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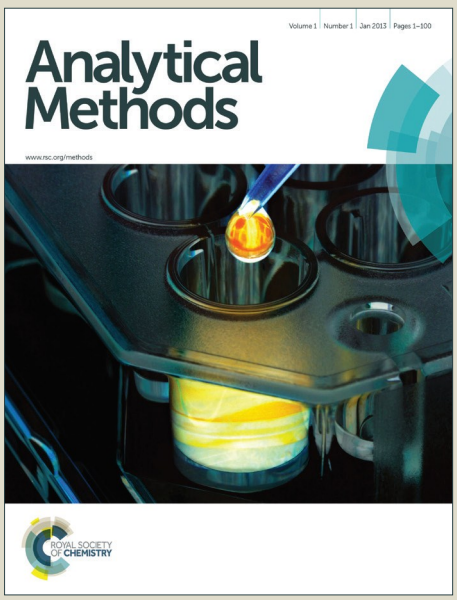

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Detection of fatty acid-binding protein 5 and small proline-rich protein 3 for forensic vaginal fluid identification by ELISA

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Abstract

Vaginal fluid identification is often required for forensic investigation of sexual assault cases. However, standardized assays for vaginal fluid identification have not been developed. Recently, we identified human fatty acid-binding protein 5 (FABP5) and human small proline-rich protein 3 (SPRR3) as characteristic vaginal fluid proteins by performing peptide mass fingerprinting. In this study, we developed enzyme-linked immunosorbent assays (ELISAs) for detecting FABP5 and SPRR3 and evaluated the specificity and sensitivity of these assays for detecting vaginal fluid. The data indicate that levels of both protein markers were significantly higher in vaginal fluids and vaginal fluid stains than in other body fluids (nasal secretions, saliva, urine, semen, blood, and sweat). The dilution limits of FABP5 and SPRR3 ELISAs equated to 0.06 μL and 0.03 μL respectively of vaginal fluid extracts, thought to be sufficient for application to real forensic samples. Furthermore, the levels of both protein markers are not lowered during the menstrual cycle. The protein markers were also detectable in menopausal samples, taken from menopause and pregnancy. The protein markers were ditected in some aged stains.FABP5 ELISA showed a better detection rate in inter

laboratory tests using simulated casework samples compare to SPRR3 ELISA. Overall, FABP5 can be more useful for the identification of vaginal fluid for forensic investigation, although both FABP5 and SPRR3 assays can potentially be useful.

Keywords: Enzyme-linked immunosorbent assay; vaginal fluid identification; fatty acid-binding protein 5; small proline-rich protein 3; forensic science

1. Introduction

Forensic anaylsis is one of the most actively growing areas of bioanalytical chemistry [1]. In particular, DNA typing is currently the most considerably developed common method for identifying individuals. In forensic biomaterial analysis, body fluids are investigated to determine the biological sources of trace evidence before DNA typing to identify the individuals involved in a crime scene. Body fluid identification is important because it helps to determine the events that occurred during a crime. The increasing significance of DNA typing has also emphasized the importance of identifying body fluids [2]. Further, blood, saliva, semen, and vaginal fluid are the most commonly found body fluids at crime scenes. The identification of vaginal fluids on objects or individuals is important in linking evidence in sexual assault cases. Vaginal fluids are often left as trace evidence on the body and clothes of the suspect, or on the surface of contraceptives left behind at a crime scene. Currently, a standard method for identifying vaginal fluid has not been established.

Previously, glycogen present in vaginal epithelial cells was used as a marker for vaginal fluids and Lugol's staining method was used to stain the glycogen of vaginal epithelial cells [3]. However, Lugol's

method is no longer used for vaginal fluid identification because male oral and urinary epithelial cells are also stained by Lugol's stain [4-7]. Recently, bacterial analysis methods have been developed for vaginal fluid identification [8-10]. However, studies have indicated that these methods fail to detect some vaginal samples [9, 10] and the influences of antimicrobial therapy have not been clarified. Additionally, the vaginal ecosystem is known to vary according to the stage of the menstrual cycle, pregnancy, use of contraceptive agents, frequency of sexual intercourse, and antibiotics [11].

Protein-based methods are routinely used for identifying body fluids in forensic institutes. For example, hemoglobin [12], prostate-specific antigen [13, 14], and amylase [15] are used as markers for blood, semen, and saliva, respectively. Previous studies identified several candidate protein markers for identifying vaginal fluids [16-21]. However, the identified protein markers do not have adequate specificity for forensic identification of vaginal fluids. Recently, Van Steendam et al. reported a method of examining vaginal fluids using nano-HPLC and ESI-Q-TOF-MS [22] with digesting potential protein markers; however, other simpler methods are required for practical forensic investigation.

