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TECHNICAL NOTE

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Variation in Nuclear DNA Concentrations During Urination*

ABSTRACT: This study examined the cellular origin and concentration of nuclear DNA in human urine. Ten subjects provided two entire, first-morning voids: one as a single specimen and one as a consecutive series of samples. The serial samples were centrifuged, organically extracted, and quantified by slot-blot analysis. Total DNA concentrations ranged from 0.02 to 21.3 ng/mL for the males and 25.0 to 96.9 ng/mL for the females. The female samples were found to contain numerous vaginal epithelial cells. DNA was detected in all of the serial samples of nine subjects; however, the DNA concentrations varied considerably. With six subjects, the DNA concentration of the first serial sample was at least three times greater than that of the entire void. DNA was only detected in the first 21% of the void from one male subject. The results of this study have implications for the collection of urine samples.

KEYWORDS: forensic science, criminalistics, forensic biology, human urine, micturition, nuclear DNA, DNA quantitation

Forensic investigations can pivot on the ability to individualize human urine. The identity of the urine specimen donor, for example, may become the point at issue in toxicological casework. Moreover, the individualization of urine stains can establish the physical link between a person and a crime scene in criminal investigations. Numerous procedures have been developed for the isolation of nuclear DNA from human urine, and DNA profiling has been achieved with RFLP and PCR-based typing systems (1–4). Presently, the ability to extract, amplify, and type urinary DNA appears to be largely limited by the initial quantity and quality of nuclear DNA (5,6).

The amount, and in particular the distribution, of nuclear DNA in human urine came into question in casework conducted by the authors. The casework included large urine stains and groups of dried urine drops, which were sampled for STR analysis. In some cases, only localized areas of the large urine stains yielded DNA. In other cases, only certain drops of a group of urine stains yielded DNA. The DNA recovered from these restricted areas was of sufficient quantity and quality to produce full STR profiles with the AmpF*l*STR® Profiler® and COfiler® PCR amplification kits (Applied Biosystems, Foster City, CA). Contrastingly, samples taken adjacent to these areas were negative for DNA. Thus, in some submissions, the DNA appeared to be distributed as irregularly spaced "hotspots." This was seen with evidentiary stains of both male and female donors. The results suggested that urinary DNA concentrations may vary spatially and temporally. Previous investigations on the DNA content of human urine have examined static concentrations, that is to say, the total DNA concentration of an entire void or portion of a void. In the present study, the temporal distribution and cellular origin of urinary DNA were investigated. Here, we report variations in DNA concentrations during the process of urination, which appears to be the first description of this property of urine. The results of this study have implications for the collection of liquid samples.

Materials and Methods

Urine Collection

Six adult males and four adult females participated in this study. A greater number of male subjects were selected because males reportedly have lower urinary DNA concentrations than females (3–7). Each subject provided two entire voids on separate days. The voids were the first of the morning. One void was collected whole in a sterile 500 mL container for microscopic examination. The other void was collected as sequential samples for DNA quantitation. For this collection, the male subjects used a series of sterile 50 mL conical centrifuge tubes, whereas the female subjects used sterile 150 mL specimen cups. The serial samples were collected by each subject urinating, from the start of the void, into the first container until a volume of $\sim$ 50 mL of urine was reached. The subject then proceeded to urinate into the second container to a volume of $\sim$ 50 mL. This process was continued with the subsequent containers until the entire void was collected. The transition from one container to the next was typically without interruption as reported by the subjects. The urine samples submitted by the female subjects were later transferred to 50 mL conical centrifuge tubes at the laboratory. (The centrifuge tubes were graduated to 50 mL, but could hold a larger volume. The 50 mL volume was exceeded in some cases.)

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Urinalysis

A preliminary study was conducted by the authors to develop a procedure for the processing of large volume, liquid samples. Initially, the urine samples were stored and centrifuged at 4°C. However, on cooling the samples to 4°C, an amorphous precipitate formed in the samples of four subjects. The precipitate was of such large amounts that it interfered with the analysis of the centrifugation pellet. The source of the precipitate was not determined; however, by processing the specimens at room temperature, after storage at 4°C, the precipitate was eliminated in the samples of three subjects, and greatly reduced in those of the fourth subject. This procedure was then followed for the samples included in this study, which were stored at 4°C for 1–6 h before processing.

