TECHNICAL NOTE

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The Effect of Pathologic Substances and Adulterants on the DNA Typing of Urine

REFERENCE: Linfert DR, Wu AHB, Tsongalis GJ. The effect of pathologic substances and adulterants on the DNA typing of urine. J Forensic Sci 1998; 43(5):1041–1045.

ABSTRACT: Human urine has not been adequately investigated as a potential source of DNA for forensic identity testing. The advent of polymerase chain reaction technology has made possible the analysis of previously undetectable levels of nucleic acids from human urine and other body fluids lacking nucleated cells. In this study, we evaluated the ability to genotype DNA extracted from adulterated urine specimens using the AmpliType® PM + DQA1 PCR amplification and typing system. Fresh, first-void male urine specimens were contaminated with household bleach, *E. coli*, human serum albumin, glucose and saponin (a strong detergent). All of the adulterated samples were typed without difficulty. Frozen male urine specimens were split into equal volumes; one aliquot was adulterated with either *E. coli* or saponin, and the other was left free of contaminants. Seventy-one percent of all frozen urine specimens tested (adulterated and unadulterated) were successfully typed using this amplification and typing system. Our data, therefore, suggest that the AmpliType® PM + DQA1 PCR amplification and typing system described is suitable for genotype analysis of adulterated fresh and frozen urine specimens.

KEYWORDS: forensic science, DNA, polymerase chain reaction, urine, polymarker, human leukocyte antigen

The testing of urine for drugs of abuse is regulated by the Substance Abuse and Mental Health Services Administration (SAMHSA), a division of the Health and Human Services (1). These guidelines apply to the testing of Federal employees, but have been largely accepted as standards for testing within the private sector as well. The regulations are very specific about how urine is to be collected, transported, tested and interpreted. Urine collection sites must be arranged so as to minimize the opportunity for adulteration or substitution. Recently voided urines are checked for volume, temperature and unusual characteristics (e.g., color or odor). Urine bottles must be sealed with tamper-evident tape and signed by the donor. All of the steps involved must be carefully documented in the appropriate custody and control (chain-of-custody) form. Some donors also have the opportunity to simultaneously submit a second (i.e., ''split'') urine sample to be sealed and sent to the testing laboratory. This second sample is not opened, but rather is held in reserve should testing at a later time be necessary. In the event of a positive result, the employee or medical review officer can opt to have the split sample sent to a second, certified drug testing laboratory. Laboratories conducting drug analyses are regularly inspected by the National Laboratory Certification Program (Research Triangle Institute, Inc.) to determine compliance with these regulations.

As part of the certification process, drug testing laboratories are responsible for maintaining the chain-of-custody documentation for each aliquot of the urine taken. In addition to the testing for the drugs of abuse, the laboratory is also charged with examining urine for evidence of adulteration. Assays for specific gravity and creatinine are helpful for determining whether or not a specimen has been diluted. Such tests cannot be used to determine if commercially available drug-free urine has been substituted for the donor's own urine. Proof that an urine has been adulterated or substituted can lead to a failure in the drug test.

Despite these precautions, breaches in chain-of-custody and claims of sample mix-ups have been litigated with reference to positive drug test results. A mechanism to identify the urine specimen along with a submitted blood sample by DNA genotyping would be helpful in addressing these issues. When a match is determined, it serves to help validate the procedures in use at urine collection stations and testing laboratories. If a match is not determined, substitution has occurred either through an administrative mix-up or intentionally by the donor at or after the time of collection. Both of these latter situations can have serious legal consequences.

Human urine has not been thoroughly researched as a usable source of DNA for forensic identity testing. Normal human urine specimens contain very few nucleated cells relative to other types of body fluids. The most common cells found in urine are leukocytes, but their concentration is minimal (300 to 500 cells/mL) (2). Epithelial cells are also present in human urine, but at low concentrations as well (2). Females tend to have high numbers of squamous cells in their urine due to vaginal contamination, resulting in a five-fold increase in female urine DNA concentration compared to male urine DNA concentration (3). The small number of nucleated cells in female and male urine provides a limited reservoir of genomic DNA for forensic identity testing.

