TECHNICAL NOTE

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Identification of Urine Specimen Donors by the PM+DQA1 Amplification and Typing Kit

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ABSTRACT: We evaluated the ability to genotype DNA extracted from urine samples, which were previously submitted for toxicological analysis, by either the AmpliType HLA DQα or the combined PM+DQA1 amplification and typing systems. Initial experiments were conducted on fresh urine, which was either processed fresh or frozen for one week at −20°C, from male and female volunteers. Although male urine is noted for containing minimal numbers of nucleated cells when compared with female urine, we were able to type these samples without difficulty. Male urine specimens that were stored frozen for one year in the Toxicology Laboratory provided sources of low concentration, poor quality genomic DNA with respect to degradation of nucleic acid. These samples, however, were also easily typed using the amplification typing kits. Our data, therefore, suggest that the PM+DQA1 amplification and typing systems described here are suitable for typing analysis of donor urine specimens.

KEYWORDS: forensic science, forensic toxicology, urine, DNA, polymerase chain reaction, Amplitype PM, HLA-DQA1, LDLR, GYPA, HBGG, D7S8, GC

Federal workplace testing for drugs of abuse is regulated by the Substance Abuse and Mental Health Services Administration (SAMHSA) (1). Many private employers have adopted similar drug testing programs as a prerequisite to gaining and maintaining employment. Because of the economic and social consequences of a positive urine drug test result, there has been an increase in the submissions of either adulterated or substituted urine specimens (2). Considerable efforts have been made by laboratories to detect the dilution of urine by using tests such as urine creatinine and specific gravity (3). The Department of Transportation has established limits of 20 mg/dL and 1.003 for these tests, respectively (4). Tests for foreign substances, such as glutaldehyde (known to invalidate many immunoassay screening tests), are also commercially available (e.g., Chimera, Research & Chemicals, Inc., Seminole, FL).

Guaranteed drug free urine for the purpose of substitution can be purchased through a number of vendors. Two procedures are currently in use for detection of substituted urine: monitoring urine temperature immediately after collection, and witnessing the collection of urine itself. Federal Guidelines currently specify that freshly voided urine must be between 90 and 100°C. Unfortunately, acceptable temperatures can be achieved if the substituted urine is stored in the axilla, vaginal cavity, or next to the scrotum just before donation (5). Even witnessed collections are not foolproof against substitution, as documented by a well-known former professional football player (6). The best procedure for verifying a urine sample as originating from one individual is to perform some sort of serologic or DNA typing analysis. These techniques can be useful in determining if misidentification or switching of samples has occurred by either the collection agency, testing laboratory, or donor.

Analysis of blood group antigens and polymorphic proteins have been used when donor identification is in question. However, the feasibility and reliability of these assays on stored frozen specimens are poor with major limitations because of low protein concentrations in urine and commonly noninformative results (7). Recent advances in molecular technologies have proven very useful to the forensic science laboratories for the analysis of nucleic acids in evidentiary samples. Identity testing has reached new limits based on extremely sensitive polymorphic genetic marker assays that do not discriminate among intact or degraded DNA, stain, smear, tissue, or body fluid specimens, and environmental contamination.

Polymorphic variation at the nucleic acid level has made possible molecular identification of biological specimens through DNA typing techniques. Southern blot/RFLP technologies require large amounts of high molecular weight DNA and are extremely laborious, while *in vitro* amplification techniques such as the polymerase chain reaction (PCR) provide the ability to analyze evidentiary samples that most often contain degraded DNA, offer increased sensitivity and shortened turn-around times (8–14).

The first PCR-mediated assay to become commercially available for analyzing DNA from evidentiary samples was the AmpliType HLA DQα typing kit (Perkin Elmer, Foster City, CA) (14–18). Recently, a combined PM+HLA DQA1 (previously described as HLA DQα) typing kit has become available (Perkin Elmer, Foster City, CA) for typing six genetic loci simultaneously. As urine drug screening programs gain popularity in the workplace and for athletic events, the temptation for substituting urine specimens becomes increasingly attractive. Thus, we wanted to establish an accurate and sensitive method for molecular identity testing of

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urine specimens for our Toxicology Laboratory. Using the PCR-mediated PM+DQA1 typing system described, we were able to DNA type urine specimens based on six genetic loci with DNA isolated from urinary epithelial cells and leukocytes as a target.

