International Journal of Biotechnology Research
Vol.11 No.2 July-December 2018

ISSN: 0974-6293

DNA Extraction for Sex Determination from Human Urinary Sediment

HEGAZI E.[1] AND OSMAN M.[2]

[1]Forensic Medicine and Clinical Toxicology Department, Faculty of Medicine, Alexandria University
[2]Genetic Engineering Institute, Menoufiya University

Urine has not been heavily studied as a potential source of DNA for forensic identification purposes. Therefore, the present study investigated the reliability of DNA extraction from human urinary sediment and its amplification by PCR for amelogenin testing and sex identification. The results were further compared with those of blood samples of the same donors.

The study was carried out on thirty adult males and female volunteers. Two samples were collected from every individual after taking informed consent; whole blood sample (by venipuncture), and urine sample.

DNA was extracted from urine and blood samples by organic phenol-chloroform method in amounts sufficient for PCR analysis.

DNA fragment patterns were indistinguishable from DNA banding patterns of blood specimens collected from the same donors.

In conclusion, extraction of DNA from human urinary sediment is applicable, and urine can be used as a reliable source material for usage as an individual discriminating material.

***Keywords**: Urine; DNA Fingerprinting; Sex Determination; Polymerase Chain Reaction.*

1. INTRODUCTION

Sex determination from dental pulp DNA was examined by loop-mediated isothermal amplification (LAMP) method, which was rapid and simple, and it should prove useful in unknown bodies of mass disasters. [1] Moreover, great interest has developed in the potential diagnostic use of DNA in plasma and serum for cancer research [2], prenatal diagnosis [3], and transplantation monitoring. [4], such cell free DNA arises from apoptosis (programmed cell death) and appears to be cleared very rapidly from the circulation. The kidney plays a role in the clearance of plasma DNA, and a proportion of the excreted DNA is thereby detectable in urine. [5]

The need for the use of urine as an identification tool may arise from a crime scene, to identify the perpetrator of a crime, or to place a victim at a particular site [6]. It may also arise in a toxicology laboratory as urine is often the sample of choice for drug screening in aviation / general forensic toxicology, workplace drug testing and doping in sport. In some instances, the origin of submitted samples may be challenged because of the medico-legal and socioeconomic consequences of a positive drug test [7].

There are evident advantages of urinary DNA-based technology compared with blood DNA. [8] Urine-based tests are absolutely non-invasive; (b) urine is non-infectious for HIV and less infectious for many other pathogens; and (c) isolation of DNA from urine is technically easier because the protein concentration is very low.

Despite these advantages, urine is not considered an ideal source of DNA due to the low concentration of nucleated cells present in human urine, and hence has not been heavily studied as a potential source of DNA for forensic identification purposes. [9]

Methods for identification of biological samples have reached a new boundary with the application

*Corresponding author: *E-mail: ???*

of the polymerase chain reaction (PCR) in DNA profiling, which made possible the analysis of previously undetectable levels of nucleic acids in body fluids. [10] Also, the use of the amelogenin gene has made sex determination much less complicated. The amelogenin gene is a single copy gene, which exists on the X and Y chromosomes; it is used to determine the sex in humans. Regions on this gene are amplified for simultaneous detection of the X and Y alleles in gender identification procedures. Presence of two amplified products indicates a male genotype, while a single amplicon implies female genotype. [11]

The aim of the present work was to determine the reliability of DNA extraction and sex identification by the amelogenin test, from human urinary sediment.

2. SUBJECTS AND METHODS

The study was carried out on thirty adult males and female volunteers. Two samples were collected from every individual after taking informed consent; whole blood sample (by venipuncture), and urine sample.

DNA was extracted from urine and blood samples by organic phenol-chloroform method.

2.1. Urine Samples

- Fresh urine was obtained from healthy volunteers (19 males and 11 females) ranging in age from 22-50 years.
- Urine samples (10 ml) were centrifuged at 1.500 rpm for 5 min after which 9.8 ml of the supernatant were discarded and the sediment was mixed with 1 ml of TNE buffer (10 mM pf Tris – HCl at pH 8.0, 1mM of Na_2 EDTA, and 100 mM of NaCl) and recentrifuged.
- Samples were then stored at -20 ^{o}C for no longer than 30 days. [12]

2.2. DNA Extraction from Urine: [12]

- Urine sediment obtained was collected into a polypropylene tube, those of females were first examined under the microscope for any sperm contamination, then 400 µL of lysing solution was added (TNE buffer at pH 8.0 containing 1% sodium dodecyl sulfate and 100 µg / mL proteinase K).
- The mixture was incubated for 4 hrs at 55^{o}C, extracted once with TE phenol (phenol saturated with TE buffer), twice with TE phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1), retaining the upper aqueous phase at each step.
- Sodium acetate was added to a final concentration of 0.3 M, and the DNA was precipitated with two volumes of chilled absolute ethanol at -20^{o} C overnight, followed by centrifugation at 15.000 rpm for 15 min.
- After washing with cold 70% absolute ethanol, the pellets were dried and resuspended in 30 µ L of TE buffer at 37^{o}C.
- Extracted DNA was quantified by spectrophotometeric analysis.