In a previous study, we evaluated a DNA typing method for urine specimens utilizing a well established typing kit (3–8). In

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Received 9 July 1997; and in revised form 2 Dec. 1997; accepted 16 Dec. 1997.

this study, we examine the potential for interference of DNA typing by adulterants which are commonly available to individuals submitting forensic urine specimens as well as substances that could be present as a result of pathologic disease.

Methods

Samples/Adulterants

First void urine (fresh urine) samples were collected from a single male volunteer in all adulterant studies to ensure uniformity of DNA concentrations obtained. These samples were collected without preservatives and processed immediately following collection. Sample volumes of 10, 20 and 30 mL were analyzed, and each sample volume was subject to DNA extraction and typing in multiple independent trials. Household bleach, *Escherichia coli*, human serum albumin (HSA), glucose and saponin were evaluated as adulterants with potential for inhibiting this typing procedure. As negative controls for the effects of the adulterants on amplification and typing, a urine specimen without the adulterant was extracted and typed for each experiment.

Household bleach (5.25% sodium hypochlorite, 94.75% inert ingredients, Nugget Distributors Inc., Stockton, CA) was added to fresh urine specimens to evaluate the effect of this adulterant on this DNA typing system. Fresh urine samples were contaminated with 0.5%, 1% and 5% (v/v) bleach in a total volume of 30 mL. The urine/bleach samples were thoroughly mixed by vigorous shaking prior to cell pelleting and subsequent DNA extraction. Each urine/bleach sample was extracted as described and typed in three independent trials.

To simulate urinary tract infections, the effect of bacterial contamination on DNA typing was performed by the addition of log phase *E. coli* to 30 mL fresh urine samples in both physiological and pathological concentrations. It is of particular interest to determine if bacterial DNA interferes with either the extraction or typing procedure. Urine samples were inoculated with 10 μL of an overnight liquid *E. coli* culture and further incubated at 37°C for 0, 1, 3 and 5 hours; these samples were placed on ice immediately following incubation. Once all incubations were complete, *E. coli* cell density was measured in each sample by absorbance spectrophotometry at 600 nm prior to cell pelleting and DNA extraction. Each urine-*E. coli* sample was extracted as described and typed in three independent trials.

In simulating proteinuria as a possible deterrent of this typing system, human serum albumin (HSA, Armour Pharmaceutical Co., Kankakee, IL) was added to fresh urine samples to achieve physiological and pathological concentrations of 10, 100, and 1000 mg/dL, in a total volume of 30 mL. Similarly, glucose (Sigma Chemical Co., St. Louis, MO) was added to fresh urine samples to achieve physiological and pathological concentrations of 10, 75, and 750 mg/dL, in a total sample volume of 30 mL. The urine-HSA and urine-glucose samples were vortexed for 20 s prior to cell pelleting and DNA extraction. These samples were extracted and amplified as described and typed in three independent trials.

Saponin (Sigma Chemical Co., St. Louis, MO), a strong detergent, was added to fresh urine specimens to evaluate its effect on this DNA typing system. Fresh urine samples were contaminated with 0.1% (w/v) saponin in a total volume of 30 mL. The urine/saponin samples were incubated for 10 to 60 min at room temperature and at 37°C. Fresh urine samples were also contaminated with 0.2% and 0.3% saponin in a total volume of 30 mL; these samples were incubated for 10 min at room temperature prior to cell pelleting and DNA extraction. A 0.1% saponin/distilled H_2O solution (total volume equaling 30 mL) was also extracted and typed to ensure that saponin, an organic extract from Quillaja bark, did not interfere with the typing system. All urine-saponin samples were thoroughly mixed prior to incubation. Cell pelleting and DNA extraction was performed immediately following incubation. The urine/saponin samples were extracted and amplified as described and typed in two independent trials.

