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Original Article

Freshwater and salt-water influence in human identification by analysis of DNA: an epidemiologic and laboratory study

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Abstract

Aim: To investigate the casuistry of drowning cases by reviewing the records from the Forensic Medicine Institute Nina Rodrigues in the city of Salvador, BA, Brazil, and to verify the potential of DNA recovery in human teeth immersed in water. **Methods**: An epidemiological survey was conducted followed by a laboratorial phase, in which 40 teeth were immersed in fresh and salt-water, the DNA was extracted by the organic method and amplified by polymerase chain reaction, using the amelogenin as initiator. The electrophoresis initially occurred in agarose gel and later in polyacrylamide gel. **Results**: In the present survey, 346 deaths from drowning were observed, most of them in salt-water (51.73%), with a predominance of male victims (86.13%) aged from 18 to 35 years-old (37.94%). Dentists identified 14.74% of the victims. DNA was recovered in 37.5% from the samples, most from teeth immersed in freshwater. Polyacrylamide gel analysis in samples that were amplified in agarose gel allowed correct gender identification in 83.3% of the cases. However, allele loss was observed in samples of two victims, jeopardizing gender determination. **Conclusions**: Dental exposure to water interfered in DNA recovery. The gender investigation using the amelogenin as initiator was effective.

Keywords: human identification, forensic dentistry, teeth, DNA, drowning.

Introduction

Forensic Dentistry has contributed to human identification for a long time. Dentists, enforced by law #5.081/66, are able to perform reports involving biological materials derived from human body under several conditions (quartered, dilacerated, carbonized, macerated, decomposed, in skeletonization and skeletonized), with the goal of establishing human identity[1].

Historically, fingerprints have been used in identification. However, in certain situations, in which the body is found in advanced stage of putrefaction, fingerprints are easily destroyed. Moreover, the technical experts frequently need to use comparative antemortem elements, such as dental records, in human body identification, but they are not always available. Before the development of molecular biology techniques, these situations would make the experts establish the victim's identification without the comparative elements. Whenever the documen-

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tation was available, but the corpses were degraded, the exams would try to recover the individual's profile by establishing the species, gender, built, age, phenotype, skin color, among other characteristics[2].

Nowadays, biomolecular resources have been employed in human identification whenever the use of traditional methods is not viable. Applying these resources allows identifying victims even without antemortem information or with deteriorated biological material in insignificant amounts, which are relatively frequent conditions in forensic analysis, especially in mass accidents[3].

In this context, some authors have emphasized the importance of gender investigation in victims' identification and the need of the joint action among several healthcare workers (physicians, dentists, psychologists, geneticists, radiologists) and professionals from related sciences (anthropologists, technical examiners, among others) in this process[4,5].

Recently reported in the media, events such as the *tsunami*, in Thailand, in 2004 and in Indonesia, in 2006, the hurricanes "Katrina" in New Orleans and "Rita" in Texas, both in 2005[6], reinforce the need of mastering DNA extraction techniques in the process of identifying skeletonized bodies or bodies in advanced state of putrefaction, under the influence of aqueous environment[7].

The practical applicability of these methods can be observed in several studies that used DNA-based techniques in the identification of victims, whose bodies had undergone the action of water[8,3]. The influence of environmental factors on DNA stability has been emphasized, since corpses or their pieces are frequently found carbonized, submersed or buried. According to Schwartz et al.[9], those factors can interfere in the amount of recovered DNA and influence in the identification process[9]. According to Bender et al.[10], this is due to the fact that the rate of DNA degradation varies according to light conditions, water content and temperature, and bacterial and fungal contamination might occur followed by microbial growth, and resulting in the physical, chemical and biological degradation of the genomic DNA[10].

Although mass accidents, including natural disasters, have been poorly documented in Brazil, records from the Ministry of Health report a significant increase in mortality rate due to external causes, among which drowning is mentioned. In this sense, the official records report the occurrence of 57,595 deaths by drowning and accidental submersion between 1996 and 2004, which represents 0.67% from the total number of deaths that occurred in the country in this period[11]. Concern is expressed when the Brazilian coastal line extension and its population concentration are considered, especially in Bahia, where, according to the Brazilian Institute of Geography and Statistics[12], there is a prevalence of coastal cities.