In a previous study, we showed that fatty acid-binding protein 5 (FABP5) and small proline-rich protein 3 (SPRR3) can be used as potential protein markers for identifying vaginal fluids [23]. FABP5 was first detected in and cloned from keratinocytes of psoriasis patients [24]. The FABP5 protein expression level is low in normal epidermis, but is high in human cultured keratinocytes and is considerably elevated in the keratinocytes of psoriasis patients [25]. Normal adult epidermis does not have detectable levels of SPRR3, however, SPRR3 is expressed in oral and epithelial tissue including tongue- and tonsil tissue [26, 27]. Adult human saliva also contains SPRR3 [28, 29] and the SPRR3 levels are considerably higher in the saliva of preterm newborns [30]. Recently, we showed that, amongst body fluids, FABP5 and SPRR3 are most abundantly expressed in vaginal fluids [23]. The expression of SPRR3 is highly elevated during the differentiation of human epidermal keratinocytes and has been considered a squamous epithelium marker [31, 32]. The proteins have the potential to be expressed in vaginal fluids regardless of menstrual cycle, menopause, and pregnancy, because vaginal fluid is known to contain a large number of squamous epithelium cells. The method using the proteins for forensic identifying vaginal fluid have not been reported.

In this study, we evaluated the expression of FABP5 and SPRR3 in various body fluids (nasal secretions, saliva, urine, semen, vaginal fluids, blood, and sweat) by developing ELISA and determining whether these ELISAs can be used as methods for the forensic identification of vaginal fluid.

2. Experimental

2.1. Sample collection and treatments

2.1.1. Sample collection

All procedures involving human volunteers were approved by the Ethical Committee of Human Genome and Genomic Analysis from the Japanese Association of Forensic Science and Technology. Samples were collected from consenting adults. Vaginal fluids (n = 14) were collected from volunteers aged 28–56 years, including two menopausal and two pregnant volunteers . Nasal secretions (n = 22), saliva (n = 31), semen (n = 17), blood (n = 20), urine (n = 30), and sweat (n = 22) were collected from volunteers aged 26–57 years. The volunteers collected vaginal fluids by swabbing the vaginal wall with sterile cotton swabs. Ten participants, except for the menopausal and pregnant volunteers, collected vaginal fluids regardless of their menstrual cycle. One participant (under thirty) collected samples once weekly for 4 weeks before pregnancy and collected a sample during pregnancy. The samples collected over the 4-week period were used to evaluate the effect of menstrual cycle on protein marker expression. Sweat drops were collected from

participants post-exercise. The sweat samples were collected from the facial region and arms by using filter paper strips. Nasal secretion samples were obtained in sterile plastic tubes by collecting expelled mucus secretions from volunteers. Blood samples were collected from the brachial vein by using VENOJECT II collection tubes (TERUMO, Tokyo, Japan). Other body fluids were collected in sterile plastic tubes. Children's saliva samples were collected from six volunteers aged between 2 months to 8 years. Collected body fluids were stored at -80°C until required for further analysis.

2.1.2. Stain preparation

Vaginal fluid stains (n = 10) were prepared as follows: Sterile cotton swabs with vaginal fluid samples were cut into approximately 5 mm × 5 mm squares and air-dried at room temperature for 1 week, 2 months, or 1 year. Sweat stains (n = 5) were prepared as follows: Filter papers with sweat samples were cut into approximately 10 mm × 10 mm squares and air-dried at room temperature for 1 week. Stains of other body fluids (n = 5 or n = 20) were prepared as follows: 5 μL of body fluids were spotted onto filter papers (approximately 10 mm × 10 mm squares) and air-dried at room temperature for 1 week. High concentrated

stains of saliva (n = 5) were prepared as follows: 25 μL of saliva samples were spotted onto filter papers (approximately 10 mm × 10 mm squares) and air-dried at room temperature for 1 week.

2.1.3 Simulated casework samples

The surface of a used condom, which was air-dried at room temperature in the volunteer's house overnight, was wiped with a sterile cotton swab. A fresh sanitary napkin which contained traces of vaginal discharge, a used pantyliner which had been incubated in a trash box for 1 week in the volunteer's house, and underwear which had been worn for one day were collected. The underwear was air-dried at room temperature for 1 week. The sanitary napkin, pantyliner, and underwear were cut into approximately 10 mm × 10 mm squares.

Vaginal fluid stains on various substrata (cotton, hemp, silk, bull leather), which were prepared by stamping a cotton swab collected vaginal fluid, were air-dried at room temperature for 1 week in the volunteer's house.

Vaginal fluid specimens were prepared by mixing with saliva and semen (n = 6), as described herein: 20 μL of saliva or semen was spotted onto sterile cotton swabs immersed in vaginal fluid samples (approximately 3 mm × 3 mm squares) and air-dried at room temperature for 1 week.