Frozen male urine specimens stored at −70°C for 4 to 6 months were obtained from the Toxicology Laboratory at Hartford Hospital and analyzed using this amplification and typing system. Each specimen was split into equal volumes, one aliquot was tested without addition of a contaminant, and the other aliquot was adulterated with either *E. coli* (incubation at 37°C for 5 h) or 0.1% (w/v) saponin (incubation at room temperature for 10 min). In some cases, both aliquots were left unadulterated. Specimen volumes ranged from 4 to 7 mL. The frozen urine, frozen urine-*E. coli* and frozen urine-saponin samples were extracted and amplified as described and typed in multiple independent trials.

DNA Extraction

DNA was isolated from urinary tract epithelial cells collected by centrifugation of urine specimens using the Puregene Genomic DNA Isolation Kit (Gentra, Minneapolis, MN). The urine specimens were centrifuged for 15 min at 2500 rpm. The resulting pellet was processed using the DNA extraction kit according to the recommendations of the manufacturer. Briefly, cells are lysed and treated with RNAse and a protein precipitating agent. The DNA located in the supernatant is precipitated with isopropanol, washed in ethanol and rehydrated in 100 μL of Tris [hydroxymethyl aminomethane]- EDTA (ethylene diamine-tetraacetic acid)buffer (pH 7.2 to 7.5). The concentration and purity of DNA samples were determined spectrophotometrically at 260 and 280 nm using a double beam spectrophotometer (Model DU650, Beckman Instruments, Fullerton, CA).

To determine the effect of the DNA extraction procedure on recovery of urine for drugs of abuse testing, 12 frozen urine specimens positive for one or more drug classes were obtained from the toxicology laboratory. Each urine specimen was subjected to the steps necessary for DNA extraction, as previously described. The supernatant from these urine samples were tested for drugs of abuse using the CEDIA assay (Boehringer Mannheim Corp., Concord, CA). The rate reactions obtained from these supernatants were compared against results of aliquots taken prior to the DNA extraction steps. The within-run precision for CEDIA on the positive control has previously been shown to range from 2.5 to 7.6% (10).

Polymerase Chain Reaction

DNA amplification was performed in 100 μL reaction volumes according to the recommendations of the manufacturer of the typing kit. 50 ng to 1000 ng of template DNA was added to the reaction mix depending on the concentration of the sample. All samples were amplified in thermal cyclers (Models 2400 or 9600, Perkin Elmer, Foster City, CA). Amplification was completed using 32 cycles, each consisting of 30 s denaturation (94°C), 30 s primer annealing (63°C), and 30 s extension (72°C). The final cycle included a 10 min extension step at 72°C; immediately following the final cycle, 5 μL of 200 mmol/L EDTA was added to stop the amplification reaction. Samples were stored frozen (−20°C) when not typed immediately following amplification.

AmpliType® PM + DQA1 Typing

We typed samples using the AmpliType® PM + DQA1 PCR amplification and typing system, a reverse dot blot assay, according to the recommendations of the manufacturer (Perkin Elmer, Foster City, CA). This kit types six individual loci which include HLA DQA1, low density lipoprotein receptor (LDLR), glycophorin A (GYPA), hemoglobin G gammaglobulin (HGGB), D7S8 and group specific component (GC). This kit subtypes the DQA1 allele 1 into a 1.1, 1.2 or 1.3 allele, and further subtypes the DQA1 allele 4 into 4.1 or 4.2/4.3 allele. The identification of DNA typing for each sample was determined independently by two laboratory technologists.

