

Body fluid identification by integrated analysis of DNA methylation and body fluid-specific microbial DNA

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Abstract Identification of body fluids found at crime scenes provides important information that can support a link between sample donors and actual criminal acts. Previous studies have reported that DNA methylation analysis at several tissue-specific differentially methylated regions (tDMRs) enables successful identification of semen, and the detection of certain bacterial DNA can allow for identification of saliva and vaginal fluid. In the present study, a method for detecting bacterial DNA was integrated into a previously reported multiplex methylation-sensitive restriction enzyme-polymerase chain reaction. The developed multiplex PCR was modified by the addition of a new semen-specific marker and by including amplicons for the 16S ribosomal RNA gene of saliva- and vaginal fluid-specific bacteria to improve the efficacy to detect a specific type of body fluid. Using the developed multiplex system, semen was distinguishable by unmethylation at the USP49, DACT1, and PFN3 tDMRs and by hypermethylation at L81528, and saliva could be identified by detection of saliva-specific bacteria, *Veillonella atypica* and/or *Streptococcus salivarius*. Additionally, vaginal fluid and menstrual blood were differentiated from other body fluids by hypomethylation at the PFN3 tDMR and the presence of vaginal fluid-specific bacteria, *Lactobacillus crispatus* and/or *Lactobacillus gasseri*. Because the

developed multiplex system uses the same biological source of DNA for individual identification profiling and simultaneously analyses various types of body fluid in one PCR reaction, this method will facilitate more efficient body fluid identification in forensic casework.

Keywords Body fluid identification · DNA methylation · Methylation-sensitive restriction enzyme-polymerase chain reaction · Bacteria

Introduction

Body fluids found at a crime scene are one of the most important pieces of forensic evidence that can provide valuable information to identify suspects or victims and also can provide important information supporting a link between sample donors and actual criminal acts [1].

Various methods to identify the type of tissues or body fluids have been continually developed in forensic science. Catalytic, enzymatic and immunologic tests have generally been used [2], and advances in forensic genetics have led to the development of methods that detect tissue-specific messenger RNA or micro RNA expression [3–5]. Recently, DNA-based body fluid identification has begun to receive attention [6, 7]. Because DNA has mainly been used as a biological source for personal identification profiling, DNA-based body fluid identification seems promising. Two DNA-based body fluid identification methods have been reported: a DNA methylation-based method [6–11] and a bacterial DNA-based method [12–17].

DNA methylation affects gene expression regulation without DNA sequence changes. Different cell types have different DNA methylation patterns [18]. Specifically, chromosome segments called tissue-specific differentially methylated regions (tDMRs) show different DNA methylation profiles according to cell or tissue type [19–21]. Body fluid identification by

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analysing the DNA methylation status of these regions has been reported as a new method in forensic science [6, 7]. An et al. [8] described a body fluid identification method using methylation-sensitive restriction enzyme-polymerase chain reaction (MSRE-PCR) and methylation SNaPshot, which can identify semen and can distinguish vaginal fluid and menstrual blood from blood and saliva. In addition, the Nucleix DSI-Semen kit (Nucleix, Tel Aviv, Israel) is a recently developed commercial kit that makes it possible to distinguish semen from other body fluids with a small amount of DNA using MSRE-PCR [9, 10]. These methods can be used to analyse aged samples by applying a small amplicon strategy and are compatible with commercially available human identity testing kits and instrumentation [8–10]. However, DNA methylation-based analysis is in its early stage of development and thus needs to be improved such that it can utilize more body fluid-specific DMRs for detailed and reliable body fluid identification.

Another DNA-based method to identify the type of body fluids uses the DNA of bacteria that are present in a specific body fluid by amplifying bacterial species-specific regions of the 16S ribosomal RNA (rRNA) gene or the 16S-23S rRNA intergenic spacer region [12–17]. In particular, amplification of hypervariable regions in the 16S rRNA gene can facilitate detection of tissue-specific bacteria [22, 23]. Forensic body fluid identification by detection of bacterial DNA has focused primarily on saliva and vaginal fluid. Saliva can be identified by the presence of *Streptococcus salivarius* and/or *Streptococcus mutans* [12–14], while vaginal fluid can be distinguished from other body fluids based on detection of *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners* and/or *Lactobacillus jensenii* [15–17]. In addition, *Gardnerella vaginalis* and *Atopobium vaginae* have been suggested as useful for vaginal fluid identification because they are associated with bacterial vaginosis, the most frequent vaginal infection in fertile women [15, 23]. However, this method is limited in that it can be used to distinguish among only a few types of body fluids containing bacteria.

The goal of the present study was to integrate DNA methylation-based analysis and bacterial DNA-based analysis to improve the efficacy of existing DNA-based body fluid identification methods. We expect integration of the two methods in a single PCR reaction will allow more efficient discrimination of body fluids since the two methods complement each other.