2.3. Blood Samples

- 10 ml whole blood samples were collected from the same individuals who volunteered for the research, after taking their consent.
- The blood was collected in two 5-ml tubes with EDTA as anticoagulant.

2.4. DNA Preparation from Blood: [13]

- To each 10 ml whole blood, 30 ml lysis buffer were added, shaken gently, incubated for 30 min on ice and centrifuged at 1200 rpm for 10 min at 4 ^{o}C.
- Supernatant was removed, and then 10 ml lysis buffer were added and centrifuged for 10 min at 4^{o} C (1200 rpm).
- Supernatant was removed and 5 ml SE buffer (NaCl and Na_2EDTA) were added to resuspend the pellet. 40 µl proteinase K and 250 µ L 20% sodium dodecyl sulfate (SDS) were added, shaken gently, and incubated overnight at 37 ^{o}C in a water bath.
- 5 ml SE-buffer and 40 ml phenol were added and shaken by hand for 10 min, followed by centrifugation at 3000 rpm for 5 min at 10 ^{o}C.
- The supernatant was transferred into a new tube and 10 ml of phenol: chloroform: isoamyl alcohol (25:24:1) were added shaken by hand for 10 min and centrifuged at 3000 rpm for 5 min at 10 ^{o}C.

- The supernatant was again transferred to a new tube and 10 ml chloroform: isoamylalcohol (24: 1) were added. Shaken by hand for 10 min, and centrifuged at 3000 rpm for 5 min at 10 ° C.
- The supernatant was finally transferred to a new tube and 300 µl of 3 M Sodium acetate (pH 5.2) was added together with 10 ml isopropanol & Shaken gently until the DNA precipitated.
- The DNA was washed in 70% ethanol and dissolved in 0.5-1 ml TE buffer overnight at 4 °C on a rotating shaker.
- DNA concentration was measured in a spectrophotometer.

2.5. PCR Amplification of the Amelogenin System: [14]

- Amplification of the X-Y homologous region was performed using the amplification primers:

  5/ -CTGATGGTTGGCCTCAAGCCTGTG-3/

  5/ -TAAAGAGATTCATTAACTTGACTG-3/

- The PCR reaction mixture included: 10 µl extracted DNA sample, 200 µM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 1.25 units of Taq DNA polymerase, 5 µL reaction buffer and 25 pmol of each X-Y primer.
- Samples were heated at 94 °C for 5 min in the first round of denaturation. Then were subjected to 32 cycles of PCR consisting of:

  1 min at 94 °C, 1 min at 52 °C and 2 min at 72 °C.

  Cycling was performed by automated DNA thermal Cycler (Gene Amp PCR System 9600, Perkin Elmer Cetus).
- After the last Cycle, the samples were incubated for an additional 15 min at 72 °C. Amplified DNAs that resulted in 977 and 788 bp products were checked on a 3% agarose gel.

3. RESULTS

DNA was successfully extracted from urinary sediments by the organic method. It was extracted from urine samples of all the volunteers (n = 30), even after a 30 day storage period. Sufficient DNA for PCR analysis was obtained from 10 ml of urine. The quantifiable amounts of DNA present were found to be greater in females (20-250 ng/ml) than in males (10-50 ng/ ml).

Microscopic precheck of female urine samples were all negative for sperm contaminations. Some samples, of both males and females, were contaminated with microorganisms (bacteria, yeasts, and protozoa) but the extracted DNA was useful for PCR amplification for the human amelogenin gene testing.

Samples were amplified by PCR and then typed for human amelogenin gene. All samples were typed without difficulty. High molecular weight DNAs with a distinct broad band were visible in all samples. Amplified bands of 977 bp on the X- chromosome, and 788 bp on the Y- chromosome could be clearly separated in all samples. (Figure 1). In all cases, the genotype corresponded with the registered sex.