Materials and Methods

Samples

Whole blood and urine samples were collected from five male and five female volunteers in the laboratory. These urine samples were processed immediately after collection. Six additional urine specimens and matching blood specimens were stored at 4°C for one week before processing. Urine from a single female donor was processed either fresh or after freezing at −20°C for one week. Eight urine samples that were stored for one week at 4°C and then frozen at −20°C for at least one year were obtained from the Toxicology Laboratory for evaluation of long-term storage on nucleic acid extraction and amplification capabilities. All urine samples were collected without preservatives.

DNA Extraction

DNA was isolated from whole blood specimens by extraction of 300 μL of EDTA collected blood using the Puregene Genomic DNA Isolation Kit (GENTRA, Minneapolis, MN). Similarly, DNA was isolated from urinary epithelial cells collected by centrifugation of urine samples. Approximately 40 mL of urine was centrifuged for 15 min at 2500 rpm. The pellet consisting of urine sediment that included cells was then processed using the DNA extraction kit according to the recommendations of the manufacturer. Briefly, cells are lysed and treated with RNase after which proteins are precipitated. DNA is precipitated from the resulting supernatant and rehydrated in buffer. Concentrations and purity of DNA samples were performed by absorbance spectrophotometry at 260 and 280 nm using a double-beam spectrophotometer (Model DU650, Beckman Instruments, Fullerton, CA).

Polymerase Chain Reaction

DNA amplification was performed in 100-μL reactions according to the recommendations of the manufacturer of the typing kit. All samples (approximately 0.5 to 1.0 μg blood DNA; 0.4 to 1.0 ng urine DNA) were amplified in thermal cyclers (Models 2400 or 9600, Perkin Elmer, Foster City, CA). DNA samples were denatured at 94°C for 30 s before PCR amplification. Amplification was accomplished using 32 cycles, each consisting of 30 s denaturation (94°C), 30 sec primer annealing (63°C), and 30 s extension (72°C). The final cycle included a 7 min extension step at 72°C.

HLA DQα and PM+DQA1 Typing

We initially typed specimens using the original HLA DQα typing system, which is based on a reverse dot blot procedure, according to the recommendations of the manufacturer (Perkin Elmer, Foster City, CA). We aso evaluated a new combined amplification/reverse dot blot typing kit (Perkin Elmer) for application to urine donor identification. This kit allows for the typing of six individual loci which include HLA DQA1 (previously referred to as DQα), low density lipoprotein receptor (LDLR), glycophorin A (GYPA), hemoglobin G gammaglobulin (HBGG), D7S8, and group specific component (GC). In this kit, the HLA DQA1 allele 4 is further subtyped into a 4.1 or 4.2/4.3 allele. Final typing determinations were performed independently by two laboratory personnel, once typing strips were processed.

Results

Traditionally, our laboratory has used a nonorganic high salt extraction protocol for extraction of whole blood and tissues (19). However, in cases in which biological specimens are very small, this procedure does not result in sufficient quantity of DNA for traditional RFLP-based molecular analysis. Because of low numbers of epithelial cells in urine samples, we used a commercially available kit designed for extraction of DNA from small specimens. We found that in ten fresh urine specimens, the concentration of extracted DNA was 5-fold higher in females (mean A_{260} = 0.0184) versus males (mean A_{260} = 0.0040) presumably because of increased epithelial cells exfoliated into urine of women. Concentrations of DNA isolated from previously frozen (one year) urine samples received from the Toxicology Laboratory were markedly decreased and of poorer quality when compared with those from fresh urine, independent of gender. An aliquot of fresh urine from a single female donor which was frozen for one week produced a 3-fold decrease in the amount of isolated DNA (A_{260} = 0.0120) when compared with an aliquot of the same urine sample processed fresh (A_{260} = 0.0357).

