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# DNA typing of urine samples following several years of storage*

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**Summary.** The possibility of typing DNA polymorphisms on urine samples was investigated in a controlled storage experiment and for samples that were 1–7 years old. Female urine samples showed a higher amount of epithelial cells and therefore a higher DNA yield. Employing the polymerase chain reaction, specific amplification results were achieved for all samples over a 6 month storage period. The microscopical examination of the samples revealed not only differing degrees of contamination with bacteria, yeasts and fungi, but also the presence of still intact epithelial cells. Only 20% of the male samples and 32% of the female samples yielded specific amplification results. By separating the human cells from the contaminating organisms prior to DNA extraction, the number of successfully typed samples could be improved to 35% of the male and 77% of the female samples. This result confirms that excess amounts of co-extracted non-human DNA can inhibit the specific amplification of human target sequences.

**Key words:** Urine – DNA-typing – Storage – PCR-inhibition – Coextraction of non-human DNA

**Zusammenfassung.** Die Typisierbarkeit von DNA-Polymorphismen an gelagerten Urinproben wurde in einem systematischen Lagerungsexperiment und stichprobenartig an 1–7 Jahre alten Proben untersucht. In beiden Untersuchungen zeigte sich bei Frauen eine erhöhte Zahl von Epithelzellen im Urin und damit eine größere Menge an extrahierbarer DNA. Mit dem Verfahren der Genamplifikation konnten für den untersuchten Zeitraum von sechs Monaten alle Proben typisiert werden. Die mikroskopische Begutachtung der mehrere Jahre alten Proben zeigte verschiedene Grade von Kontamination mit Bakterien, Hefen und Pilzen, aber auch das Vorhandensein von intakten Epithelzellen. Nur 20% der Urinproben von Männern und 32% der Proben von Frauen ergaben spezifische Amplifikationsergebnisse. Durch die Trennung von humanen Zellen und kontaminierenden Organismen vor der DNA-Extraktion konnte diese Quote auf 35% der männlichen und 77% der weiblichen Proben erhöht werden. Dieses Ergebnis zeigt, daß ein hoher Anteil koextrahierter nicht menschlicher DNA einen hemmenden Effekt auf die spezifische Amplifikation humaner Zielsequenzen hat.

**Schlüsselwörter:** Urin – DNA-Typisierung – Lagerung – PCR – Hemmung – Koextraktion nicht humaner DNA

## Introduction

The necessity to identify human urine samples arises in various situations, e.g. verification of mixed-up samples in clinical laboratories, or in doping control and drug screening programs, where substitution and manipulation of evidence can occur. The analysis of several serological polymorphisms in urine has been reported since 1965 [7]. Although some of the proteins were remarkably stable (e.g. Gc could still be typed after 6 months [10]), the number of blood group systems typable on urine is limited.

The presence of nucleated cells in human urine implied the application of DNA typing methods. Gasparini et al. (1989) [6] reported the amplification of a DNA sequence linked to cystic fibrosis using urine samples, suggesting cells from urine as easily obtainable material for genetic testing. The amount of urine necessary for DNA typing with multilocus probes according to Roewer et al. (1990) [13] is 200 ml. Brinkmann et al. (1992) [4] reported a short investigation on the possibility of typing different DNA polymorphisms using urine samples. In one case it was possible to analyse the polymorphism D2S44 using Southern blotting and the probe YNH24 on DNA from 10 ml of urine, but in the other cases the DNA yields were insufficient for single locus probes and required PCR amplification.

To evaluate the chances of identifying urine samples through DNA typing in the abovementioned circumstances, it has to be ensured that it is possible to achieve reliable results on stored samples. To test the influence

* Dedicated to Prof. Dr. M. Staak on the occasion of his 60th birthday

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of age on the ability to type DNA from urine samples, urine from different donors was used for controlled storage experiments over a time period of 6 months. By testing urine samples from the archives of the toxicology department that were several years old, one inhibitive parameter was identified, and a method to overcome PCR inhibition was developed.

## Materials and methods

Approximately 1 l of urine, which had been collected over a 24 h period from each of 4 individuals (2 male, 2 female), was divided into 20 ml aliquots and stored at 4°C over a period of 6 months. Duplicate DNA extraction and subsequent amplification was carried out every 2 weeks. Several aliquots were stored frozen at −20°C. Additional urine samples stored for longer periods at 4°C were acquired from the toxicological department of our institute. A total of 84 urine samples (20 ml) from 42 males and females were tested with storage periods ranging from 1 to 7 years. Reference blood samples were available in only a few cases.

