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TECHNICAL NOTE

CRIMINALISTICS

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Evaluation of Tamm-Horsfall Protein and Uroplakin III for Forensic Identification of Urine

ABSTRACT: In this study, Tamm-Horsfall protein (THP), a major component of urinary protein, and uroplakin III (UPIII), a transmembrane protein widely regarded as a urothelium-specific marker, were evaluated for forensic identification of urine by ELISA and/or immunohistochemistry. THP was detected in urine, but not in plasma, saliva, semen, vaginal fluid, or sweat by the simple ELISA method developed in this study. In addition, most aged urine stains showed positive results. The urine specificity of THP was confirmed by gene expression analysis. Therefore, as reported previously, ELISA detection of THP can be used as a presumptive test for urine identification. UPIII was specific for immunohistochemical staining of cells in centrifuged precipitate of urine. However, ELISA and RT-PCR for UPIII were not specific for urine. UPIII may be applicable for forensic urine identification by immunohistochemistry.

KEYWORDS: forensic science, urine identification, Tamm-Horsfall protein, uroplakin III, enzyme-linked immunosorbent assay, immunohistochemical staining, messenger ribonucleic acid, reverse transcription-polymerase chain reaction

Identification of urine stains is important in cases of strangulation because locations with such stains may be helpful in identifying where, for example, strangulation victims were murdered. In forensic casework, urine stains are characterized by presumptive tests for urea and uric acid. Determination of urea from urine stains has been performed by coloration tests, such as bromothymol blue and urease test (1), *para*-dimethylaminocinnamaldehyde test (2), and dimethylglyoxime and thiosemicarbazide test (3). However, these tests are not specific for urine because other body fluids, such as sweat, and some cosmetics also contain urea at relative high concentrations (4). Determination of uric acid has often been performed by monitoring the disappearance of UV absorption of uric acid at 293 nm catalyzed by uricase (5–7). This method is sensitive for urine but is influenced by contaminants, such as drugs, which have UV absorption near 293 nm. In addition, bird feces also contains uric acid at relative high concentrations. Therefore, it is necessary to develop a more specific and sensitive method for identification of human urine.

Tamm-Horsfall protein (THP) is a high molecular weight glycoprotein, which is a major component of urinary protein (8). Previous reports outlined the forensic identification of urine stains by radioimmunoassay (9) and by enzyme-linked immunosorbent assay (ELISA, 10) for THP. However, these methods have not generally been used in forensic casework.

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On the other hand, urine contains exfoliated urothelial cells derived from the ureter, bladder, and renal pelvis mucosa. Therefore, assays for urothelial cell-specific components may be applicable to forensic identification of urine. The apical plaque of mammalian urothelial umbrella cells is formed by the asymmetric unit membrane (AUM) (11,12), which contains highly conserved urothelial differentiation-related cell surface glycoproteins, i.e., uroplakin Ia, Ib, II, and III (13,14). In these glycoproteins, uroplakin III (UPIII) is regarded as a urothelium-specific marker and is widely used for diagnosis of tumors and determination of urothelial differentiation (15–19). Therefore, UPIII may be useful for forensic identification of urine and urine stains.

Therefore, this study was performed to evaluate the utility of THP and UPIII for forensic identification of urine and urine stains. THP and UPIII protein in urine and urine stains were determined by ELISA and/or immunohistochemistry. In addition, the specificities of THP and UPIII for urine were evaluated by comparison with blood, saliva, semen, vaginal fluid, and sweat. Furthermore, to verify the urine specificity of THP and UPIII, gene expression analysis was also performed by RT-PCR.