Results

DNA isolated from 10, 20 and 30 mL fresh male urine specimens was quantitated using absorbance spectrophotometry at 260 nm. Mean concentrations obtained suggest the absence of DNA or concentrations below the sensitivity of spectrophotometric detection (Table 1). The DNA was of low purity as determined by the absorbances at 260 and 280 nm. Despite low concentrations of detectable DNA, 100% of the DNA isolates from the 10 mL urine specimens were typable by the AmpliType® PM + DQA1 PCR amplification and typing kit. Only 96% and 92% of the DNA isolated from the 30 mL and 20 mL urine specimens, respectively, were amplifiable and typable.

DNA isolated from fresh male urine specimens adulterated with household bleach, *E. coli,* glucose, human serum albumin or saponin were quantitated using absorbance spectrophotometry at 260 nm. Minimal levels of DNA or concentrations below the sensitivity of spectrophotometric detection were demonstrated (Table 2). For the majority of the adulterated samples, the purity of DNA isolates was low as determined by absorbances at 260 and 280 nm. All DNA samples isolated from fresh urine specimens contaminated with household bleach, *E. coli,* glucose, human serum albumin or saponin were typable by the AmpliType® PM + DQA1 PCR amplification and typing kit. The 0.1% saponin/distilled H_2O solution, which was extracted and amplified for the purposes of a negative control, was not typable by this system.

DNA isolated from frozen male urine specimens was also quantitated using absorbance spectrophotometry at 260 nm. Except for the frozen urine-saponin samples, the purity and concentrations of isolated DNA was low as indicated by mean absorbancies at 260 and 280 nm (Table 3). Of the 48 frozen urine specimens (both adulterated and not) tested, 71% of these samples were typable by the AmpliType® PM + DQA1 amplification and typing kit despite the low concentrations and poor quality of the DNA. Sixty-seven percent of the DNA isolates from the frozen urine samples and 80% of the DNA isolates from the adulterated frozen urine samples were successfully typed (Fig. 1). Interestingly, 85% of the DNA isolates from the frozen urine samples (both adulterated and not) were successfully typed for at least the PM markers.

Table 4 shows the results of testing urine specimens before and after DNA extraction for drugs of abuse. There was no significant difference in the ΔmAU/min (rate reaction) values caused by centrifugation, as the change in values were within the precision of the CEDIA method. These data suggests that none of the drugs tested were co-precipitated or sequestered within the proteins, cellular elements and/or DNA pellet. Urine specimens can be returned to the drug testing laboratory, retested if necessary, to confirm the presence of drugs after being subjected to DNA analysis. It is important to note, however, that the DNA has been removed from the original urine specimens on the chain-of-custody documents.

TABLE 1—*Absorbancies from fresh urine samples.*

Fresh Urine	Mean 260 nm	Std. Dev. 260 nm	Range 260 nm	Mean 260/280	n
10 mL	0.0001	0.0055	0.0118(−):0.0046	1.233	10
20 mL	0.0050	0.0089	0.0043(−):0.0108	1.359	12
30 mL	0.0000	0.0028	0.0052(−):0.0036	1.254	12