2.2. Enzyme-linked immunosorbent assay

2.2.1. Reagents

Goat polyclonal antibody against human FABP5 (anti-FABP5) purified by affinity chromatography was purchased from R&D systems (R&D systems, MN, USA). Mouse monoclonal antibody against human SPRR3 (anti-SPRR3) purified by affinity chromatography was purchased from Abnova (Abnova, Taipei, Taiwan). Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Sigma Aldrich, MO, USA) and HRP-conjugated goat anti-mouse IgG (KPL, MD, USA) were purchased.

Lyophilized anti-FABP5 antibody was dissolved in 75 μL of distilled water, and diluted (1:500) with 0.05% Tween-20 in phosphate-buffered saline (PBST). Anti-SPRR3 antibody was diluted (1:1,000) in PBST. The HRP-conjugated rabbit anti-goat IgG was diluted (1:1,000) with PBST. Finally, HRP-conjugated goat anti-mouse IgG was diluted (1:5,000) with PBST for detecting body fluid samples, and was diluted (1:2,500) with PBST for detecting dry stain samples.

Recombinant FABP5 (ATGen, Seongnam, Korea) and recombinant SPRR3 (CUSABIO, Wuhan,

China) were refined by LC. Then, the concentrations were quantified using the 2D-Quant kit (GE Healthcare, Amersham, UK).

2.2.2. Sample preparation

Body fluid samples were prepared by cutting cotton swabs with vaginal fluid into approximately 5 mm × 5 mm square pieces and then extracting the sample with 100 μL of 0.05 M bicarbonate buffer (BCB, pH 9.6). Body fluids and vaginal fluid extracts were diluted in BCB by using dilutions in the range from 1:100 to 1:6,400. Filter papers with sweat samples were cut into approximately 10 mm × 10 mm squares and extracted with 250 μL of BCB by pipetting, and the extracts were diluted in BCB by using dilutions that ranged from 1:2 to 1:64.

Stain samples were prepared by extracting vaginal fluid stains or mixed stains with 100 μL of BCB by pipetting. The extracted samples (5 μL) were diluted (1:100) with BCB on ice. Other body fluid stains, high concentrated saliva stains, and casework samples were extracted with 250 μL of BCB by pipetting on ice. The sample extracts (250 μL) were centrifuged at 7,900 g for 3–5 min at 4°C. The

supernatants were diluted with BCB by using dilutions that ranged from 1:2 to 1:64.

Recombinant SPRR3 was diluted from 0.3 ng/mL to 30 μg/mL and recombinant FABP5 was diluted from 3 ng/mL to 100 μg/mL by BCB.

2.2.3. ELISA

Diluted samples (50 μl per well) were added to 96-well multitier plates (SUMILON MS7296F, Sumitomo Bakelite, Tokyo, Japan) and incubated at 37°C for 1 h. Each well was blocked with 200 μL of Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) at 37°C for 1 h. The wells were washed three times by using 250 μL of PBST per well and 50 μL of diluted anti-human FABP5 or anti-human SPRR3 was added to each well. The plates were then incubated at 37°C for 1 h. The plates were washed three times with 250 μL of PBST per well and then incubated with 50 μL of diluted HRP-conjugated rabbit anti-goat IgG or HRP-conjugated goat anti-mouse IgG per well at 37°C for 1 h. The plates were washed five times with 250 μL of PBST per well and 50 μL of TMB + Substrate Chromogen (Dako Cytomation, CA, USA) was added to each well and incubated at room temperature for 3 min. Color development was stopped by additional of 50

μL of 1 M H_2SO_4. Absorbance was measured at a wavelength of 450 nm by using Molecular Devices SPECTRA max PLUS 384 (Molecular Devices, CA, USA). Each absorbance value was normalized by subtracting the primary antibody blank absorbance value. Standard curves from FABP5 and SPRR3 ELISAs were obtained using the recombinant proteins.

2.2.4. Inter laboratory testing

A portion of the simulated casework samples (sanitary napkin, pantyliner, and underwear) and two urine stains (5 μL of urine were spotted onto approximately 10 mm × 10 mm square filter papers and air-dried at room temperature for 1 week) were sent to the National Research Institute of Police Science (NRIPS) in cold storage. ELISA procedures were as described in 2.2.2. and 2.2.3.

2.3. Detection of bacterial ribosomal RNA genes

DNA samples were extracted from cotton swabs with collected vaginal fluids (n = 14), semen (n = 5), and urine (n = 5) using an EZ1 DNA Investigator Kit (Qiagen, Hilden, Germany) and BIO ROBOT EZ1 (Qiagen).

The procedure for PCR amplification and agarose gel electrophoresis was performed as previously described [10].

2.4.Statistical analysis

The ELISA data were analyzed to determine statistically significant differences by using one-way ANOVA with Scheffé's multiple-comparison test for each dilution ratio. The cut off values of the ELISAs were obtained by receiver operating characteristic (ROC) curve analysis for the highest absorbance values of each body fluid sample in the dilution range from 1:100 to 1:6,400.