Materials and Methods

Reagents

Sheep purified immunoglobulin against human THP was purchased from Biogenesis (Poole, U.K.). Rabbit purified immunoglobulin against human UPIII was purchased from Abcam (Cambridge, U.K.). Horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, and HRP-conjugated rabbit anti-sheep IgG were purchased from Promega (Madison, WI), Sigma (St. Louis, MO), and Zymed Laboratories (San Francisco, CA), respectively. A mouse monoclonal antibody against UPIII

(clone AUI), 10% normal rabbit serum, biotin-conjugated goat anti-mouse IgG+IgA+IgM, peroxidase-conjugated streptavidin, 4-amino-9-ethyl carbazole (AEC) solution, and Mayer's hematoxylin solution were all purchased from Nichirei Bioscience (Tokyo, Japan). Other reagents used in this study were of research grade and purchased from Wako Pure Chemical Industries (Osaka, Japan).

Collection of Urine and Other Body Fluids

Urine samples were collected from 16 volunteers (13 men and 3 women). Saliva, semen, vaginal fluid stains, and sweat (n = 5–6) were also collected from volunteers by general noninvasive methods. Blood (n = 5) was collected from the brachial vein. Some urine and other body fluids were obtained from the same participants. For immunohistochemical staining and ELISA, the samples were stored at −20°C until use. Vaginal fluid stains, which were obtained by wiping the vaginal mucosa with a cotton swab, were cut into 5 × 5-mm squares and extracted with 100 μL of 0.01 M phosphate-buffered saline (PBS, pH 7.2). For extraction of total RNA, the samples were stored at −80°C and used within 1.5 months.

Aged urine stains (n = 16) were prepared as follows: 1 mL of urine was spotted onto cotton cloth (urine stains were approximately 13 cm in diameter), air-dried, and stored at room temperature for 3 years.

All procedures involving human subjects were approved by the Institutional Review Board of the National Research Institute of Police Science.

Sample Preparation and Dilution for ELISA

For determination of THP, urine, other body fluids, and vaginal fluid extract were diluted from 1:10 to 1:1024 with 0.05 M bicarbonate buffer (BCB, pH 9.6) directly. Urine stains were cut into 2 × 2-cm squares (corresponding to approximately 30 μL of urine) and extracted with 300 μL of BCB. Urine stain extract was diluted from 1:1 to 1:1024 with BCB. For determination of UPIII, urine, other body fluids, and vaginal fluid stain extract were concentrated and cleaned up by centrifugal filter devices (Centricon or Microcon YM-30; Millipore, Billerica, MA) as follows: samples in a volume of 200 μL and 0.05 M BCB were mixed and applied onto the sample reservoir. The mixtures were then centrifuged at 8500×g for Microcon YM-30 or 750×g for Centricon. Concentrate was washed with BCB and collected. Concentrated and cleaned up samples (approximately 15 μL) were diluted 1:20 to 1:1280 with BCB.

ELISA

Aliquots of 50 μL of each diluted sample were added to each well of 96-well microtiter plates (Sumilon-7196F or 7296F; Sumitomo Bakelite, Tokyo, Japan) and incubated at 37°C for 1 h. Then, each well was blocked with 200 μL of 1% BSA in PBS at 37°C for 1 h. Sheep-purified immunoglobulin against human THP was diluted 1:500 with 0.05% Tween 20 in PBS (PBST), 50 μL was added to each well, and incubated at 37°C for 1 h. HRP-conjugated rabbit anti-sheep IgG was diluted 1:5000 with PBST, 50 μl was added to each well, and incubated at 37°C for 1 h. For determination of UPIII, rabbit purified immunoglobulin against human UPIII diluted 1:100 and HRP-conjugated goat anti-rabbit IgG diluted 1:5000 were used as the primary and secondary antibody, respectively. For anti-UPIII, incubation was performed at 4°C overnight. Each well was washed three times with 250 μL of PBST between each step. A volume of 50 μL of *ortho*-phenylenediamine dissolved at 0.5 mg/mL in McIlvaine buffer (0.1 M citrate-phosphate buffer, pH 5.0) containing 0.06% H_2O_2 was added to each well and incubated at room temperature for 5 min. Color development was stopped by addition of 100 μL of 1 M H_2SO_4. Absorbance at 490 nm was measured by spectrophotometry (Powerwave 200; Bio-Tek, Winooski, VT). Samples with absorbance above 0.2 were considered positive in each dilution ratio. This threshold was determined in consideration of nonspecific absorbance. Statistical analysis was performed by one-way ANOVA with Scheffé's multiple-comparison test.