TABLE 2—*Absorbancies of adulterated fresh urine samples.*

Adulterant	Mean 260 nm	Std. Dev. 260 nm	Range 260 nm	Mean 260/280	n
Bleach 0.0%	0.0173	0.0190	0.0045:0.0391	1.306	3
Bleach 0.5%	0.0048	0.0043	0.0017:0.0097	1.698	3
Bleach 1.0%	0.0026	0.0014	0.0011:0.0038	1.101	3
Bleach 5.0%	0.0027	0.0008	0.0018:0.0033	1.603	3
E. coli 0 h	0.0044	0.0053	0.0012:0.0105	1.424	3
E. coli 1 h	0.0029	0.0013	0.0016:0.0042	1.193	3
E. coli 3 h	0.0084	0.0019	0.0062:0.0099	2.422	3
E. coli 5 h	0.0230	0.0212	0.0048:0.0463	1.554	3
Glucose 0 mg/dL	0.0053	0.0005	0.0050:0.0059	1.821	3
Glucose 10 mg/dL	0.0096	0.0083	0.0026:0.0187	1.38	3
Glucose 75 mg/dL	0.0071	0.0044	0.0030:0.0117	1.373	3
Glucose 750 mg/dL	0.0116	0.0060	0.0071:0.0184	1.365	3
HSA 0 mg/dL	0.0062	0.0040	0.0034:0.0108	1.226	3
HSA 10 mg/dL	0.0042	0.0007	0.0036:0.0049	1.411	3
HSA 100 mg/dL	0.0032	0.0023	0.0006:0.0050	2.337	3
HSA 1000 mg/dL	0.0050	0.0027	0.0023:0.0076	1.305	3
Saponin 0.1%, (RT)	0.0050	0.0008	0.0044:0.0056	1.993	2
Saponin 0.1%, (37°C)	0.0047	0.0017	0.0035:0.0059	2.363	2
Saponin 0.2%, (RT)	0.0011	0.0004	0.0008:0.0013	3.9(−)	2
Saponin 0.3%, (RT)	0.0011	0.0011	0.0003:0.0019	0.9497	2

TABLE 3—*Absorbance spectrophotometry of unadulterated and adulterated frozen urine samples.*

Samples/Adulterants	Mean 260 nm	Std. Dev. 260 nm	Range 260 nm	Mean 260/280	n
Frozen	0.0211	0.0579	0.0118(−):0.2317	1.34	33
Frozen/0.1% Saponin	0.0064	0.0057	0.0039(−):0.0147	1.87	9
Frozen/*E. coli*	0.0571	0.0517	0.0054(−):0.1197	1.15	6

Discussion

Specimen processing and breaches in the documentation of chain of custody have been important issues in several cases where urine has been positive for drugs of abuse. One of the most important early cases was National Treasury Employees Union vs. von Raab (U.S. District Court, Louisiana, #86-3422, 11/14/86). An employee of the U.S. Customs Service was discharged because of a positive urine drug test. The court found in favor of the employee on grounds that the testing was an invasion of privacy, and that the accuracy of the testing procedure (including specimen identification, storage, handling, and preparation) was ''. . . fraught with dangers of false positive readings . . .'' Such testing was conducted prior to enactment of the Federal drug testing guidelines. This ruling, however, was vacated on appeal (U.S. Court of Appeals, #86-3833, 4/22/87) and the U.S. Supreme Court, on the grounds that ''the drug-testing program is not so unreliable as to violate due process of law,'' and that ''Customs also employs elaborate