3. Results

3.1. Specificity and sensitivity of ELISA for detecting adult body fluids

The expression of FABP5 and SPRR3 in adult body fluids was evaluated by ELISA. All absorbance values were measured using the 450 nm wavelength. The FABP5 absorbance values were higher for vaginal fluids than those for other body fluids (Figure 1A). Furthermore, the FABP5 absorbance values of diluted vaginal fluids (1:3,200) were higher than the FABP5 absorbance values of other body fluids. The FABP5 absorbance values of vaginal fluid samples at dilutions ranging from 1:100 to 1:3,200 were significantly higher than those of the other body fluids (nasal secretions, saliva, semen, urine, blood, and sweat; $p < 0.01$). The data also revealed that there was moderate inter-individual variation in FABP5 expression.

Similarly, SPRR3 ELISA data show that vaginal fluids had higher absorbance values than those of other body fluids (Figure 1B). Furthermore, the SPRR3 absorbance values of diluted vaginal fluids (1:1,600) were higher than those of other body fluids. The SPRR3 absorbance values of vaginal fluids at dilutions that ranged from 1:100 to 1:6,400 were significantly higher than those of the other body fluids (nasal secretions,

saliva, semen, urine, blood, and sweat; $p < 0.01$). The data also revealed that there was moderate inter-individual variation in SPRR3 expression.

To validate the significance of FABP5 and SPRR3 as markers the ROC curves were plotted (Figure 2). The exclusion of absorbance values (no false positives and maximum true positives) were calculated as 0.28 (30 ng/ mL) for FABP5 and 0.10 (160 ng/ mL) for SPRR3 by ROC analysis. In FABP5 ELISA, the values that were lower than 0.28 yielded positive results only for the vaginal fluid samples and all other body fluids tested negative (Table 1). The limit of dilution for FABP5 ELISA was determined to be 0.06 μL (50 μL of 1:800 dilution) by the comparison of the average absorbance (Aav) values in each dilution ratio with the cut-off absorbance value, and the Aav value at the dilution limit was 0.48. In SPRR3 ELISA, if absorbance values under 0.10 were excluded, 12 (including the two samples from menopause and pregnancy) out of 14 diluted vaginal fluid samples showed positive results, and other body fluid samples showed negative results. The limit of dilution for SPRR3 ELISA was determined to be 0.03 μL (50 μL of 1:1,600 dilution) and the Aav of vaginal samples was 0.10.

3.2. Influence of Menstrual cycle

Both FABP5 and SPRR3 were detected by ELISA. Diluted vaginal fluid samples that were collected once weekly were used for the assay. The ELISA data revealed that all samples had significantly high absorbance values for both protein markers regardless of the stage of menstrual cycle. Furthermore, all samples had higher absorbance values than the aforementioned cut-off values for each protein marker.

3.3. Protein marker expression in saliva of children

Both FABP5 and SPRR3 were measured by performing ELISA by using 50 μL of diluted saliva obtained from children. None of the samples showed significant absorbance. Furthermore, all samples had absorbance values that were lower than the aforementioned cut-off values for each protein marker.

3.4. Stains

3.4.1. Specificity and sensitivity of ELISA for body fluid stains

Both FABP5 and SPRR3 ELISAs were performed using 50 μL of diluted extract obtained from 1 week-stains of various adult body fluids. The FABP5 absorbance of vaginal fluids was higher than that of other body

fluids (Figure 1C). Furthermore, the FABP5 absorbance values of diluted vaginal fluids (1:16) were higher than those of other body fluids. The absorbance values of vaginal fluid samples at dilutions ranging from 1:1 to 1:8 were significantly higher than those of other body fluids (nasal secretions, saliva, semen, urine, blood, and sweat; $p < 0.01$). The SPRR3 absorbance of vaginal fluids was higher than that of other body fluids (Figure 1D). Furthermore, the SPRR3 absorbance values of diluted vaginal fluids (1:32) were higher than those of other body fluids. The absorbance values of vaginal fluid samples at dilutions ranging from 1:1 to 1:4 were significantly higher than those of other body fluids (nasal secretions, saliva, semen, urine, blood, and sweat; $p < 0.01$).

The FABP5 and SPRR3 ELISAs were performed using 50 μL of diluted vaginal fluid extracts of obtained from stains that were aged for various time-periods (1 week, 2 months, and 1 year). The numbers of samples that tested positive for each protein marker are shown in Table 2. Both FABP5 and SPRR3 were detectable in one-year-old stains of vaginal fluid.

The limit of dilution for 1-week-old stains for FABP5 ELISA was determined to be 1.25 μL (50 μL of 1:4 dilution) and the Aav of vaginal samples was 0.33. The limit of dilution for SPRR3 ELISA was determined to be 12.5 μL (50 μL of 1:4 dilution), and the Aav of vaginal samples was 0.15.