Immunohistochemical Staining

Urine (20 or 1 mL), other body fluids (100 μL), and vaginal fluid stain extract were centrifuged at 320×g for 10 min. The precipitates were stored at −20°C until use. Centrifuged precipitates of urine and other body fluids were diluted with PBS and smeared onto silanized glass microscope slides (Dako, Glostrup, Denmark). The slides were air-dried and then treated with 3% H_2O_2 in methanol for 15 min to block endogenous peroxidase activity. The smears were incubated with 10% normal rabbit serum for 10 min, followed by incubation with mouse monoclonal antibody against UPIII at 4°C overnight. The slides were then treated with biotin-conjugated goat anti-mouse IgG+IgA+IgM for 10 min followed by peroxidase-conjugated streptavidin for 5 min and then stained with AEC as the substrate. After immunohistochemical staining, the slides were counterstained with hematoxylin and mounted with aqueous mounting medium. To confirm the specificity of immunohistochemical staining for UPIII, controls were subjected to staining according to the same protocol but without primary antibody.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from blood, urine, saliva, semen, and vaginal fluid stains using an RNeasy Mini kit (Qiagen, Hilden, Germany). Contaminating DNA was digested with DNase I using an RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. Reverse transcription of total RNA was performed as described previously (20).

Gene Expression Analysis for THP and UPIII

Gene expression analysis was performed as reported previously (20). TaqMan probes (Applied Biosystems, Foster City, CA) were used for gene expression analysis for THP and UPIII. Target genes were THP (Hs00358451_m1, 87 bp), UPIIIA (Hs00199590_m1, 58 bp), and 18S rRNA (Hs99999901_s1, 189 bp). Probes for THP and UPIII were designed to overlap exon/exon junctions to avoid positive reactions with contaminating DNA. These probes were labeled with FAM as a reporter dye and with TAMRA as a fluorescent quencher at the 5′ and 3′ ends of the oligonucleotides, respectively. The 18S rRNA probe was labeled with VIC at the 5′ end and with Minor Groove Binder at the 3′ end of the oligonucleotide.

RT-PCR conditions for THP, UPIII, and 18S rRNA consisted of an initial denaturing step at 95°C for 10 s, followed by 45 cycles of 95°C for 5 s and 60°C for 20 s.

Comparison of expression levels of target genes among body fluids was performed in a linear range of cycle threshold (Ct) value (Ct = 15–27) of 18S rRNA amplification. The cut-off Ct value of target genes was determined as 40. To compensate for the different amounts of total RNA added to reverse transcription reactions, the Ct values of target genes were normalized relative to that of 18S rRNA as the delta Ct value (dCt). The dCt value of each sample was calculated by subtracting the Ct value of 18S rRNA from that

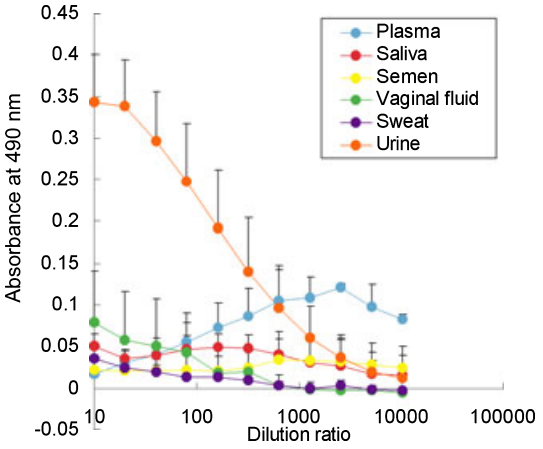

FIG. 1—*Specificity of ELISA detection of Tamm-Horsfall protein in urine among body fluids. Body fluids were diluted at 1:10 to 1:1024 with bicarbonate buffer, added to each well of microtiter plates, and incubated at 37°C for 1 h. After blocking, samples were incubated sequentially with sheep anti-THP and HRP-conjugated rabbit anti-sheep IgG at 37°C for 1 h. Ortho-phenylenediamine solution was added to each well and incubated at room temperature for 5 min. Color development was terminated by addition of 1 M of H_2SO_4 and absorbance was measured at 490 nm. Data represent means ± SD calculated from 5–16 samples.*