We first evaluated our ability to type specimens with the original HLA DQα typing kit. DNA extracted from whole blood was easily

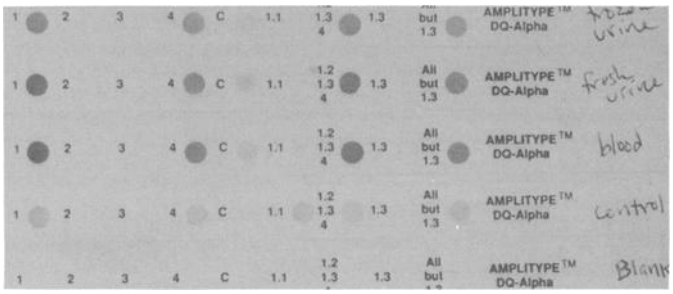

FIG. 1—*Typing strips for HLA DQα comparing female DNA from blood, fresh urine, and urine frozen for one week. The DNA shows the same typing results (1.2, 4) as expected from the same individual.*

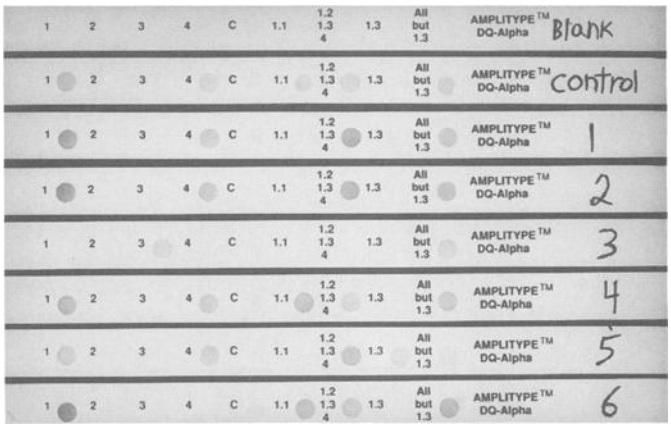

FIG. 2—*Typing strips for HLA DQα comparing fresh male urine (1), fresh female urine (2), and male urine frozen for one year (3–6). Individuals 1 and 2 share the same typing results (1.2, 4), indicating a need for further discrimination using other genetic loci. Individuals 3–6 show different typing results (3, 3; 1.1, 4; 1.3, 4; 1.1, 1.2, respectively).*

TABLE 1—*Absorbance spectrophotometry and PM+DQA1 results from fresh urine and blood matching samples.*

Case number	Urine		Blood		Typing	
	260 nm	260/280	260 nm	260/280	DQA1	PM
95-56 (female)	0.186	0.246	0.151	1.987	1.1, 2	AA,AB,AC,AA,BC
95-57 (female)	0.149	0.330	0.113	2.093	3, 3	AA,AB,AA,AB,AA
95-58 (female)	0.106	0.338	0.498	1.894	1.3, 1.2	AA,AA,AB,AA,CC
95-59 (female)	0	—	0.443	1.909	1.1, 1.1	BB,AB,AB,AA,AB
95-60 (female)	0	—	0.145	2.042	1.1, 3	AB,AA,AB,AB,AC
95-61 (male)	0.232	0.396	0.246	1.875	1.2, 1.2	AB,AB,AB,AA,AC

typed using this protocol (Fig. 1). In addition, DNA isolated from fresh female urine samples was also easily typed, whether processed fresh or after being frozen at −20°C for one week (Fig. 1). In an initial evaluation of sensitivity, we were able to type samples with as little as 400 pg of target DNA that was quantitated by absorbance spectrophotometry.

To evaluate typing capabilities with the HLA DQα kit further, we isolated DNA from urine specimens that were frozen at −20°C for one year. Although the quantity and quality of DNA were poor as determined by absorbancies at 260 and 280 nm, these samples were also easily typed (Fig. 2). Dilution experiments of a single long-term frozen urine DNA sample resulted in typing being obtained in as low as a 1:100 dilution of the original isolated DNA. The accurate quantitation of this sample was not possible with spectrophotometry as the absorbance (260/280 nm) ratio was 0.3. However, this did indicate that the typing is not dependent on the purity of the DNA sample. As control specimens in these studies, DNA isolated from fresh urine, male and female, was also typed (Fig. 2). Interestingly, the two volunteer donors from the same laboratory were found to have the same typing results with the HLA DQα kit (Fig. 2).