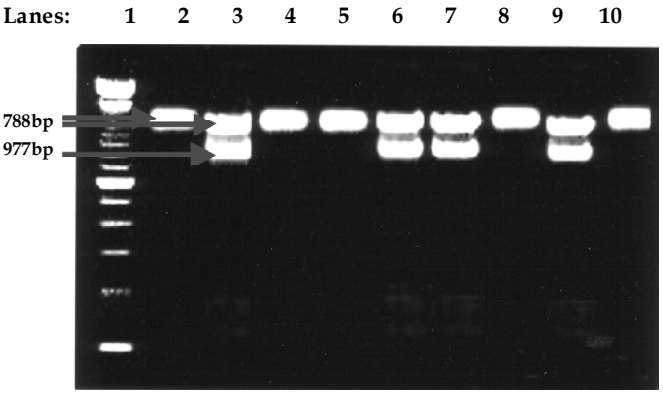

Figure 1: Amplification of the X-Y homologous gene amelogenin in urine samples. Lanes 2, 4, 5, 8 and 10 show one band at 977 bp indicating female genotype. Lanes 3, 6, 7, and 9 show two bands at 977 bp and 788 bp indicting male genotype

DNA extracted from urine samples was compared with that extracted from whole blood samples. The DNA fragment banding patterns were indistinguishable from DNA banding patterns of blood specimens collected from the same individuals.

4. DISCUSSION

The use of biological samples and bodily fluids as sources of DNA for identification has been thoroughly investigated and documented. [15] Urine, however, has not been heavily studied as potential source of DNA for forensic identification purposes. Therefore, the present work was

conducted to determine the reliability of DNA extraction from human urine and the possibility of applying the amelogenin test for sex identification from urine samples.

Reliable extraction of DNA was obtained with all urine samples, amounts being greater in females (20-50 ng/ml) than in males (10-50 ng/ ml). This is explained by the large differences between the amount of epithelial cells present in male and female urine. Females are more likely to have a higher number of epithelial cells present in their urine, vaginal cells being the chief contributors. [9] This agrees with the work of Yokota *et al*. (1998), who were able to extract sufficient amounts of DNA used for PCR (ranging from trace levels to 20 µg/ 10 mL urine) [12].

DNAs from microorganisms including; bacteria, yeast or protozoa, did not result in any problems in PCR amplification. Linfert *et al*., (1998) [10] also had no difficulty in typing adulterated urine samples, and this was because amplification was specific for the human DNA components. Although urine may contain proteins that can potentially interfere with PCR, such as albumin, hemoglobin and bilirubin, phenol-chloroform extraction can remove these substances [12]. Because sperm contamination could cause a misdiagnosis in X-Y identification of women, a microscopic pre-check of urine sediments was performed to prevent this situation.

In the present study, DNA extracted from urine was easily used for PCR-based investigations. Results of urine sex typing using the amelogenin test were identical with those of the whole blood samples and the registered sex of volunteers. Thus the present results demonstrated a genotype success rate of 100 %. Van der Hel *et al.* (2002), [16] however, demonstrated a success rate of 89.3% but their research was conducted on samples 15-25 years old.

Failures in genotyping of male individuals due to mutations originating in the Y-homologue of the amelogenin gene have been reported by Kashayap *et al*. (2006). [17] They calculated the overall failure rate among the Indian population to be 0.23%. Further studies in this area are desired. Also, further studies are needed in the field of mixed biological traces, as amplification can fail to reflect the true quantitative ratio between mixture components. [18].

Results obtained demonstrate that DNA can cross the kidney barrier, which agree with Botezatu *et al*., (2000) [8] who concluded that the kidney barrier is at least partially permeable to polymeric DNA. Lo *et al*., (1999) [5]estimated that 0.5 – 2% of the free DNA that passes through the blood stream crosses the kidney barrier and is excreted in urine. The molecular weight of DNA isolated from urine was high enough to allow its use for genetic analysis. Mechanisms by which such DNA can cross lipid bilayers have been proposed, yet, more research is needed to clarify it. [19]

In conclusion, urine collection is a much easier procedure than other sampling procedures. It can be used as a reliable sample for DNA extraction and sex identification. Urinary sediment can be easily used for genetic analysis, and DNA obtained from urine can be used for PCR despite the presence of adulterants and microorganisms. Furthermore, urine can be stored up to 30 days before DNA extraction and give excellent results. Thereby, urine can be used as a source material for PCR- based investigation.

Acknowledgement

The authors would like to express their gratitude to Professor Dr. Laila Abdel Meguid, Professor of Forensic Medicine and Clinical Toxicology, for her great assistance and valuable suggestions.

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