Materials and methods

Samples

Blood, saliva and semen from 20 males and vaginal fluid and menstrual blood from 14 female volunteers were collected using procedures approved by the Institutional Review Board

of Severance Hospital, Yonsei University in Seoul, Korea. The 34 donors provided written informed consent after the goals and procedures of the study were explained. During sample collection, it was determined that 2 of the 20 male volunteers had undergone a vasectomy. Blood was collected in a syringe, and 200 μ L aliquots were stored frozen. Saliva and freshly ejaculated semen were collected in a microcentrifuge tube and plastic cups, respectively, and 200 μ L aliquots of each were stored frozen. Vaginal fluid and menstrual blood were collected using sterile cotton swabs and allowed to dry at room temperature. Dried swabs were stored frozen until use.

DNA was extracted from each aliquot of blood, saliva and semen using a QIAamp[®] DNA Mini Kit (QIAGEN, Hilden, Germany) and from each swab of vaginal fluid and menstrual blood using a QIAamp[®] DNA Investigator Kit (QIAGEN) following the manufacturer's instructions. Extracted DNA was quantified using a Quantifiler[®] Duo DNA Quantification Kit (Applied Biosystems, Foster City, CA, USA).

Selection of markers and primer design for the development of multiplex PCR

For DNA methylation-based body fluid identification, two tDMRs for the USP49 and DACT1 genes were selected as a semen-specific unmethylation markers and the PFN3 tDMR was selected as a vaginal fluid and menstrual blood-specific hypomethylation marker [7, 8]. A previously reported semen-specific methylation marker, L81528 [6], was also added for more accurate identification of semen. To determine the methylation status of these four tDMRs, primers flanking the *Hha* I recognition sites (GCGC) of the four tDMRs were designed using the Primer 3 program (<http://frodo.wi.mit.edu/primer3/>). The amplicon sizes were smaller than 150 bp and one forward or reverse primer was labelled with the fluorescent dye FAM (Table 1).

For body fluid identification based on the detection of bacterial DNA, *Streptococcus salivarius* [12–14] and *Veillonella atypica* [24] were selected as saliva-specific bacteria, and *L. crispatus* and *L. gasseri* [15–17] were selected as vaginal fluid-specific bacteria. Primers for the amplification of body fluid-specific bacterial DNA were designed to target species-specific regions of the 16S rRNA gene using the Primer 3 program and the probe match program of the Ribosomal Database Project (<http://rdp.cme.msu.edu/>). The amplicon sizes were 98–127 bp, and one forward or reverse primer was labelled with the fluorescent dye TAMRA.

Primers for the amplification of amelogenin, D3S1358, an amplification control, and a digestion control were also designed using the Primer 3 program and were labelled with FAM or HEX fluorescent dye. The artificial DNA templates of the amplification and digestion controls for PCR success and restriction enzyme digestion, respectively, were obtained by PCR amplification of the 481-bp portion of the pCR[®]2.1-TOPO[®] vector (Invitrogen, Carlsbad, CA, USA) [8].

Table 1 Multiplex PCR primers for simultaneous amplification of body fluid-specific DMRs and bacterial 16S ribosomal RNA genes

Marker	Forward primer sequences (5'>3')	Reverse primer sequences (5'>3')	Conc. (μM)	Amplicon size (bp)
USP49	GTAGCAGGTGTTGCCAGGTT	FAM-CCCTCCCTACCTCACGCAGA	1.0	107
DACT1	FAM-CACTCCTCCCCTGCTGTCTA	GATAAACTGGGCCTTGACCA	0.7	118
L81528	FAM-CTTCTGGGGCGACTACCTG	AGTCAGCCTCATCCACACTGA	0.4	128
PFN3	CCTGGCAGCCTCTAGACTCA	FAM-GGGCCAAATAAACTGTGACC	0.15	137
Amelogenin	CCCCTTTGAAGTGGTACCAGAG	FAM-GCATGCCTAATATTTTCAGGGAATAA	0.25	81, 84
D3S1358	GAGCAAGACCTGTCTCATAGA	HEX-TCAACAGAGGCTTGCATGTAT	0.27	83–111
Amplification control	CTGTTCTTCTAGTGTAGCCGTAGTT	HEX-CAACCCGGTAAGACACGACT	0.15	131
Digestion control	GGTGAAGATCCTTTTTGATAATCT	HEX-TTTGTTTGCAAGCAGCAGAT	1.0	144
<i>V. atypica</i>	TTAATAGACGGAAGCGAAACC	TAMRA-CCGCAGTATGCTGACCTGC	0.12	127
<i>S. salivarius</i>	TAMRA-TACCGCATAACAATGGATGAC	TTACCTCACTACTAGCTAATACAACG	0.2	98
<i>L. crispatus</i>	TAMRA-TGCCCATAGTCTGGGATAC	CATCCCATAGCGACAGCTTA	0.2	107
<i>L. gasseri</i>	TAMRA-GACGGTAATTACTTAGAAAGTCACGG	CTTATTGAACCGCCTGCACT	0.4	117