*DNA extraction.* DNA was extracted from all 20 ml samples of urine. After the first sedimentation (10 min, 1500 × *g*) 50 µl aliquots of the pellets were used for microscopical examination. The pellets were resuspended in 5–20 ml physiological saline at 50°C and centrifuged again from 10 mins at 3000 × *g*. In cases where the majority of the sediment consisted of ureate crystals, they were dissolved by incubating the samples at 50°C for 30 min before the centrifugation step. The supernatant was discarded and the pellet was incubated with DNA-lysis buffer and proteinase K [8]. After incubation, the samples were extracted twice with phenol/chloroform, once with water-saturated n-butanol and concentrated using Centricon 100 concentrators (Amicon). After the concentration step the retentate was washed twice with 2 ml of TE-buffer (0.01 ml Tris HCl pH 8.0, 0.001 *M* EDTA).

*Removal of fungi and bacteria.* Contaminated samples were centrifuged as above and microscopically examined. Each pellet was incubated at 50°C with physiological saline as above, but prior to the second centrifugation the samples were filtered through 53 µm pore size nylon gauze (HC-53my, Schweizer Seidengazefabrik, Thal). The fabric was rinsed with additional saline. After 10 mins at 3000 × *g*, the supernatant was discarded and the sediment was resuspended in 1–2 ml physiological saline and transferred to 1.5 ml Eppendorf tubes. The lids of the tubes had been cut off and pierced in the middle leaving only the outer ring. The tubes were now closed by placing a 20 µm pore size nylon gauze (P-20my, Schweizer Seidengazefabrik, Thal) over the opening and pressing the outer ring of the lid down. The tubes were then inverted, placed in a 14 ml centrifuge tube on top of a second Eppendorf tube and centrifuged for 5 min at 1500 × *g*. The 20 µm mesh retains the human epithelial cells, which are subsequently removed by opening the tube, adding DNA-lysis buffer, again closing the tube with the net, shaking the tube and applying additional lysis buffer on the top. Using this procedure 42 urine samples of different ages were extracted parallel to 42 aliquots, that were extracted without removal of contaminants.

*DNA quantitation.* The DNA yields, and the quality of the samples, were determined by yield gel electrophoresis. Human DNA content was quantified by dot blot analysis using the S&S Minifold dot blot apparatus (Schleicher & Schuell) and the "Human DNA Quantitation System" from GibcoBRL according to the manufacturers instructions.

*DNA amplification.* The amplification of the HVR-region 3′ of the apolipoprotein B gene was carried out in a Bioexellence DNA incubator (Biozym), using the following parameters:

Reaction mix:
2U Taq polymerase (Promega), 0.5 µ*M* each primer, 150 µ*M* dNTPs, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 10 m*M* TrisHCl pH 9.0, 0.1% Triton X 100, 8 µg BSA.

Primer sequences [2]:
5′ TGGAAACGGAGAAATTATGGAGG 3′
5′ CCTTCTCACTTGGCAAATACAATT 3′

*Temperature cycles.* 94°C − 1 min, 58°C − 1 min, 72°C − 4 min, 29 cycles. The amplification results were tested on 10 cm, 6% polyacrylamide gels, 0.45 mm thick, cast on Gelbond (Biozym). The electrophoresis was run for 1 h at 15 mA using a discontinuous buffer system as described by Allen et al. (1989) [1]. DNA fragments were detected by silver staining according to Allen et al. (1989) [1]. For allele designation the samples were run on 20 cm, 5% polyacrylamide gels, 0.75 mm thick with 0.08 *M* Tris formate buffer in the gel and 0.28 *M* Tris borate buffer in the agarose plugs. Electrophoresis time was 3 h, with 25 mA held constant by adjusting the Watt setting every 20 min. The allele designation followed the nomenclature from Rand et al. (1992) [12] and was done using an allele cocktail that had been compared to a reference allele cocktail kindly provided by the Institute of Legal Medicine Münster.

## Results

### *Microscopical examination*

The fresh urine samples showed low amounts of ureate and calcium oxalate crystals, and few or no leucocytes. There was a considerable difference in the sediment between males and females: urine from females contained more epithelial cells, which were often associated, and more bacterial cocci than the male samples. Over the 6 month controlled storage period the microscopical examination revealed a degeneration of the epithelial cells to isolated nuclei. There was also an increase of yeast cells, which varied for the different donors, and after 5 months one sample began to show filamentous mycelia.