For two-months-old stains, the limit of dilution for FABP5 ELISA was determined to be 25.0 μL (50 μL of 1:2 dilution) and the Aav of vaginal samples was 0.40, and the limit of dilution for SPRR3 ELISA was determined to be 12.5 μL (50 μL of 1:4 dilution) and Aav of vaginal samples was 0.12.

The dilution limits for both assays were lower for detecting protein markers in stain-extracted samples than that for vaginal fluid samples. The FABP5 assay had high detection rate for vaginal fluids and for 1-week-old stains of vaginal fluids (Table 1 and 2).

3.4.2. Protein marker expression in high concentrated saliva stains

The expression of FABP5 and SPRR3 was detected by ELISA, using 50 μL of eluted high concentrated saliva stains. The data showed that the saliva stain samples had undetectable protein marker expression.

3.4.3. Simulated casework samples

ELISAs for FABP5 and SPRR3 were performed using 50 μL of extracts from casework samples. The condoms surface sample, sanitary napkin, pantyliner, and underwear contained protein markers at detectable expression levels (Table 3). FABP5 and SPRR3 were detected in stamped samples on various substrata (cotton, hemp, silk), however only FABP5 was detected in the stamped bull leather sample (Table 4). FABP5 was detected in all mixed samples, and SPRR3 was detected in some mixed samples (Table 5).

3.4.4. Inter laboratory testing

FABP5 was detected in all the simulated casework samples (sanitary napkin, pantyliner, and underwear) sent to NRIPS; the urine stains (n=2) showed no FABP5. No samples showed SPRR3 (Table 3).

3.5. Detection of bacterial ribosomal RNA genes

To assess the advantage of FABP5 and SPRR3 ELISAs over previously reported methods, bacterial ribosomal RNA gene detection was performed. The results are shown in Table 6.

4. Discussion

This study initially assessed FABP5 and SPRR3 ELISAs as forensic vaginal fluid identification methods. ELISAs for FABP5 and SPRR3 detection were highly specific and sensitive and could efficiently discriminate vaginal fluid samples from other biological fluids based on the protein marker expression (Figure 1 and Table 1). The ELISA data for both protein markers indicate that the detection rate of both assays for 1-week-old stains of vaginal fluids and vaginal fluids was comparable (Table 1 and 2), although the sensitivity of detection for both assays was decreased for the 1-week-old stain samples. In addition, FABP5 and SPRR3 were detectable in aged stains (2 months and 1 year). These results indicate that this method has potential application for forensic identification of vaginal fluids.

The exclusion absorbance values were fixed by ROC analysis for body fluid samples. In forensic investigation, fewer false positives (high specificity) might be more important than a high detection rate of a body fluid. Therefore, the minimum false positive and maximum true positive absorbance values were selected as cut off values. All vaginal fluid samples in FABP5 ELISA and 12 out of 14 vaginal fluid samples

in SPRR3 ELISA yielded positive results by using these values. For stain samples, the values yielded positive results only for vaginal fluid stains and negative results for all other body fluid stains. Therefore, the values could be useful for discriminating vaginal fluid samples.

The concentrations of exclusion absorbance values in this study were lower than those of limit of detections (FABP5: 5 μL/mL, SPRR3: 20 μL/mL) in LC/MS [23]. FABP5 ELISA was approximately 170-fold high sensitive than LC/MS, and SPRR3 ELISA was 120-fold high sensitive. Therefore, the ELISAs may be more useful than LC/MS, because forensic samples are generally small amount. Moreover, the standards for LC/MS are not available at present.

To evaluate the effect of menstrual cycle on protein marker expression, vaginal fluid samples were obtained during various stages of the menstrual cycle and the expression of FABP5 and SPRR3 was assessed. The data showed that FABP5 and SPRR3 expression was not influenced by menstrual cycle. However, this finding should be re-evaluated by using a larger sample population.

Previous studies have reported that FABP5 and SPRR3 expressions are significantly elevated during the differentiation of human epidermal keratinocytes and that SPRR3 can be used as a marker for squamous epithelium [31-33]. Thus, the SPRR3 expression in vaginal fluids may be related to the large numbers of squamous epithelial cells in the vaginal fluid. The SPRR3 expression data also indicated that there were some inter-individual differences in SPRR3 expression and that two samples did not test positive for SPRR3. The samples with undetectable SPRR3 were reassessed by using another antibody dilution and showed detectable SPRR3 expression with the changed antibody dilution (data not shown). Thus, SPRR3 expression was considerably low in two of the samples.

