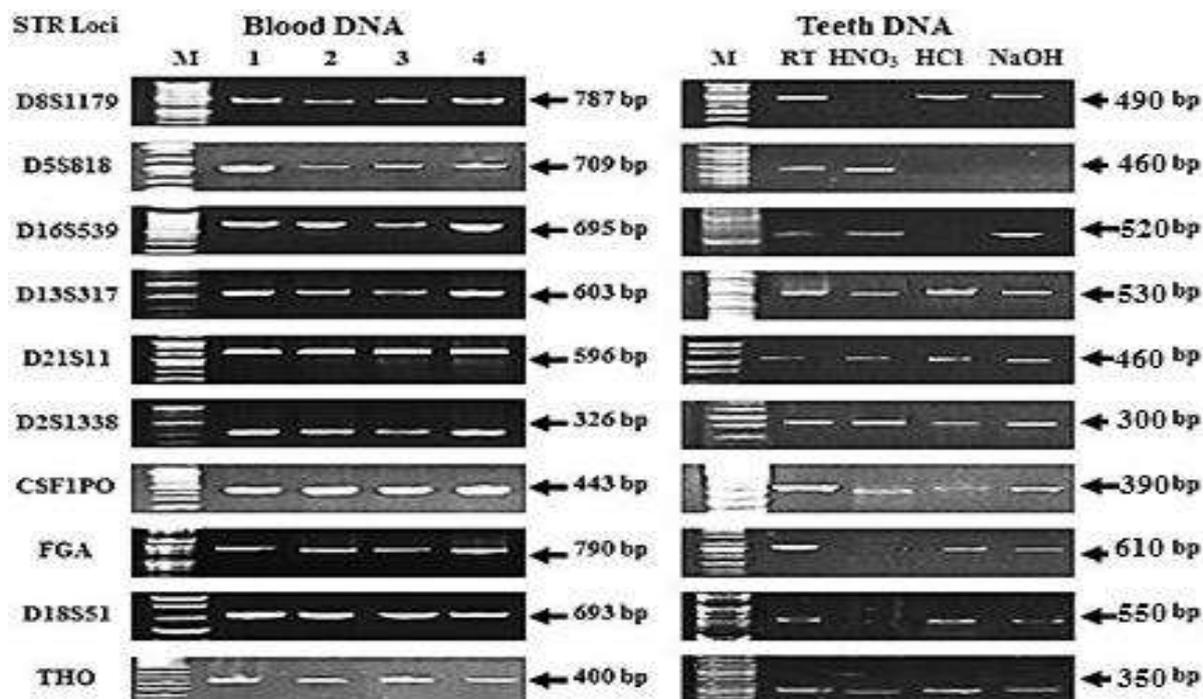


Figure 1: Electrophoresis pattern of 10 STR loci for teeth immersed in various chemical conditions (HNO<sub>3</sub>, HCl, NaOH). Amplification products were run on a 1% agarose gel, 70 V, 20mA, for 60 min. Amplified STR Loci from blood samples are given on the left panel whereas those from teeth samples are given on the right panel. For more details about the length of the various STR bands, please refer to Table 1.

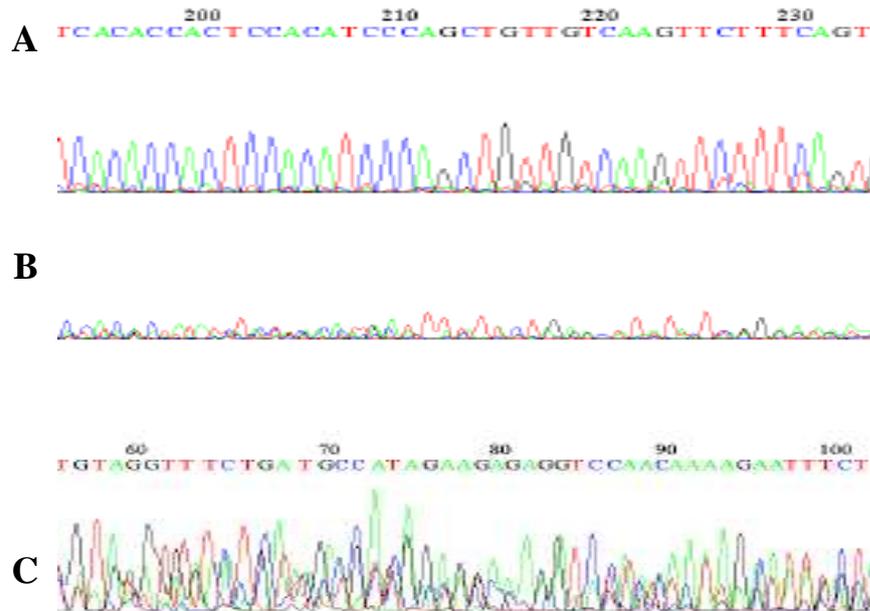


Figure 2: Histograms of STR loci DNA sequence traces of teeth immersed in various chemical solutions (A) HNO<sub>3</sub>, (B) HCl, (C) NaOH.