The older samples showed different degrees of contamination with bacteria and fungi, that were not correlated to storage time. While two 4-year-old samples were well preserved, several 1-year-old samples contained a great amount of mycelia. The oldest samples examined were 7 years old and still contained intact epithelial cells. Figure 1 shows an example of a 7-year-old sample from a female. Next to the fungal mycelia several single yeast cells, a few ureate crystals and intact epithelial cells are visible.

### *DNA extraction and amplification*

The DNA extraction method using proteinase K, phenol/chloroform extraction and Centricon concentration was chosen subsequent to a comparison of different methods e.g. chelex extraction [15], because it gave more consistent results. The amount of total human DNA that could be extracted from 20 ml of fresh urine ranged from 20–40 ng for males and 400–800 ng for females. Over the storage period of 6 months the DNA yields dropped to 1–2 ng for male and 10–20 ng for female urine samples. Even after 6 months of storage the extracted DNA in the samples was of high molecular weight but this could not be confirmed for all samples, since 10 ng of DNA is the de-

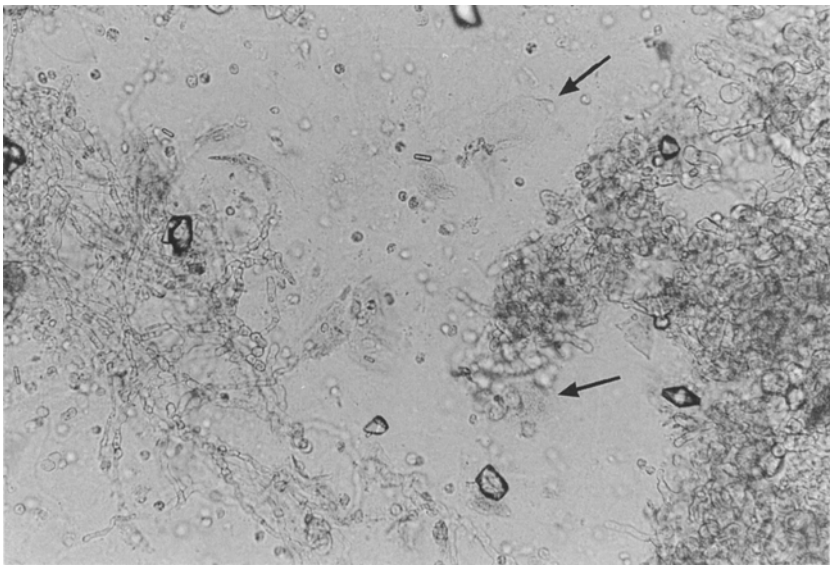

**Fig. 1.** Urine sediment, female sample, stored at +4°C for 7 years. Arrows indicate intact epithelial cells

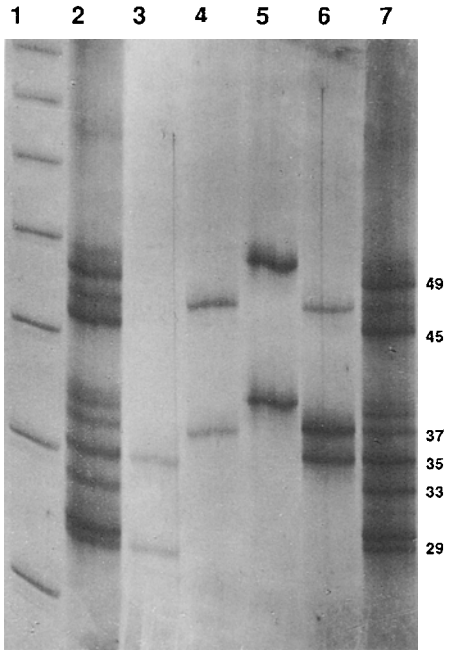


**Fig. 2.** Electrophoretic separation of Apo B amplification products. Lane 1: 123bp ladder; lane 2,7: allele cocktail; lane 3–6: urine samples after 4 months storage time. The weaker third allele in lane 6 derives from sperm cells that were present in the female urine sample