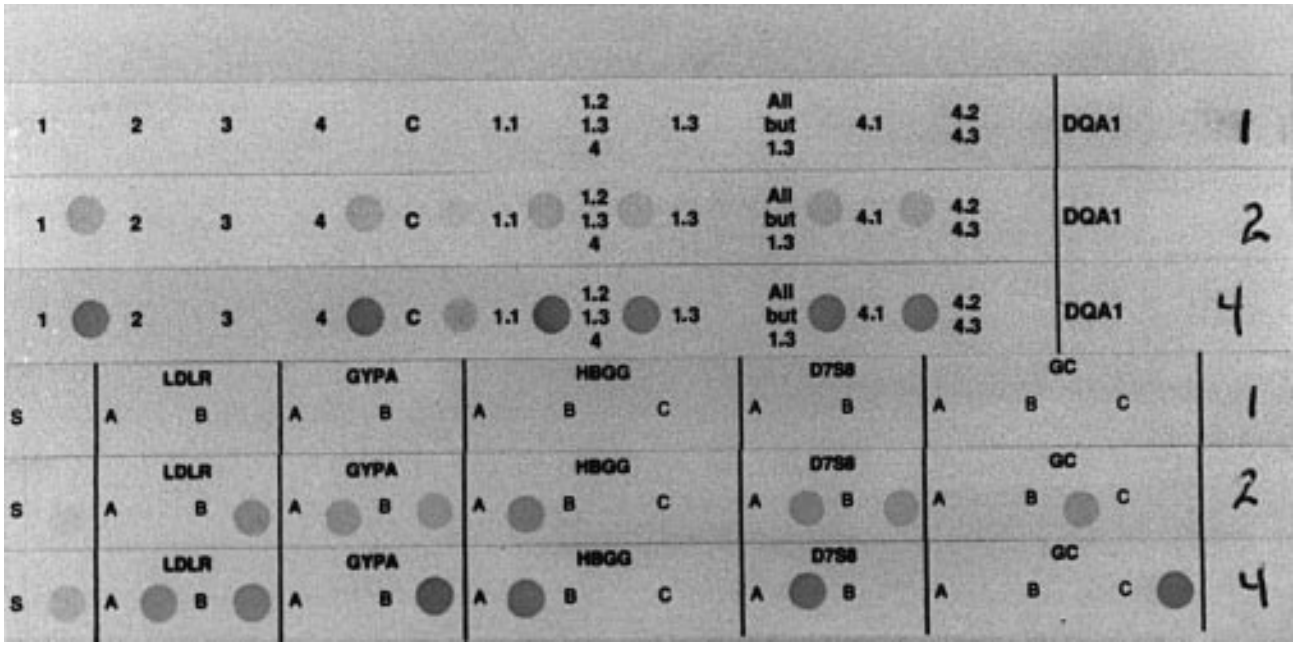

FIG. 1—*Representative PM + DQA1 typing strips demonstrating typing of adulterated frozen male urine. 1, blank; 2, kit control DNA (DQA1: 1.1, 4.1; PM: BB, AB, AA, AB, BB); 4, frozen male urine adulterated with* E. coli *(DQA1: 1.1, 4.1; PM: AB, BB, AA, AA, CC).*

TABLE 4—*Results of drug testing after extraction of DNA from urine.*

Specimen No.	Drug Class	Pre-extraction (ΔmAU/min)	Post-extraction (ΔmAU/min)	Change (%)
1	cocaine metabolite	429	420	2.1(−)
2	cocaine metabolite	791	792	0.1
3	cocaine metabolite	463	486	4.7
4	cocaine metabolite	339	331	2.4(−)
5	cocaine metabolite	785	779	0.8(−)
6	cocaine metabolite	701	703	0.3
	opiates	1258	1249	0.7(−)
7	opiates	608	620	1.9
8	opiates	1232	1256	1.9
9	marijuana metabolite	203	203	0.0
10	marijuana metabolite	212	207	2.4(−)
11	amphetamines	315	340	7.3
12	benzodiazepines	640	654	2.3

chain-of-custody procedures to minimize the possibility of falsely positive readings.''

There have been other cases whereby the reliability of chain-of-custody procedures have been questioned. In Potts vs. Velasco, (Court of Appeals, Missouri, #69039, 8/3/96) an employee charged that the collection station failed to seal his urine container, allowing his sample to be switched with another (thereby leading to a false positive result). In O'Connor vs. SmithKline Bio-Sciences Laboratories, Inc., (Appeals Court, Mass., #93-P-707, 4/21/94), the plaintiff claimed that sample switching had occurred because the chain-of-custody form was not properly signed. In Frank vs. Department of Transportation (Federal Circuit Court of Appeals, #9303510, 9/16/94), a sealed and initialized sample was left unattended for ''about a minute.'' In each of these cases, the courts ruled that the collection agencies were not negligent as there were no proof that these chain-of-custody breaches led to tampering and negligence with the positive drug test.