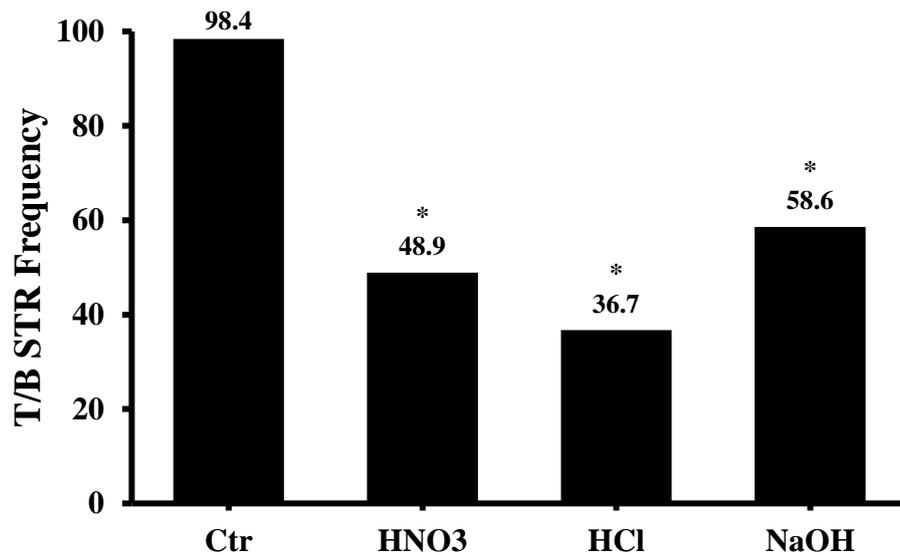


Figure 3: Effect of various chemical solutions (HNO<sub>3</sub>, HCl and NaOH) on STR loci frequency demonstrated by the ratio of teeth / blood.

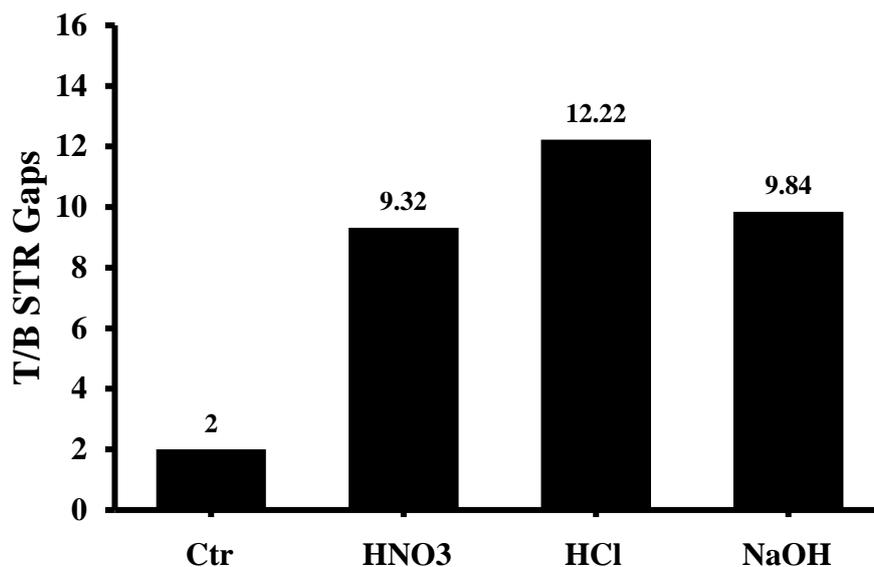


Figure 4: Percentages of gaps in sequences of STR loci of teeth samples immersed in various chemical solutions (HNO<sub>3</sub>, HCl and NaOH).

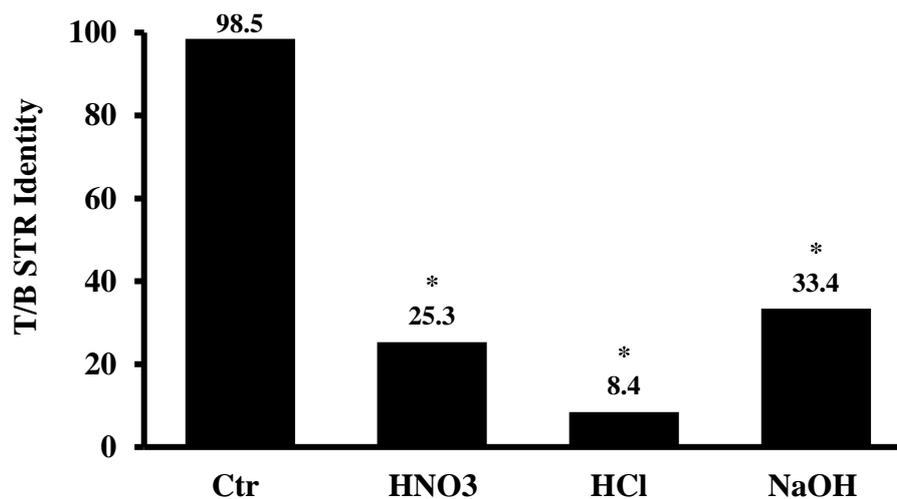


Figure 5: Mean percent identity among sequences of STR loci of teeth samples immersed in different chemical solutions (HNO<sub>3</sub>, HCl and NaOH)