The present study aimed at understanding the casuistry in situations involving drowning cases, and verifying the potential of DNA recovery from teeth immersed in water.

Material and methods

An epidemiological survey was conducted with records from the Forensic Medicine Institute Nina Rodrigues in Salvador, BA, Brazil, between 2003 and 2005. Initially, information such as number of deaths by drowning, gender, age, type of water in which the body was submerged (freshwater or salt-water) and the contribution or not of Forensic Dentistry in the victims' identification was collected. Later, a research laboratorial phase was carried out. The sample was composed by 40 teeth from 20 individuals (two from each victim). Saliva in MGM® cards was collected from oral mucosa swabs. The teeth were submitted to a physicochemical process for tooth-cleaning, through scaling with curettes and 70% alcohol-wash and then immersed in fresh and salt-water for one month, according to Schwartz et al.[9]. After the immersion, the tooth crown was sectioned[13] and pulverization was done according to the method proposed by Sweet and Hildebrand[14], adapted to the use of a mortar and pestle, and manual storage by immersion in liquid nitrogen.

DNA extraction from the saliva stored on cards was performed with those fragments using a FTA kit. The protocol to extract DNA from the teeth was based on the organic method described by Hochmeister et al.[15]. The region for amplification by polymerase chain reaction (PCR) was the amelogenin. The sequences of initiators were sense: ACCTCATCCTgggCACCCTgg (21BP) and anti-sense: AggCTTgAggCCAACCATCAg (21BP). The following cycling protocol was used: denaturation at 96° C for two minutes, followed by ten denaturation cycles at 94 °C for one minute, hybridization at 64 °C for one minute, elongation at 72 °C for one minute and 30 seconds and final elongation at 72 °C for ten minutes.

The PCR products were initially analyzed in agarose gel at a 1.5% concentration. Electrophoretic separation occurred in 100 V and 60 mA for 40 minutes. Later, the samples that amplified in the agarose gel were submitted to electrophoresis in silver-stained 8% polyacrylamide gel.

Results

The review of the records referring to the total of deaths occurred between 2003 and 2005 showed a prevalence of only 2.7% deaths resulting from drowning. The records also revealed a higher prevalence of death in male individuals and young adults (37.94%), as seen in **Figures 1** and **2**.

Regarding the type of water in which the bodies were submerged, salt-water prevailed, followed by freshwater and swimming pool water, except for 2003, when the first two categories were inversed (**Figure 3**). When evaluating Forensic Dentistry contribution to victim identification, little participation of the forensic dentist was observed (**Figure 4**).

During the laboratorial phase, the DNA was initially by electrophoresis in 1.5% agarose gel (**Figure 5**). In this phase, genetic material recovery was observed in 37.5% of the dental samples, with a predominance of teeth immersed in freshwater (45%) over teeth immersed in salt-water (30%). All saliva samples used as controls presented positive amplification.

The analysis of polyacrylamide gel in 27 samples (15 teeth and 12 saliva samples), from 12 victims with positive amplifica-

tion in agarose gel, allowed the visualization of the amelogenin different alleles, enabling the correct identification of gender in 83.3% of the cases (ten individuals). Moreover, an adequate correlation was observed between band intensity and the type of material used in the exam (tooth or saliva) (**Figure 6**). However, loss of alleles in the polyacrylamide gel was observed in the samples of two individuals, jeopardizing gender identification in these cases.

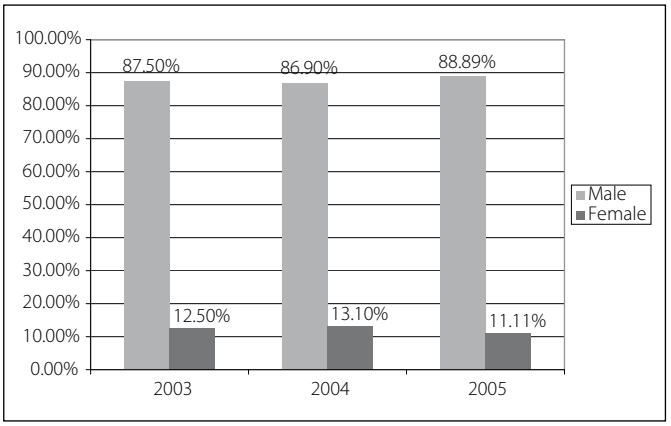

Figure 1. Percent distribution of drowning victims according to gender, Salvador, 2003 to 2005.