The void submitted whole was thoroughly mixed and then transferred to 50 mL conical centrifuge tubes. The aliquots were spun at 2753 × g for 30 min, at 25°C, with an Allegra 6R Centrifuge (Beckman Coulter Inc., Fullerton, CA). The resultant pellets were sampled and applied to microscope slides. The samples were examined as unstained preparations by phase contrast microscopy, and as stained preparations by bright-field microscopy, at ×200 and ×400 magnification. The cytological stain used was hematoxylin and eosin.

The sequential samples were processed as follows: first, the volume of each sample was measured using volumetric pipettes, and the first sample of each series was tested for the presence of blood with the Kastle–Meyer test. (The Kastle–Meyer test was laboratory prepared [2.0 g phenolphthalein, 20.0 g potassium hydroxide, and 500 mL distilled water refluxed with 20.0 g powdered zinc for 2–3 h, followed by the addition of 100 mL ethanol], and could detect blood diluted 10,000 times.) The samples were then spun at 2753 × g for 30 min, at 25°C, with an Allegra 6R Centrifuge (Beckman Coulter Inc.). The resultant supernatant was removed from each sample, and the pellet was extracted by an organic procedure. The organic procedure included: (1) a cell lysis step (~ 12 h incubation at 56°C in 400 µL of stain extraction buffer [10 mM Tris, 10 mM EDTA, 100 mM NaCl, 39 mM dithiothreitol, 2% SDS, 20 µL of 10 mg/mL proteinase K, and pH 8.0]), (2) a phenol/chloroform/isoamyl alcohol extraction step, and (3) a Centricon® YM-100 (Millipore Corp., Billerica, MA) concentration and recovery step. The cell lysis step of the procedure began on the day of receipt of the urine samples. The lysis buffer was added directly to the pellets in the 50 mL centrifuge tubes, and the resultant digests were later transferred to 2 mL microcentrifuge tubes.

Following the organic extraction, the DNA samples were quantitated by slot blotting. The QuantiBlot Human DNA Quantitation Kit (Perkin Elmer, Foster City, CA) was used, with the provided D17Z1 primate-specific probe, in combination with the Bio-Dot SF Microfiltration Apparatus (Bio-Rad Laboratories Inc., Hercules, CA). The slot-blot procedure followed the manufacturer's recommendations. Visualization of the bound DNA probe was by chemiluminescence with ECL™ Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ) and a Kodak X-OMAT™ LS film (Eastman Kodak Co., New Haven, CT). Each DNA extract was quantified once. A 0.04 ng human DNA standard was detected with each slot-blot assay performed in this study.

Results

Questionnaire

The questionnaire results were used in this study for general comparisons; therefore, the data will be summarized here. The male and female subjects were 27–46 and 27–44 years of age, respectively. Reportedly, the subjects were in good health. Moreover, none of the subjects reported conditions that may have complicated the analysis of the voids. For example, none of the subjects reported the possibility of blood, pus, or spermatozoa in their urine samples. For the DNA quantitation study, each subject provided the time interval between the tested void and the preceding void. The interval ranged between 5.7 and 8.5 h for the male subjects, and between 7.5 and 9.5 h for the female subjects.

Urinary Sediment Analysis

The amount and composition of the urinary sediment varied between subjects. Collectively, the material identified microscopically included epithelial cells, urinary casts, oval fat bodies, granular bodies, crystals, amorphous sediment, and bacteria. Erythrocytes and leukocytes were not found in any of the preparations. Overall, the types of epithelial cells present in the sediment samples could be identified based on morphological and staining characteristics. Nucleated squamous epithelial cells were found, in large numbers, in the sediment samples of the majority of the female subjects. Contrastingly, the sediment samples of the majority of male subjects showed few cells. The cells identified in the male samples were predominantly transitional epithelial cells. Transitional epithelial cells line the ureters, urinary bladder, and part of the urethra. Renal tubule cells of the kidneys were found in samples from some of the male subjects, but their occurrence was rare. Transitional and renal tubule cells were also present in samples from some of the female subjects.

Urinary DNA Quantitation

The voids submitted for DNA analysis tested negative for the presence of blood by the Kastle–Meyer test. The amount of urinary sediment, after centrifugation at room temperature, ranged from 1.4 to 3.3 g per void for the male subjects and from 1.6 to 2.5 g per void for the female subjects. The average volume of the sequential samples was 41.0 mL for the 10 subjects. The volume and DNA concentration of each serial sample is given in Table 1 for the male subjects and in Table 2 for the female subjects. Each sample set is listed in consecutive order. Additionally, the calculated total volume and DNA concentration of the sequentially sampled voids are given in Table 3. The DNA concentration of the entire void (sequentially sampled) was determined by summing the amount of DNA in each serial sample, and then dividing the total by the sum of the sample volumes. Also given in Table 3 is the DNA concentration of the serial sample of each subject's void that includes the halfway point of the volume of the void. These samples were used to approximate midstream, clean-catch urine specimens for comparative purposes.