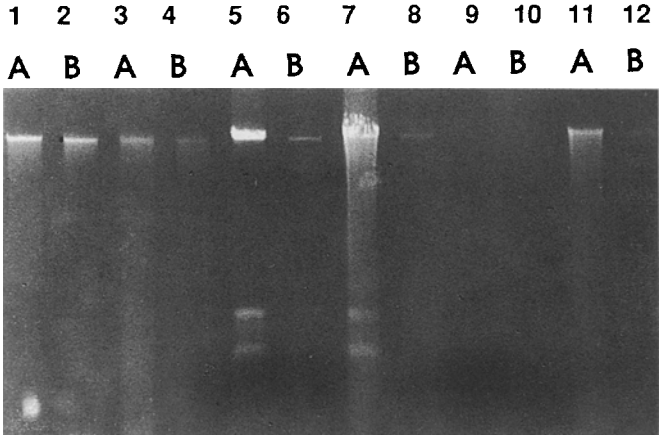


**Fig. 3.** DNA-yield gel. A: normal DNA extraction; B: DNA extraction after removal of contamination; lanes 1–6: female urine samples; 1,4 and 7 years old; lanes 7–12: male urine samples; 1,4 and 7 years old. The low molecular weight DNA in lanes 5 and 7 disappeared after removal of contamination

tection limit for ethidium bromide stained agarose gels. For female urine samples the amplification of 1/10 of total DNA showed good results for the entire 6 month storing period. For the male samples it was necessary to employ 1/5 of total DNA for a successful amplification. It was important to include BSA in the amplification reaction, otherwise a loss of amplification signals occurred. Using these conditions it was possible to obtain specific alleles for all 4 individuals over the 6 month storage period. Figure 2 shows a PAGE gel for the samples after 4 months. The sample in lane 6 contains 3 alleles and the weaker fragment could be identified as deriving from sperm cells contained in the urine sample. DNA extraction of samples stored for 6 months at −20°C showed yields equivalent to fresh samples and no problems with amplification.

Yield gel electrophoresis for the year-old samples following normal extraction showed up to 5 µg high molecular DNA and additional low molecular weight bands. After the filtration steps the electrophoretically determined yields dropped. Figure 3 shows a typical yield gel. The parallel samples are designated A/B, where B is the sample where contamination had been removed. These samples show less DNA and the absence of low molecular weight bands. The specific quantitation of human DNA gave results from 50 ng to less than 1 ng of human DNA. In most cases the removal of contamination caused a loss of human DNA. According to the dot blot and yield gel results the ratio of human to non-

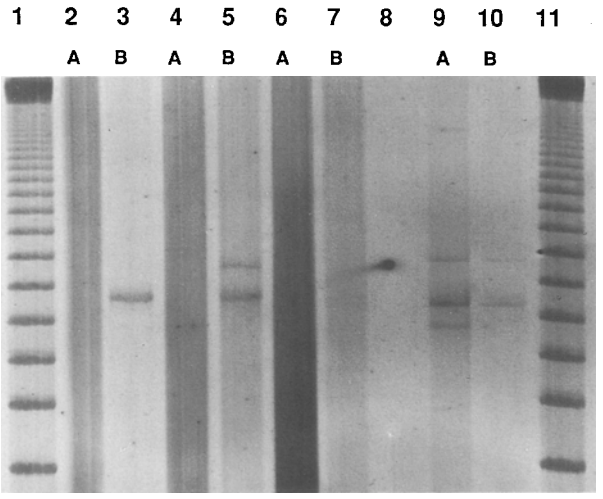


**Fig. 4.** Amplification test gel. A: normal DNA extraction, B: DNA extraction after removal of contamination; lanes 1,11: 123bp ladder; lane 8 empty; other lanes contain urine samples after different storage time: lanes 2,3: 7 years; lanes 4,5: 4 years; lanes 6,7: 1 year; lanes 9,10: 4 years. The sample in lane 9 shows an additional third band that must derive from unspecific amplification of the contaminating DNA

human DNA was up to 1:10,000 for highly contaminated samples.

For amplification 2/5 of the total DNA were employed. Figure 4 shows an amplification test gel using samples 2, 4, 5 and 7 years old. A 3-band pattern, as shown in lane 9, was not counted as specifically amplified. After the filtration steps the third band disappeared (Lane 10). Without removal of contamination prior to DNA extraction only 32% of the female samples and 20% of the male samples could be typed. There was no correlation between the results and age of the samples. Therefore the results for all storage periods are combined in Table 1. After removal of contamination the number of successfully amplified samples could be improved to 77% for the female samples. The number of specific results (35%) and the improvement rate after filtration was lower for male samples.

## Discussion

The additional epithelial cells in urine from females stem from the vaginal tract, as do the sperm cells that could in some instances be found. The higher amount of cells for females is the explanation for the sex specific difference in DNA yields. The decrease of DNA yields for the stored samples is in correlation with the decrease of the number of cells revealed by the microscopical examination. Since even after years of storage a certain amount of epithelial cells remained intact, it is not surprising that the extracted DNA was still of high molecular weight. The decrease in the amount of human DNA could only be detected through hybridization with a human specific probe [17] because of the parallel increase of bacteria and yeast cells. This is probably the reason for the different DNA yields reported by Brinkmann et al. (1992) [4] and Pötsch et al. (1993) [11], who employed fluorimetric quantification methods.