TABLE 1—*The result of ELISA detection for Tamm-Horsfall protein in urine, urine stains, and body fluids.*

Body Fluids	*n*	Absorbance (Mean ± SD)*	Number of Positive Samples†
Urine	16	0.343 ± 0.057‡	16
Aged urine stain	16	0.554 ± 0.315	14
Plasma	5	0.017 ± 0.006	0
Saliva	6	0.051 ± 0.015	0
Semen	6	0.022 ± 0.015	0
Vaginal fluid	5	0.079 ± 0.063	0
Sweat	5	0.035 ± 0.015	0

*Data represent absorbance at 490 nm in dilutions of 1:10 for body fluids and 1:1 for extracts of aged urine stains.

†Absorbance at 490 nm >0.2 in dilution of 1:10 for body fluids and 1:1 for extracts of aged urine stains.

‡Absorbance at 490 nm was significantly higher than plasma, saliva, semen, vaginal fluid, and sweat ($^{\ddagger}p < 0.01$, one-way ANOVA with Scheffé's multiple-comparison test).

of the target gene under conditions of equal amplification efficiency between 18S rRNA and the target gene. Therefore, a smaller dCt value indicates a higher level of target gene expression. On the other hand, when expression of the target gene was below the cut-off value (Ct > 40), the dCt value can be set to 25 (20,21).

Results

ELISA for THP and UPIII

ELISA for detection of THP was performed in 50 μL of each diluted sample. As shown in Fig. 1, although absorbance values were moderately different among individuals, urine showed high absorbance values compared with other body fluids even in dilution of 1:160. Table 1 showed the results of ELISA detection of THP at dilutions of 1:10 for body fluids and 1:1 for extracts of aged urine stains. As a result, absorbance values of urine at dilutions of 1:10 were significantly higher than those of plasma, saliva, semen, vaginal fluid extract, and sweat ($p < 0.01$). In addition, all 16 diluted urine samples and 14 of 16 samples of aged urine stain extracts showed positive results (absorbance at 490 nm >0.2) on ELISA for detection of THP. On the other hand, as shown in Table 1, THP was not detected in plasma, saliva, semen, vaginal fluid extract, and sweat other than urine.

ELISA for detection of UPIII was also performed for urine and other body fluids, which were concentrated and cleaned by ultrafiltration. Although urine showed positive results at a dilution of 1:640, saliva, semen, and sweat showed cross-reactivity to rabbit purified immunoglobulin against human UPIII (data not shown).

Immunohistochemical Staining for UPIII

The results of immunohistochemical staining using anti-UPIII antibody for the precipitate of urine are shown in Fig. 2*A*. Some