Discussion

The human urinary system consists of the kidneys, ureters, urinary bladder, and urethra. The uriniferous tubules of the kidneys and the passages of the urinary tract are lined with an epithelium. The superficial cells of the epithelial linings undergo exfoliation, and can be found in the urinary sediment of normal individuals (8–10). In this study, the microscopic examination revealed numerous squamous epithelial cells in the sediment samples of most of the female subjects. These cells probably represent contamination from vaginal and vulvar sources as a result of the collection procedure. The samples from the male subjects

TABLE 1—*Volume and DNA concentration of the serial urine samples from the male subjects.*

	Serial Sample															
	1		2		3		4		5		6		7		8	
Subject	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.
A	64.0	0.1	64.0	0	64.0	0	51.0	0	47.0	0	10.0	0	—	—	—	—
B	38.4	1.4	45.0	0.1	41.3	0.2	44.4	0.1	41.4	0.03	—	—	—	—	—	—
C	35.0	0.08	34.0	0.06	37.8	0.02	22.0	0.09	—	—	—	—	—	—	—	—
D	43.0	157.0	40.0	6.8	48.6	4.2	47.0	4.3	44.2	4.6	48.0	2.8	48.0	1.3	49.0	0.4
E	50.4	0.09	49.4	2.1	46.5	0.8	53.0	0.5	51.0	0.5	45.4	0.4	—	—	—	—
G	46.0	0.1	48.8	0.02	50.0	1.4	48.4	2.1	47.0	2.2	44.3	1.5	49.5	2.0	—	—

Vol., volume of urine aliquot in mL; conc., DNA concentration in ng DNA/mL urine.

presumably better represent the cellular and DNA contributions of the urinary organs. However, in this study, the microscopic examination was performed on one void and the quantitative analysis was performed on another void. Therefore, cells extraneous to the male urinary system proper cannot be excluded as possible DNA sources in the quantitation study, because the cellular composition of the urine samples was not directly examined. This analytical approach was adopted because of the reported scarcity of cells in normal urine, particularly with male subjects (4,6,7).

In general, the voids from the male subjects showed fewer cells and lower amounts of DNA than those from the female subjects. A difference between the sexes in urinary DNA concentrations has been observed in other studies (3,4). In the present study, the calculated total DNA concentration of the sequentially sampled voids ranged from 0.02 to 21.3 ng/mL for the males and from 25.0 to 96.9 ng/mL for the females (Table 3). By comparison, Vu et al. (3) reported DNA yields of 4–60 ng for male subjects and 14 to >200 ng for female subjects, from a 1 mL sample of the subjects' fresh, midstream, clean-catch specimens. In the present study, the DNA yield of a midstream, clean-catch specimen can be estimated for each subject from the data obtained for a serial sample that includes the halfway point of the volume of the void. Following this approach, the estimated midstream DNA yields ranged approximately from 0 to 5 ng/mL for the male subjects and from 4 to 62 ng/mL for the female subjects (Table 3). These ranges are lower than those of Vu et al. The differences between the two results may represent human variation; however, there may be other contributing factors, such as, differences in the effectiveness of the extraction procedures. Importantly, the research of Vu et al. is cited here because the authors specified the portion of the void that was analyzed. This information is necessary because our study indicates that urinary DNA concentrations can vary during the course of urination. For comparisons of yields to be relevant, therefore, the collection and sampling methods of the urine specimens need to be equivalent, i.e., the part of the void collected (beginning, middle, end, or whole) should be specified in reports and publications to allow for the comparison of DNA yields.

As stated previously, our research indicates the possibility of intraurination variation in DNA content. With six subjects (A, B, D, G, H, and I) in this study, the DNA concentration of the first serial sample was at least three times greater than that of the entire void. Specifically, the male subjects showed a 4–7-fold difference, whereas the female subjects showed a 3–11-fold difference. In the case of male subject A, DNA was only detected in the first 21% of the void. With male subject E, the second serial sample showed the highest DNA concentration of the set, which was three times greater than that of the entire void, and four times greater than that of an estimated midstream sample. We estimate that with five subjects (B, D, F, H, and I), the DNA concentration of the first serial sample would have been at least five times greater than that of a midstream sample. Specifically, we estimate a 7–34-fold difference for the male subjects, and a 5–31-fold difference for the female subjects in this group.