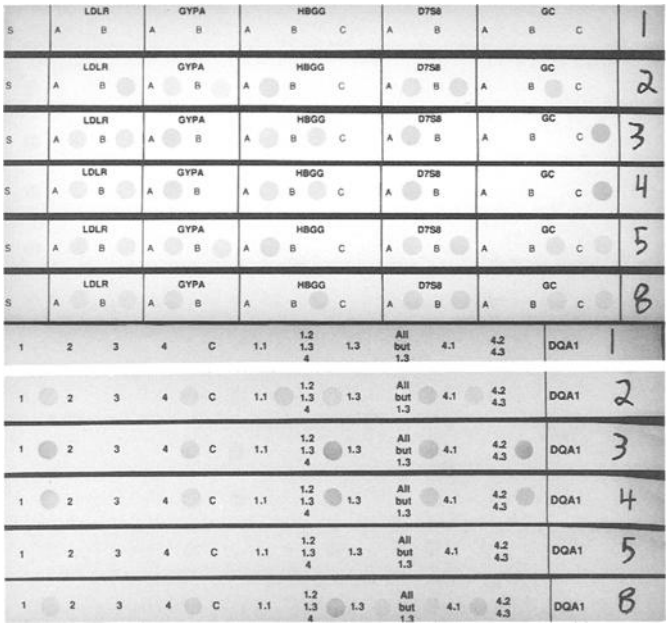

FIG. 3—*Typing strips for the PM(A)+DQA1 (B) system. 1, blank; 2, kit control DNA; 3, fresh female urine (PM: AB,AA,AB,AA,CC; DQA1: 1.2, 4.2/4.3); 4, female urine one week frozen (PM: AB,AA,AB,AA,CC; DQA1: 1.2, 4.2/4.3); 5, fresh male urine (PM: AB,AB,AA,AB,BC; DQA1: 1.2, 4.1); 8, male urine one year frozen (PM: AB,AA,BB,AB,BC; DQA1: 1.3, 4.1).*

We extended our analysis to include results with a newly available PM+DQA1 typing kit. Fresh (male and female), short-term (one week) frozen (female), and long-term (one year) frozen (male) urine specimens were easily typed with this system (Fig. 3A and B). In this combined kit, the new DQA1 typing strips contain additional subtypes of the 4 allele (4.1, 4.2, or 4.3). In the case of the two volunteers who typed the same with the original kit, this subtyping distinguished their individuality (Fig. 3B). Although the photograph of typing results for specimen #5 (Fig. 3B, HLA DQA1) is not ideal, typing results were visible at the time of processing. We have no explanation for the faintness of these signals as the typing of this same specimen is shown in Fig. 2 #1. In addition, each of six individuals contributed a fresh urine and blood specimen which was correctly typed using both the DQA1 and PM typing strips despite poor quality DNA preparations (Table 1). Urine samples 95-59 and 95-60 showed no evidence of DNA being present by absorbance spectrophotometry at 260 nm, yet both samples were capable of being typed with results that matched the donors blood typing (Table 1).

Discussion

Human urine specimens as a potential source of DNA for forensic identity testing has not been explored to the extent of applications for a forensic toxicology laboratory. It is well known that normal human urine specimens contain few nucleated human cells (up to 400 cells/mL urine). Epithelial cells (i.e., renal tubular, transitional urothelial, and squamous) are commonly found in low numbers in normal urine specimens (20). The high numbers of squamous epithelial cells in female urine specimens indicates vaginal contamination (20). More common are leukocytes that do not exceed 300–500 cells/mL. All cells included, the source of DNA in urine samples remains small and is potentially abrogated by contaminating materials in the urine sediment.