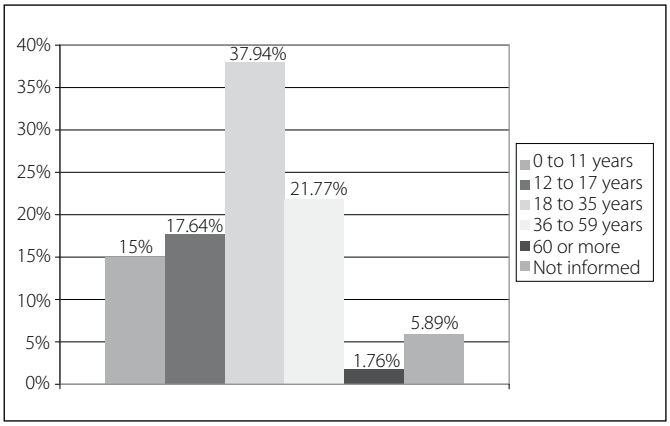

Figure 2. Percent distribution of drowning victims according to age, Salvador, 2003 to 2005.

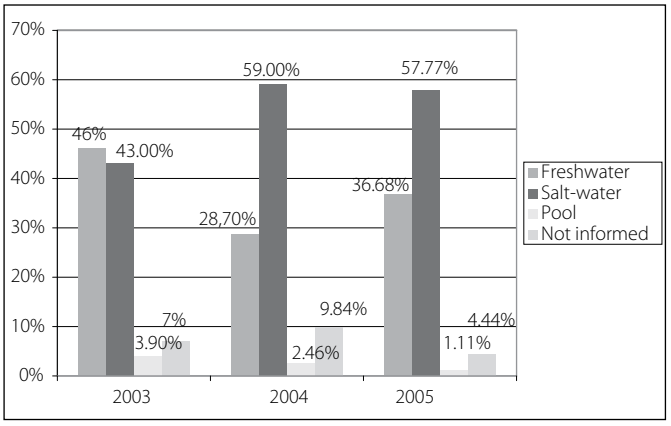

Figure 3. Percent distribution of drowning victims according to the place where the body was found, Salvador, 2003 to 2005.

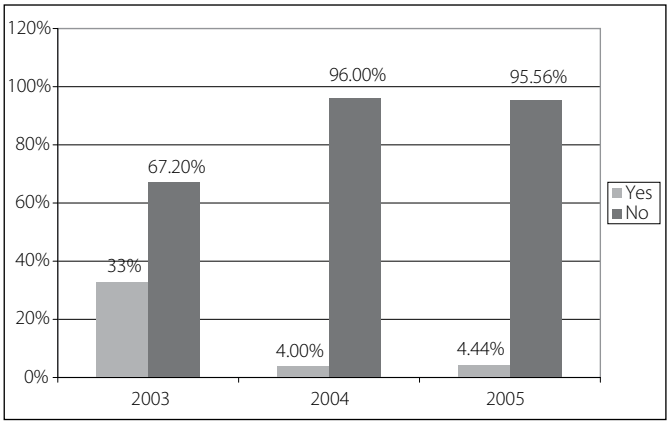

Figure 4. Percent distribution of drowning cases according to the contribution of forensic dentistry in victim identification, Salvador, 2003 to 2005.