The differences in DNA concentrations between initial samples, midstream samples, and entire voids may significantly affect the ability to genotype human urine. This assertion is best illustrated by the results obtained from the serial samples of subject A. A target amount of 1.5 ng DNA for amplification would require 75 mL of the entire (mixed) void from subject A, whereas only 13 mL of the subject's initial sample would be needed to obtain the targeted amount. Moreover, a midstream catch sample from this subject would not have yielded DNA by our methods.

Our preliminary findings suggest that the ability to recover nuclear DNA from liquid urine samples can be optimized, in the majority of cases, by collecting the first portion of the void. Collecting the initial void, as a toxicological sample, may increase the likelihood of nuclear DNA typing in cases of questioned donorship. This approach may be particularly useful with specimens from male subjects, which have been problematic in the experience of the authors and other investigators (4,7). Before its

TABLE 2—*Volume and DNA concentration of the serial urine samples from the female subjects.*

	Serial Sample																			
	1		2		3		4		5		6		7		8		9		10	
Subject	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.	Vol.	Conc.
F	27.5	101.3	26.2	39.1	30.7	12.8	31.4	4.3	36.5	5.5	30.5	11.1	—	—	—	—	—	—	—	—
H	36.2	279.7	37.5	360.0	40.0	126.6	36.2	10.2	41.0	61.7	30.5	48.4	52.4	18.1	47.5	13.3	41.7	12.6	—	—
I	15.0	450.0	12.5	202.5	20.0	5.2	37.5	16.9	33.5	16.1	35.0	7.7	37.5	14.4	45.0	11.7	40.0	21.1	51.0	10.3
J	47.5	9.5	42.5	19.8	56.0	30.1	42.5	59.6	45.5	27.8	47.5	17.8	42.5	29.8	48.7	10.8	18.0	19.2	—	—

Vol., volume of urine aliquot in mL; conc., DNA concentration in ng DNA/mL urine.

TABLE 3—*Calculated total volume, midstream DNA concentration, and total DNA concentration of the sequentially sampled void for each subject.*

Subject	Sex	Void (mL)	Midstream (ng DNA/mL)	Void (ng DNA/mL)
A	M	300.0	0	0.02
B	M	210.5	0.2	0.3
C	M	128.8	0.06	0.06
D	M	367.8	4.6	21.3
E	M	295.7	0.5	0.7
F	F	182.8	4.3	26.7
G	M	334.0	2.1	1.3
H	F	363.0	61.7	96.9
I	F	326.0	14.4	40.7
J	F	390.7	27.8	25.0

implementation, however, further research is needed to evaluate fully the advantages of this collection method. Moreover, the issue of sample donorship may not be significant in the casework of a laboratory to necessitate a change in collection method.

In forensic casework, the authors have collected samples from a large bloodstain or from a series of blood drops where the samples did not give equivalent amounts of DNA. That is to say, the concentration of DNA was not uniform across the large bloodstain or between the serial blood drops. In these cases, decomposition was the likely cause of the unequal concentrations of DNA. The stains showed various indications of degradation including low DNA yields and partial STR profiles. As previously stated, the present study was initiated by the irregular distribution of DNA seen within large urine stains, or between serial drops, in casework submissions. These stains, however, did not show evidence of degradation. One possible explanation for this finding may be provided by the results of this study, i.e., the heterogeneous distribution of nucleated cells, and therefore DNA, in the excretion of urine may be continued in the formation of urine stains. However, urine stains were not the subject of this investigation, and the distribution pattern of DNA in urine stains remains to be elucidated.

In the present study, the first void of the morning was examined. This void presumably has one of the longest interurination intervals of daily voids. Reportedly, most people urinate about four to six times each day, and mostly during the daytime (8). The frequency of urination may be one factor that affects the amount of exfoliated cells in the urinary tract, and consequently urinary DNA concentrations. Therefore, other daily voids need to be tested to determine the extent of intra- and intervoid variation in DNA content. Lastly, our preliminary findings indicate that urinary DNA can exhibit different distribution patterns; however, additional research is needed to fully evaluate this phenomenon and its implications to forensic casework.

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