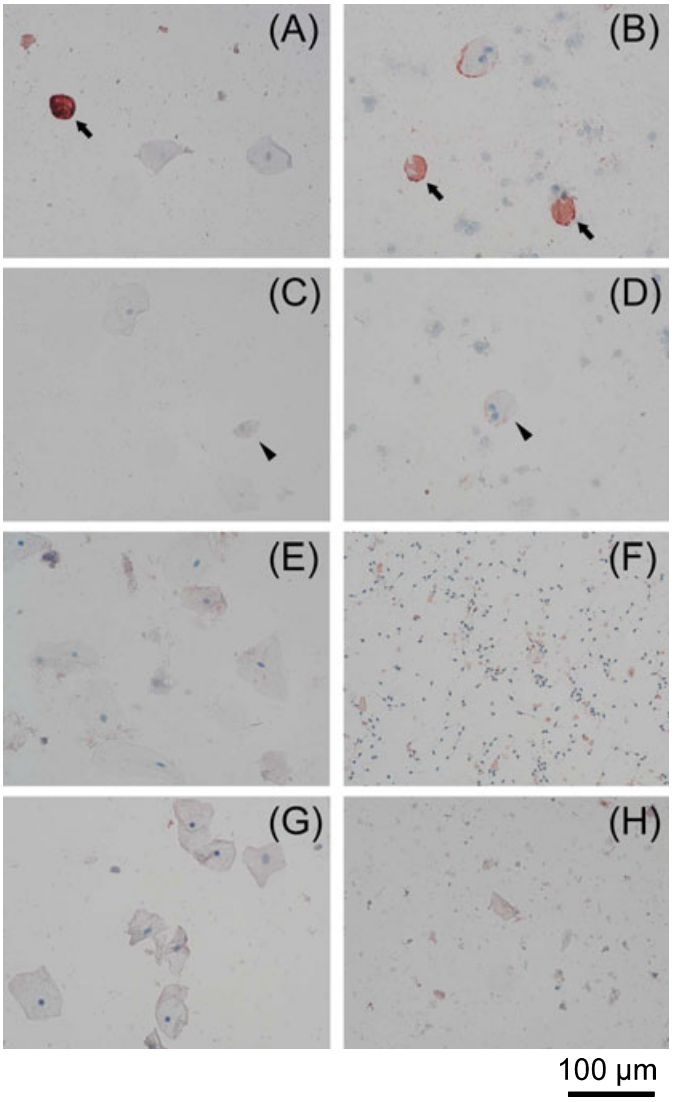

FIG. 2—*Immunohistochemical staining for uroplakin III to the urothelial cells and body fluids. Urine precipitate (A) and human bladder mucosa smear (B) were incubated with mouse monoclonal antibody against UPIII, HRP-conjugated goat anti-mouse IgG, and visualized with 3-amino-9-ethyl carbazole. Cell nuclei were counterstained with Mayer's hematoxylin solution. Controls consisted of urine precipitate (C) and human bladder mucosa smear (D) incubated without anti-UPIII antibody. The positively stained cells were marked with arrows, and unstained cells in control sample were marked with arrowheads. Immunohistochemical staining for UPIII in saliva (E), semen (F), vaginal fluid stain extract (G), and sweat (H). Centrifuged precipitates of saliva, semen, and sweat and vaginal fluid stain extract were stained with mouse monoclonal antibody against UPIII.*

cells were stained strongly with mouse monoclonal antibody against UPIII. These cells showed similar staining to multinuclear cells in the smear of human bladder mucosa, which was used as a positive control (Fig. 2*B*). On the other hand, the precipitate of urine and smear of human bladder treated without anti-UPIII antibody showed no staining (Fig. 2*C* and *D*).

As shown in Table 2, all and 13 of 15 samples showed positive results for immunohistochemical staining against UPIII for precipitates prepared from 20 and 1 mL of urine, respectively. In 2 of 15 samples, it was difficult to detect the stained cells in the precipitate of 1 mL of urine because few cells were collected from these urine samples.

Immunohistochemical staining for UPIII was also performed in saliva, semen, vaginal fluid stain extract, and sweat (n = 3–4). Positive results were not obtained in any body fluids except urine (Fig. 2*E–H* and Table 2).

Gene Expression Analysis for THP and UPIII

The levels of THP mRNA in urine varied among individuals (Fig. 3*A*). In addition, THP mRNA level was below the cut-off value (Ct >40) in one of the urine samples (Fig. 3*A*, Urine-1). On the other hand, UPIII mRNA was detected at similar levels in all samples of urine (Fig. 3*B*).