In contrast, in Blappert vs. Department of Police (Court of Appeals, Louisiana, #94-CA-1284, 12/15/94), a police officer was dismissed because of a positive test for marijuana. They argued that the collection site was busy, and that the officer's urine sample was placed upon a counter with several other samples before being labeled, leading to a sample mix-up. The Civil Service Commission and Louisiana Court of Appeals reinstated the officer with back pay because the department was unable to produce a representative from the collection site to refute the claims.

In each of these cases, comparison of genotypes from the urine and a sample of the donor's blood could have avoided the litigations that occurred. DNA testing of urine protects both the laboratory from unwarranted litigations, and the donor from wrongful results. SAMHSA currently does not have provisions for the testing or transportation of submitted urine for DNA identity testing. However the court can order urine to be sent to DNA testing laboratories. In DrugScan vs. Pena (U.S. District Court, Penn., #04-CV-2296, 6/29/94) a federal judge in Pennsylvania upheld a Nevada judge's order that urine be sent to a DNA laboratory for identity testing.

With the advent of polymerase chain reaction technology, analysis of previously undetectable levels of nucleic acids has become feasible. As little as 1 ng of template genomic DNA can be used to amplify and detect allelic sequence variations in the human genome (9). We amplified minimal quantities of template genomic DNA extracted from fresh and frozen male urine specimens utilizing the AmpliType® PM + DQA1 PCR amplification and typing kit. Following amplification, the amplicon was used in a reverse dot blot assay to determine allele type of the samples for select genetic markers. Using this approach, we have successfully typed fresh and frozen male urine specimens as well as fresh and frozen adulterated male urine specimens.

DNA isolated from various amounts of fresh male urine was typable by this procedure in greater than 90% of these specimens. This typing was possible despite the lack of purity and low concentrations of isolated DNA. This confirms the sensitivity of PCR-based technologies and its application to identification of specimens containing degraded DNA. Addition of common adulterants

and agents found in pathological conditions did not inhibit DNA typing of the specimen. Interestingly, typing signals were strongest for specimens adulterated with *E. coli* or saponin despite low purity and DNA concentration of the initial specimen. This suggests that *E. coli* and/or its DNA acts as a carrier for low concentrations of human genomic DNA and that saponin may more efficiently act in the lysing of the few urinary tract epithelial cells which are present. Alternatively, *E. coli* may also increase the lysing of urinary tract epithelial cells resulting in a greater yield of genomic DNA for amplification.

DNA isolated from frozen male urine was typable by this procedure in greater than 70% of these specimens (despite the lack of purity and low concentrations of isolated DNA). There are several possibilities why the frozen urine specimens did not type as effectively as the fresh urine specimens. Freeze-thawing of urine results in the destruction of urinary tract epithelial cells which are present in the urine, thus limiting the possible source of genomic DNA. More likely, however, the volumes of the frozen urine specimens (4 to 7 mL) tested were significantly less than the fresh urine specimens (10 to 30 mL). Thus, the number of urinary tract epithelial cells from these specimens was proportionately lower. In addition, all fresh urine samples were first void whereas this may not have been the case for collection of frozen urine specimens originally submitted for drugs-of-abuse testing. In either case, DNA isolated from these samples was below the sensitivity of spectrophotometry and, in non-typable cases, may have been so low as to be below the sensitivity of the amplification and typing assay. Interestingly, when the frozen urine specimens were not successfully typed, both aliquots (adulterated and unadulterated) were untypable. This suggests that the specimens were lacking nucleated cells as a source of DNA, rather than the adulterants inhibiting the amplification and typing procedure. Although we did not test all possible adulterants and endogenous substances that could be present in normal or pathologic urine, our study suggests that if DNA is present in quantities sufficient to extract and amplify, it can be genotyped using the PM + DQA1 assay without significant interference by these substances.

Acknowledgments

The authors wish to thank the Medical Review Officers Certification Council Research Initiative Grant Program for funding of this project. Special thanks are given to D. Anamani, L. Chaffee, K. Hodges, D. Roscioli and R. Johnson for their technical assistance.

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