The amplification result of the systematic storage experiment showed reliable results for at least a 6 month storage period at +4°C. Therefore the stability of DNA polymorphisms is at least comparable to the results for Gc and ABO blood groups in urine [5, 10]. In practice, the suspicion of sample mix-up or manipulation can arise after completion of biochemical testing, which takes several weeks. Over this time period DNA typing can be safely employed for identity testing. Even for male samples 1/5 of total DNA was sufficient for ApoB amplification. That means that after quantitation, at least 4 PCR-based DNA polymorphisms can be typed from 20 ml of urine. This number, and therefore the discrimination chance, can possibly be increased using the more sensitive STR (short tandem repeat) polymorphisms [3]. The presence of sperm cells in female urine poses a possible problem for the interpretation of results. Pötsch et al. (1993) [11] described the stability of spermatozoa in urine samples and stress the importance of the microscopical examination of the urinary sediment. Another approach to solving this problem would be the use of differential lysis for DNA extraction [8].

The microscopical examination and the DNA yields for the urine samples that had been stored for several years showed the same sex-specific difference in cell content and DNA yields as the fresh samples. The degree of contamination with yeast cells, bacteria and mycelia did not correlate to the storage time, which is apparently due to the individual variability between the samples. The number of successfully amplified samples was increased after removal of the contaminating organisms prior to DNA extraction, even though the necessary filtration steps caused a loss of human cells. The latter is thought to be the cause for the reduced increase of typing results for the male samples. Starting with a low amount of intact epithelial cells, the number of cells after the filtration process was insufficient.

The fact that the removal of contaminating organisms leads to improved typing results, verifies the assumption

**Table 1.** Apo B – amplification results for urine after 1–7 years of storage

| | Gender of donors | | | |
|---|---|---|---|---|
| | ♀ | | ♂ | |
| Parallel samples | Normal extraction | Removal of contamination | Normal extraction | Removal of contamination |
| Number of samples | 22 | 22 | 20 | 20 |
| Specifically amplified | 7 (32%) | 17 (77%) | 4 (20%) | 7 (35%) |

that the presence of, in this case, non-human DNA is inhibitive to the PCR reaction. According to our experience, even the presence of high amounts of human DNA containing the target sequence can result in weaker amplification signals. A mixing experiment of 10 ng of human DNA with various amounts of bacteriophage Lambda-DNA revealed the failure of amplification after addition of 6 µg or more of Lambda-DNA (data not shown). Stefan and Atlas (1988) [16] described the successful amplification of a bacterial DNA sequence before a background of 20 µg of DNA from different bacteria. Their target sequence, a repetitive 1 kb fragment, amounted to 0.3 pg starting sequence, while in our mixing experiment 10 ng human DNA contains only 0.0001 pg of a single copy, heterozygote 691 bp Apo B allele.

The inhibitive effect of high amounts of non-target DNA can be explained by the complexity of the primer annealing process during the first cycles of the PCR reaction [14]. At first, the primers have to scan the genomic template for the specific annealing sites. After the first few cycles, the previously amplified target sequence becomes the preferred template for further synthesis [14]. If the desired target sequence represents only a small fraction of the total DNA, the collision frequency between primers and annealing site is greatly reduced. For mixed samples, this can result in the failure to amplify the lower represented alleles in the first few cycles, and because of the presence of amplified alleles of the major component, later on in allelic drop out. This is confirmed by Gyllenstein et al. (1992) [9] who reported HLA-DQα amplification results for mixed samples, where the lower represented allele could still be typed if it amounted to 0.1–1% of total DNA. This ratio could be greatly improved by employing primers that were specific for the lower represented allele and therefore did not result in inhibitive amounts of amplification products from the other alleles.

When dealing with mixed samples it is therefore advantageous to be able to separate the genetically different cells before the DNA extraction. This of course requires morphological or biochemical disparity between the cells, such as the thiol-rich protein structure of sperm nuclei which is the basis for differential lysis of sperm and epithelial cells [8], or as in our case the diverse cell sizes of bacteria, yeasts, fungi and human cells. By removing these non-human DNA sources, we have been able to reduce the ratio of nonhuman to human DNA in the amplification setup, thereby greatly improving amplification results.

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