A previous study reported high SPRR3 expression in the saliva of preterm newborns [30]. The expression of protein markers in the saliva of children was assessed in this study to evaluate the specificity of the SPRR3 ELISA for discriminating children's saliva samples from vaginal fluid samples. The six saliva samples obtained from children did not have significant absorbance values and therefore tested negative for SPRR3. In addition, SPRR3 has also been reported to be expressed in adult saliva. Thus, the expression of

SPRR3 was determined in highly concentrated saliva stains. The data indicate that SPRR3 was not detectable in the five adult saliva stains. Thus, SPRR3 can be used to discriminate vaginal fluid stains from saliva stains. Our previous study suggest the expressions of FABP5 and SPRR3 in vaginal fluid are characteristic higher than in other body fluids at almost the same volume [23], although the expression in other tissues were reported previously [24-30].

To evaluate the effect of menopause and pregnancy on protein marker expression, vaginal fluid samples were obtained from two menopausal volunteers and two pregnant volunteers, and the expression of FABP5 and SPRR3 was evaluated. The data showed that the protein expression levels were not influenced by the condition of menopause and pregnancy. The high expression levels of these protein markers may serve as the physiological function of defense against external pathogens [23]. However, the sample population used in the present study was small, and thus, further studies are required to clarify the influence of menopause and pregnancy on protein marker expression.Vaginal fluid samples are often collected as a body fluid mixed with saliva or semen in sexual assault cases. Therefore, this study affirmed

the potential of FABP5 and SPRR3 ELISA for identifying vaginal fluid in a mixture, but with FABP5 showing a higher detection rate than SPRR3 ELISA (Table 5). To clarify the potential of these methods with different materials and environmental effects, different materials which were air dried in the volunteers' homes were tested. As a result, FABP5 was detected in all material samples, however SPRR3 was not detected in the bull leather sample. The reasons for the limited detection of SPRR3 have not been fully revealed. The presence of microbial flora and proteinases in saliva and semen might be one reason for its limited detection. Additionally, leather may take longer to dry than cloth, concealing SPPR3 because vaginal bacteria express proteinases [34, 35]. Digestion of the epitope may easily affect the detection, because the anti-SPRR3 antibody is monoclonal, but the anti-FABP antibody is polyclonal. In forensic investigation, inspectors should bear this information in mind, although further studies are needed to clarify these influences.

The forensic potentials of FABP5 and SPRR3 were affirmed by an inter-laboratory test (Table 3). SPRR3 was not detected in any simulated casework samples. The difference of SPRR3 detection between the

two laboratories is unclear, however. SPRR3 was also shown to have low absorbance depending on the differences of material and mixing with saliva and semen. The delivery process and/or transportation may influence the different results between laboratories, because the test at NRIPS was performed almost 2 weeks after our own.

In analysis of simulated casework samples, the detection rate of FABP5 ELISA was higher than that of SPRR3 ELISA (Table 3). The reasons for the difference are not revealed. Further studies are needed to clarify why the detection rate of SPRR3 ELISA is decreased by mixing with saliva and semen. FABP5 was detected in all samples. Therefore, FABP5 ELISA may be more useful for investing a sample suspected of mixing body fluids

Recently, microbial-based methods for identifying vaginal fluids have been reported [8-10]. To assess the advantage of FABP5 and SPRR3 ELISA, bacterial ribosomal RNA gene detection was performed (Table 6). *L. jensenii* and *A. vaginae* were detected only in vaginal fluid samples. However, *L. jensenii* was previously reported to be detected in female urine as it is similar to *L. crispatus.* Therefore, the specificity of

FABP5 and SPRR3 ELISA may be higher than *L. jensenii* and *L. crispatus* detections. The detection rate of FABP5 and SPRR3 ELISA for vaginal fluids is higher than any potential markers of bacteria. If using three markers at the same time, more than one marker could be detected in 13 out of 14 vaginal samples at least. In this case the bacterial methods might be unable to discriminate female urine and vaginal fluid, although the detection rate was similar to that of FABP5 and SPRR3 ELISA. Therefore, FABP5 and SPRR3 could be a better specific marker for the detection of vaginal fluid. *L. jensenii* was detected in two menopausal samples. We additionally tested the influence of pregnancy on bacterial detection methods. We had collected another sample before pregnancy from one of the pregnant volunteers. In results, no bacterial marker was detected in the pregnant sample, although *L. crispatus* was detected in the sample taken before pregnancy. It is important to note that the composition of the vaginal flora changes according to the menstrual cycle, pregnancy, use of contraceptive agents, frequency of sexual intercourse [36, 37], and antibiotics; these changes likely involve the loss of lactobacilli in the vagina [11, 37]. FABP5 and SPRR3 have the potential to unaffected by the vaginal flora change, because the proteins are detectable in vaginal fluids regardless of menstrual cycle, menopause, and

pregnancy, and vaginal fluid is known to contain a large number of epithelial squamous cells. Further studies are required to clarify the influence of these matters in a large sample population and a greater diversity.