Urine specificity of THP and UPIII mRNA was examined using blood, saliva, semen, and vaginal fluid (n = 5–6). The results indicated that THP mRNA was specific for urine, with the exception of one semen sample (Fig. 3*A*). On the other hand, UPIII mRNA was detected in all urine samples at relative high levels; however, some blood, semen, and vaginal fluids also contained UPIII mRNA (Fig. 3*B*).

Discussion

In this study, to evaluate THP and UPIII as markers for forensic identification of urine and urine stains, ELISA was used for detection of THP and UPIII, and immunohistochemical staining for UPIII was performed. In addition, to verify the urine specificity of THP and UPIII, gene expression analysis was also performed by RT-PCR.

Application of THP for forensic identification of urine stains was reported previously (9,10). However, these methods have not generally been used in forensic casework. In this study, ELISA detection of THP from urine and urine stains was re-examined by a simple ELISA method developed with commercially available reagents. In addition, the urine specificity of THP in forensic identification of body fluids was evaluated by gene expression analysis of THP. The results of ELISA detection of THP indicated that although variability of the levels of THP was seen in urine samples, THP was specifically detected in urine but not in blood, saliva, semen, vaginal fluid extract, or sweat (Fig. 1 and Table 1). In addition, all urine samples and 14 of 16 samples of aged urine stains showed positive results on ELISA detection of THP. Furthermore, the results of gene expression analysis showed that expression of THP mRNA was specific for urine, except in one semen sample (Fig. 3*A*). However, as both urine and semen pass through the urethra, semen is likely to be contaminated with urine. Therefore, as reported previously (10), ELISA detection of THP can be used as a sensitive presumptive test for forensic identification of urine as well as aged urine stains. The method for detection of THP by ELISA used in this study may be useful for forensic casework because of its simplicity, without the requirement of any pretreatment, such as solubilization or deaggregation. In addition, all reagents are commercially available and the result can be obtained within 5 h. In

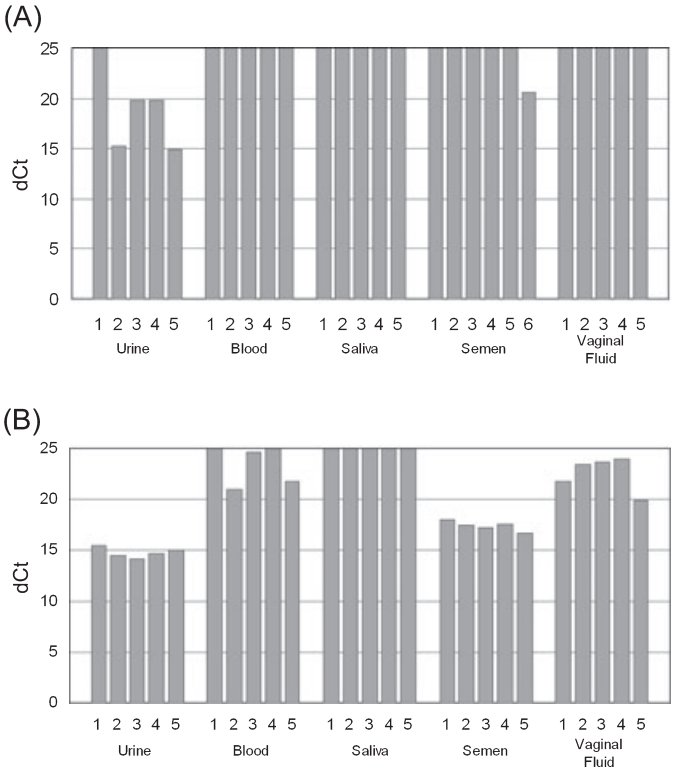

FIG. 3—*Comparisons of Tamm-Horsfall protein and uroplakin III mRNA levels among body fluids. THP (A) and UPIII (B) mRNA levels in body fluids. Total RNA was isolated from body fluids* (n = 5–6) *and reverse transcribed. RT-PCR was performed for THP, UPIII, and 18S rRNA (internal standard) using TaqMan probes. Expression levels of THP and UPIII in each body fluid were compared with dCt values, calculated by subtracting the Ct value of 18S rRNA from that of the target gene. Smaller dCt value indicates higher expression of the target gene. A dCt value of 25 indicates the cut-off value of target gene expression.*