Our evaluation of this potential source of DNA for donor verification demonstrates the utility of this method for the forensic toxicology laboratory and forensic identity testing laboratories in general. PCR-mediated allelic typing was successful on urine specimens processed immediately and after various times of storage at −20°C using the DQα and PM+DQA1 typing kits. We were also able to type correctly 6/6 specimens using matched blood and urine specimens from the same donors. Of particular significance was the success of DNA extraction from the urine of males after long-term storage, although the isolated DNA was of poor quality. In women, large numbers of squamous epithelial cells are slothed into urine as the result of vaginal contamination, and thus, a higher yield of extracted DNA is achieved. The cellular content of urine from males is considerably lower. Nevertheless, there are sufficient numbers of cells present in these urines to perform the PM+DQA1 typing.

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References

(1) Department of Health and Human Services, Mandatory guidelines for federal workplace drug testing programs, Notice Fed Register, 1994;59:29908–31.
(2) More people drinking fluids to avoid testing positive, Drug Det Report, 1993;3:5.
(3) O'Connor E, Ostheimer D, Wu AHB. Limitations of forensic urine drug testing by methodology and adulteration, A.A.C.C. Ther Drug Monit-Toxicol Update, 1993;14:275–96.
(4) Department of Transportation. Procedures for transportation workplace drug testing programs: final rule and notice of conference. Fed Register, 1989;54:49853–76.
(5) Person NB, Ehrenkranz JRL. Fake urine samples for drug analysis: hot, but not hot enough. JAMA, 1988;259:841.
(6) Taylor L, Faulkner DLT. Living on the edge. Times Books, New York, 1987;158.
(7) Holland MM, Roy R, Fraser MD. Serological and DNA methods for the identification of urine specimen donors In: Handbook of workplace drug testing. Liu RH, Goldberger BA, editors. A.A.C.C. Press, Washington, DC, 1995;168–76.
(8) Brinkman B, Rand S, Bajanowski T. Forensic identification of urine samples, Int J Leg Med 1992;105:59–61.
(9) Medintz I, Chiriboga L, McCurdy L, Kobilinsky L. Restriction fragment length polymorphism and polymerase chain reaction-HLA DQα analysis of casework urine specimens. J Forensic Sci 1994;39:1372–80.
(10) Brinkman B, Rand S, Wiegand P. Population and family data of RFLP's using selected single- and multi-locus systems. Int J Leg Med 1991;104:81–6.
(11) Tsongalis GJ, Coleman WB, Esch GL, Smith GJ, Kaufman DG. Identification of human DNA in complex biological samples using the Alu polymerase chain reaction. J Forensic Sci 1993;38:961–7.
(12) Hochmeister MN, Budowle B, Borer UV, Rudin O, Bohnert M, Dirnhofer R. Confirmation of the identity of human skeletal remains using multiplex PCR amplification and typing kits. J Forensic Sci 1995;40:701–5.
(13) Shibata D, Kurosu M, Noguchi TT. Fixed human tissues: A resource for the identification of individuals. J Forensic Sci 1991;36: 1204–12.
(14) Comey CT, Budowle B, Adams DE, Baumstark AL, Lindsey JA, Presley LA. PCR amplification and typing of the HLA DQα gene in forensic samples. J Forensic Sci 1993;38:239–49.
(15) Erlich H, Bugawan T, Begovich A, Scharf S. Analysis of HLA class II polymorphisms using polymerase chain reaction. Arch Pathol Lab Med 1993;117:482–5.
(16) Walsh PS, Fildes N, Louie AS, Higuchi R. Report of the blind trial of the cetus AmpliType HLA DQα forensic deoxyribonucleic acid (DNA) amplification and typing kit. J Forensic Sci 1991;36:1551–6.
(17) Herrin G, Fildes N, Reynolds R. Evaluation of the AmpliType PM DNA test system on forensic case samples. J Forensic Sci 1994;39:1247–53.
(18) Fildes N, Reynolds R. Consistency and reproducibility of the AmpliType PM results between seven laboratories: Field trial results. J Forensic Sci 1995;40:279–86.
(19) Tsongalis GJ, Kaufmann WK, Wilson SJ, Friedman KJ, Silverman LM. Rapid screening of p53 mutations with a sensitive heteroduplex detection technique. Clin Chem 1994;40:485–6.
(20) Haber MH. Urine. In: Clinical laboratory medicine, McClatchey KD editor, Williams and Wilkins, Baltimore, MD 1994;513–48.

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