FABP5 may be more useful for forensic identification of vaginal fluids, because FABP5 showed better results than other methods in our simulated casework samples. The results of FABP5 and SPRR3 assays may be skewed if the samples are taken from cancer patients [38-45]. Thus, to improve reliability, we suggest using two protein markers for identifying vaginal fluids if possible, because SPRR3 also has a high specificity and detection rate for vaginal fluid.

5. Conclusion

This study proposed novel methods for identifying vaginal fluids. A standard method has not been established to identify vaginal fluids and stains for forensic investigation. FABP5- and SPRR3-specific ELISAs have high specificity and sensitivity for vaginal fluids and can be used to detect aged vaginal fluid stains. Overall, the assays developed in this study have potential applications in the identification of vaginal fluids for forensic investigation.

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Table 1

Comparison of FABP5 and SPRR3 ELISA specificity for vaginal fluid detection

Body fluids	Number	Number of positive samples	
		Detection of FABP5*	Detection of SPRR3*
Nasal secretion	22	0	0
Saliva	31	0	0
Semen	17	0	0
Vaginal fluid	14	14	12
Urine	30	0	0
blood	20	0	0
Sweat	22	0	0

*Absorbance measured at 450 nm wavelength that was higher than 0.28 for FABP5 or higher than 0.10 for SPRR3.

Table 2

Detection of FABP5 and SPRR3 in vaginal fluid stain samples

Aged periods	Sample number	Number of positive samples*	
		FABP5	SPRR3
1 week	10	10	8
2 months	10	7	8
1 year	2	2	1

* Absorbance measured at 450 nm wavelength that was higher than 0.28 for FABP5 or higher than 0.10 for SPRR3.

Table 3

FABP5 and SPRR3 ELISA results of simulated casework samples from two laboratories

Samples	FSL Okayama		NRIPS	
	FABP5	SPRR3	FABP5	SPRR3
Surface of condom (overnight)	+	+	NT	NT
Sanitary napkin (fresh)	+	+	+	-
Pantyliner (1 week)	+	+	+	-
Underwear (1 week)	+	+	+	-
Urine 1 (1 week)	-	-	-	-
Urine 2 (1 week)	-	-	-	-

NT, not tested; FSL Okayama, Forensic Science Laboratory of Okayama Prefectural Police H.Q.; NRIPS, National Research Institute of Police Science. Absorbance measured at 450 nm wavelength that was higher than 0.28 for FABP5 or higher than 0.10 for SPRR3.

Table 4

Detection of FABP5 and SPRR3 from vaginal fluid stains on different materials

Materials	FABP5	SPRR3
Cotton cloth	+	+
Hemp cloth	+	+
Silk cloth	+	+
Bull leather	+	-

Absorbance measured at 450 nm wavelength that was higher than 0.28 for FABP5 or higher than 0.10 for SPRR3.

Table 5

Detection of FABP5 and SPRR3 in mixed samples

Samples	Sample number	Number of positive samples*	
		FABP5	SPRR3
Vaginal + Saliva	**6**	**6**	**2**
Vaginal + Semen	**6**	**6**	**2**

* Absorbance measured at 450 nm wavelength that was higher than 0.28 for FABP5 or higher than 0.10 for SPRR3.

Table 6

Detection of bacterial ribosomal RNA genes from body fluids

Body fluids	Sample number	*L. crispatus*	*L. jensenii*	*A. vaginae*
Female urine	**5**	**4**	**0**	**0**
Semen	**5**	**0**	**0**	**0**
Vaginal fluid	**14**	**4**	**8**	**6**

Fig. 1 Specificity of ELISA for detection of FABP5 (A) and SPRR3 (B) in body fluids and for detection of FABP5 (C) and SPRR3 (D) in 1 week-old-stains

Body fluids were diluted with bicarbonate buffer by using dilutions ranging from 1:100 to 1:6,400, 1 week-old-stain extracts were diluted from 1:2 to 1:64. Absorbance values are presented as means (± S.D.). The one-way ANOVA with Scheffé's multiple-comparison test analysis indicates that the absorbance values of vaginal fluids and stains were significantly higher than those of other body fluids and stains (**$p < 0.01$, *$p < 0.05$).

Fig. 2 Receiver operating characteristic (ROC) curves of FABP5 (A) and SPRR3 (B) ELISA in body fluid samples

ROC analyses were performed using the absorbance values of FABP5 and SPRR3 ELISAs. Area under the curve (AUC) and standard error of the AUC were 1.00 and 0.00 respectively for FABP5, and 0.99 and 0.01 respectively for SPRR3.