TABLE 2—*The results of immunohistochemical staining for uroplakin III to urine and other body fluids.*

Body Fluids	*n*	UPIII-Positive*	UPIII-Negative	Difficult to Detect	Percentage of UPIII-Positive
Urine (20 mL)	15	15	0	0	100
Urine (1 mL)	15	13	0	2	86.7
Saliva	4	0	4	0	0
Semen	3	0	3	0	0
Vaginal Fluid	3	0	3	0	0
Sweat	4	0	4	0	0

*Cells were stained with mouse monoclonal antibody against uroplakin III.
UPIII, uroplakin III.

previous studies, although it was reported that detection levels of THP were negligible in animals except some monkeys (9,10), we confirmed that some canine urine might be cross-reacted with anti-THP antibody used in this study (data not shown). Species specificity should be investigated in detail for various animals in ELISA detection of THP developed in this study.

On immunohistochemical staining for UPIII, the cells in the urine precipitates showed strong staining with mouse monoclonal antibody against UPIII. As these cells showed no staining without primary antibody, the specificity of mouse monoclonal antibody to UPIII for urothelial cells in urine was confirmed (Fig. 2*C* and *D*). On the other hand, although saliva, semen, vaginal fluid, and sweat showed some background staining, no cells in these body fluids were stained strongly with mouse monoclonal antibody against UPIII (Fig. 2*E–H*). That is, no cross-reactivity was observed in the cells of these body fluids. Therefore, the results of this study indicated that immunohistochemical staining for UPIII is specific for urothelial cells in urine. However, on ELISA detection of UPIII, cross-reactivities were detected in saliva, semen, and sweat (data not shown). Similarly, the results of gene expression analysis showed that UPIII mRNA is detected not only in urine but also in some samples of blood, semen, and vaginal fluid (Fig. 3*B*). Although UPIII is widely used as a urothelium-specific cell surface marker for diagnosis of tumors and urothelial differentiation (15–19), it was previously reported that UPIII mRNA was expressed in prostatic glandular epithelium (22). Although UPIII may not be localized at the cell surface of nonurothelial cells, it may be expressed at low levels in the nonurological system and be present in several body fluids. The results of this study suggested that UPIII may be useful for forensic identification of urine by immunohistochemical staining. In the case of drug abuse testing, it is necessary to confirm the sample submitted by the suspect is urine. As centrifuged supernatant liquid is used for drug abuse testing, the precipitate of the sample may be subjected to immunohistochemical staining for UPIII for identification of urine.

There have been a number of recent reports of identification of blood, semen, saliva, vaginal fluid, and menstrual blood using mRNA markers detected by single or multiplex RT-PCR (20,21,23–25). However, target genes for identification of urine have not been reported. The results of gene expression analysis performed in this study indicated that although gene expression of THP was specific for urine, THP mRNA was not detected in one fresh urine sample (Fig. 3*A*). On the other hand, UPIII mRNA was detected not only in urine but also in some blood, semen, and vaginal fluid samples (Fig. 3*B*). Although THP protein is expressed in renal tubules and secreted in urine (26), THP mRNA is thought to be expressed in renal tubules but to be secreted in urine at only very low levels. For this reason, in some urine samples, THP is thought to be detectable at the protein level but difficult to detect at the mRNA level. For mRNA-based identification of urine, further investigations are required to identify better target gene(s) than THP and UPIII.

In conclusion, the results of this study indicated that ELISA detection of THP can be used as a presumptive test for forensic urine identification because THP was confirmed as a urine-specific protein marker. In addition, UPIII may be applicable in forensic urine identification by immunohistochemical staining.

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