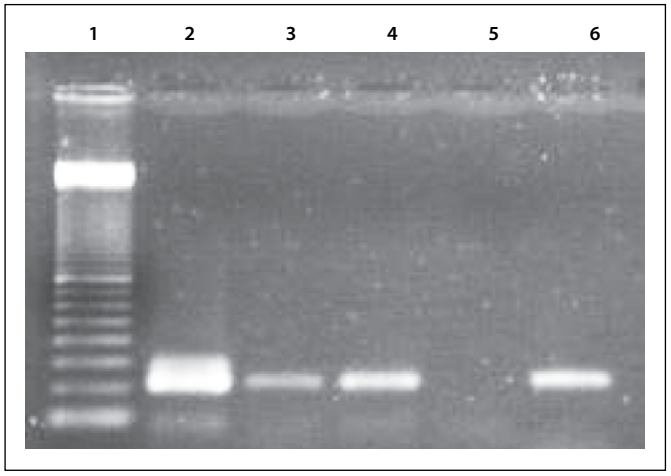

Figure 5. Photograph in 1.5% agarose gel stained with ethidium bromide, after AMEL locus amplification. Samples: 1 = 100 pb marker; 2 = saliva; 3 = tooth submerged in salt-water; 4 = tooth submerged in freshwater; 5 = PCR negative control and 6 = PCR positive control.

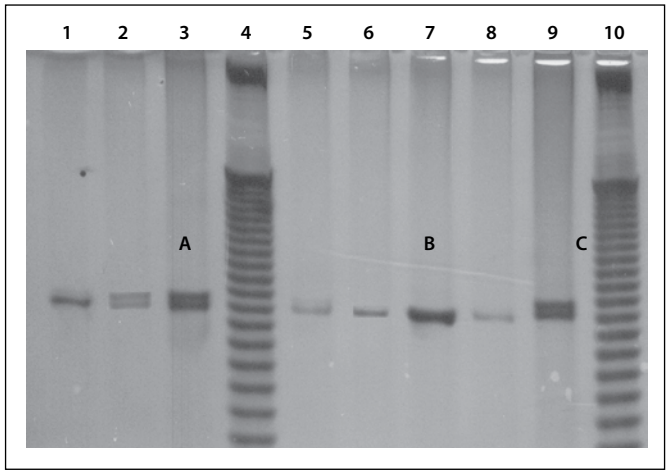

Figure 6. Photograph in 8% polyacrylamide gel stained with silver. Samples: 1, 5 and 8 = tooth submerged in salt-water; 2 and 6 = tooth submerged in freshwater; 3, 7 and 9 = saliva; 4 and 10 = 10 pb standard. A: male; B: female; C: no gender identification.

Discussion

The mortality rates due to external causes in Brazil, among which drowning, have increased significantly in the last few years, reaching the second place in Brazilian mortality statistics[16]. Thus, it is necessary to know the population profile that have mostly contributed to this statistics, in such a way that planners and executors of government policies are able to define, in concrete basis, actions that should be a priority, in order to contemplate prevention and attention to those victims.

In this sense, the findings of the present epidemiological survey showed prevalence of 2.7% of deaths by drowning in the city of Salvador. Baptista et al.[17] and Martins and Andrade[18] have found 26 and 19.5% of deaths by drowning in Portugal and in Paraná/Brazil, respectively. These discrepant percent values can be attributed to the methodological differences in the studies and their duration.

Regarding the distribution of deaths by drowning, the findings of the present study agree with the literature[19,20] regarding the most prevalent gender (male) and age group (adults).

The findings of a 12-year survey of deaths by drowning stated that 63% of the cases occurred in freshwater[18], while another study[19] found a predominance of death cases in salt-water. In the present research, most cases occurred in salt-water, which is probably due to the fact that 95% of the water in the Brazilian territory is formed by salt-water[20].

There is a low prevalence of Forensic Dentistry contribution in the identification of victims by drowning in the present study. Forensic dentists participated in only 14.74% of the cases within three years. This result is probably due to the lack of Forensic experts in Bahia during the period of this study, since the first public selection for these positions occurred in 2006[21].

The results of the review of the records referring to the total of deaths in the Forensic Medicine Institute served as basis for the next phase of the present study: the laboratorial. Considering the recent world casuistry of mass accidents involving the aquatic environment, and the consequent need of mastering the DNA-based technique in the process of identifying victims that were under the influence of water, teeth were immersed in fresh and salt-water in order to simulate cases of forensic investigation.

In this sense, one of the first steps to proceed with the victim identification is gender investigation[4], usually performed according to anatomical characteristics from male and female genitals. However, when forensic examination has to be done in skeletonized corpses or bodies in advanced state of putrefaction, bones and teeth are, many times, the only materials available and identification can be performed by molecular biology techniques, using amelogenin analysis[22].