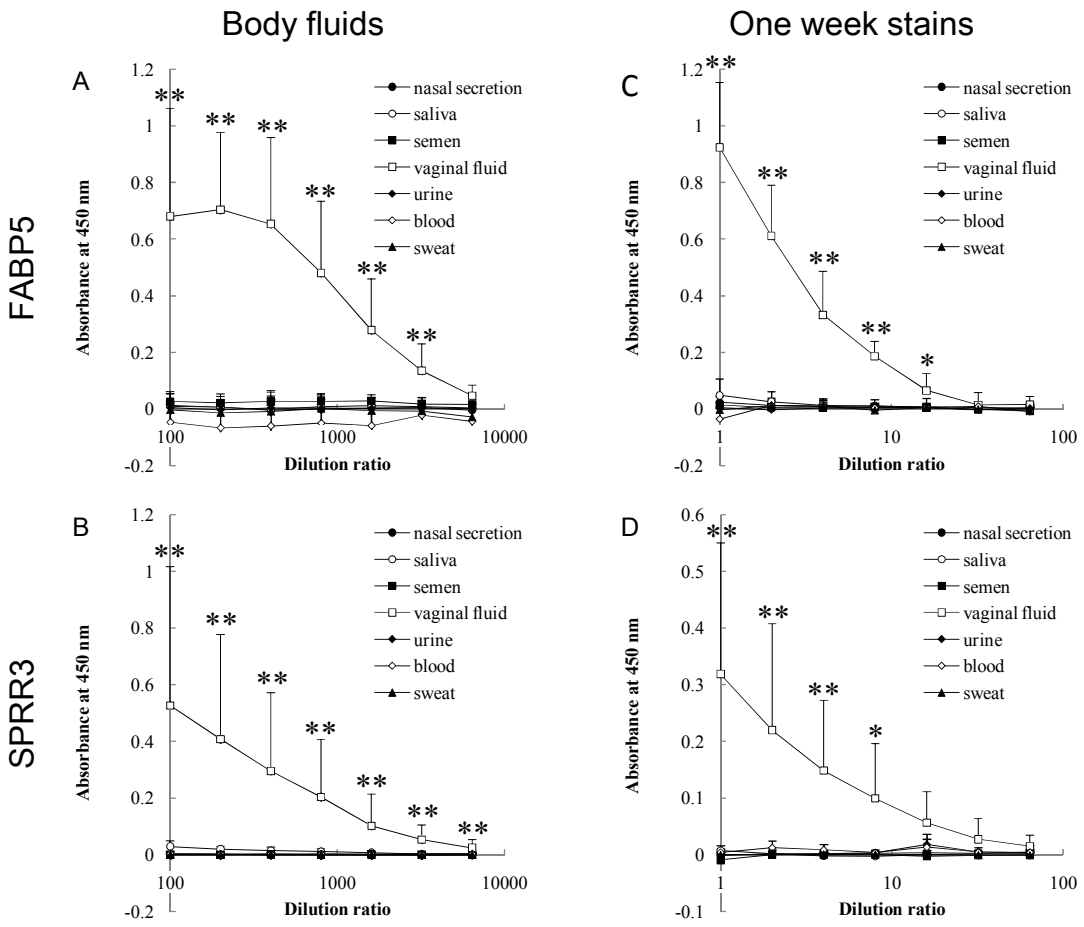

Figure 1

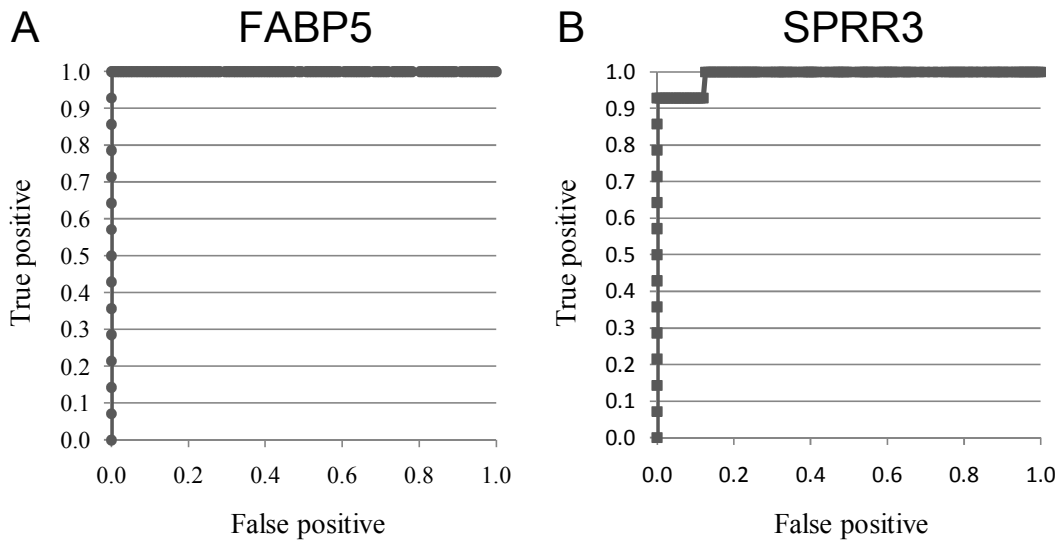

Figure 2