In the present paper, the analysis by electrophoresis in agarose gel showed only one DNA region for gender investigation (amelogenin). Genetic material recovery was possible in only 37.5% of the teeth, demonstrating that the water interfered directly in DNA preservation. On the other hand, DNA from all saliva samples used as controls was successfully amplified. There was greater DNA degradation in salt-water (70%) compared to freshwater (55%), probably due to the chemical composition of both aqueous conditions. Both types of water present ions such as: calcium, magnesium, sodium, potassium, bicarbonate, chloride, sulfate, nitrate, among others. Traces of lead, copper, arsenic, manganese and a large spectrum of organic compounds from decomposition of organic matter of animal and vegetal origin can also be found. In addition, in some cases, residues from agricultural fields and disposal of effluents from domestic and industrial origin are present, varying from humic acids to synthetic organic compounds such as detergents, pesticides and solvents[20].

It was not possible to determine the mechanism by which the water affects DNA recovery. However, some components present in aquatic ecosystems have already been reported as being able to degrade genetic material (microbial growth, humidity)[10] or inhibit the Taq DNA polymerase enzyme (humic acid). This enzyme is responsible for the incorporation of free nucleotides, which will form a new fragment of the DNA molecule. Thus, its inhibition is a hindrance to PCR[23].

The analysis of the agarose gel does not allow gender identification due to a slight difference in base pairs[6] present in men and women, when the amelogenin is used as a region. In this sense, Mukherjee and Biwas[24] have suggested the use of polyacrylamide gel in those cases because it promotes a better band separation and, consequently, allele visualization. Running polyacrylamide gel was performed in samples that amplified in agarose gel, enabling the accurate visualization of the amelogenin alleles, and identifying correctly the gender of ten victims. However, although gender determination using amelogenin is a recognized and scientifically accredited methodology, loss of alleles in the polyacrylamide gel was observed in the samples of two individuals. Pinheiro[1] comments on this possibility when he states that sometimes DNA degradation, instead of impeding the results, can cause the visualization of one allele, instead of two, and the disappearing of the allele with larger dimension is more frequent. Hence, when old vestiges are analyzed and homozygous profile is obtained by some systems, one should be careful using the results because they might be from a heterozygous profile. An alternative to solve this problem was proposed by Steinlechner et al.[25], while studying the genetic profile of 29,432 men stored in an Australian database, they verified the absence of PCR product specific of the amelogenin related to chromosome Y in six individuals. After changing the methodology and analyzing eight short tander repeat (STR) from the same chromosome, the complete genetic profile of five victims was obtained, confirming their male genotype. The error rate in gender investigation using the amelogenin test in the present work was 0.018%.

A factor that may lead to DNA non-amplification is the quality of the forensic biological sample. The insignificant amount of biological material to DNA extraction may result in absence of the target sequence in the fraction used for the reaction or the same can be degraded, not allowing DNA amplification by PCR[24]. In an attempt to minimize the effects of this degradation and even as legal endorse-

ment to avoid the exam result to be questioned, it is necessary that forensic laboratories keep the chain-of-custody of the samples. The records must be readily accessible and followed since data collection until the final disposition, in such a way to warrant that every precaution has been taken to avoid falsification, break, loss or contamination of the samples. In addition, inadequate procedures that may lead to sample contamination, by the lab investigators and forensic professionals, may result in erroneous interpretation of the genetic profile[26]. Therefore, the maintenance of good laboratorial practices is essential and specific training on DNA-based identification techniques is mandatory.

The following conclusions may be draw from the obtained results: the epidemiological survey allowed outlining the population profile that most contributed to the statistics surveyed, during a three-year period in the Forensic Medicine Institute of Salvador; there was a predominance of adult male victims exposed to the action of salt-water; tooth exposure to water interfered directly in DNA recovery; despite its well-established efficacy, recognition and credibility, gender investigation by amelogenin, as every procedure that uses biological molecular resources, requires a careful interpretation of the results by the researcher, therefore, the observation of all the criteria related to the maintenance of the chain-of-custody and DNA analysis are important tools in the processes of human identification and must be used with caution and considered within a set of varied evidences.

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