Restriction enzyme treatment and multiplex PCR

Before conducting multiplex PCR, 1 ng of genomic DNA was digested with *Hha* I in a 10-μL reaction containing 10 U of *Hha* I (New England Biolabs, Ipswich, MA, USA), 1 μL of Gold ST*R 10× Buffer (Promega, Madison, WI, USA) and 1 μL of artificial DNA template (equivalent to 1 ng of genomic DNA) at 37 °C for 30 min, and then the enzyme was heat-inactivated by incubation at 65 °C for 20 min. Multiplex PCR was carried out in a 20-μL reaction volume containing 10 μL of enzyme-digested DNA, 2.0 U of AmpliTaq Gold® DNA Polymerase (Applied Biosystems), 1 μL of Gold ST*R 10× Buffer, 5 % dimethyl sulphoxide (Sigma-Aldrich Inc., St. Louis, MO, USA) and 0.12–1.0 μM of each primer (Table 1). PCR cycling was conducted on a PTC-200 DNA engine (MJ Research, Waltham, MA, USA) under the following conditions: 95 °C for 11 min; 28 cycles of 94 °C for 20 s, 59 °C for 60 s, and 72 °C for 30 s; and a final extension at 60 °C for 60 min. One microlitre of amplification product in a mix containing 20 μL HiDi formamide (Applied Biosystems) and 0.2 μL LIZ-500 size standard (Applied Biosystems) was denatured by incubation at 95 °C for 5 min immediately followed by 3 min on ice, and run on an ABI 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. All resulting electropherograms were analysed using GeneMapper® ID software v.3.2 (Applied Biosystems). The threshold for a positive signal was set to 100 relative fluorescent units.

Sensitivity test and analyses of aged samples, mixtures and artificial casework samples

Sensitivity testing was carried out using a series of diluted DNA (1 ng, 500 pg, 250 pg, 125 pg, 62 pg, 31 pg and 15 pg) derived from saliva, semen and vaginal fluid samples from

two volunteers per body fluid. One microlitre of each concentration was tested in triplicate to evaluate the minimum quantity required to obtain the predictable body fluid profile.

DNA obtained from body fluid samples that had been exposed to ambient temperature for 75 days in the previous report [8] were used as an aged sample test of our detection method. These samples were analysed under the same conditions except for the use of 29 amplification cycles.

In addition, vaginal swabs spotted with 5 or 10 μL of semen and with 5 or 10 μL of saliva, and spots of saliva and semen mixed at ratios of 1:1 and 1:2 (i.e. 10 μL saliva and 10 μL semen, and 10 μL saliva and 20 μL semen) on sterile cotton tissues were left to dry overnight for a mixture test.

For a casework validation, some typical artificial casework samples were collected from anonymous contributors. Three post-coital vaginal samples and a post-coital penile sample were obtained with a sterile cotton swab or clean tissue paper, and a skin swab sample was obtained from a kissing site.

DNA from all aged samples, mixtures and artificial casework samples were extracted using a QIAamp® DNA Investigator Kit (QIAGEN) following the manufacturer's instructions. In some cases which have mixed semen and vaginal secretions, differential DNA extraction protocol provided in the QIAamp® DNA Investigator Kit (QIAGEN) was also used.

Results and discussion

Multiplex PCR system development

To facilitate body fluid identification using DNA, a multiplex PCR system was developed by integrating the method that detects DNA methylation status at several tDMRs and

the method that checks for the presence of body fluid-specific bacteria.

The developed multiplex PCR system comprised 12 amplicons containing four tDMRs and four bacterial DNA targets, and each amplicon was labelled with FAM, TAMRA or HEX. The FAM fluorescence signal represented amelogenin typing results and DNA methylation status at the four tDMRs (USP49, DACT1, L81528 and PFN3) to identify semen and to differentiate vaginal fluid and menstrual blood from blood and saliva. The TAMRA fluorescence signal represented the presence of *L. crispatus* or *L. gasseri* in vaginal fluid and the presence of *Streptococcus salivarius* or *V. atypica* in saliva, and the HEX fluorescence signal represented the D3S1358 typing results and amplification of controls providing confirmation of PCR success and complete restriction enzyme digestion (Fig. 1).

This multiplex PCR system, which is based on MSRE-PCR, has some improvements in comparison with previously reported methods because it allows simultaneous detection of bacterial DNA with human genomic DNA. In order to integrate the bacterial DNA detection method with DNA methylation analysis using MSRE-PCR, differences in the methylation sites of human genomic DNA and bacterial DNA were considered. Human DNA methylation generally occurs at the C⁵ position of cytosines that are followed by a guanine while bacterial DNA methylation occurs at the N⁶ position of adenines in the sequence 5'-GATC-3' and at the C⁵ position of the cytosines in the sequences CCAGG and CCTGG [25, 26]. Therefore, the MSRE *Hha* I recognition site, GCGC, is always unmethylated in bacterial DNA. In the developed multiplex system, however, bacterial DNA segments not containing the *Hha* I restriction site were targeted and thereby the amplification of bacterial DNA was not influenced by MSRE treatment.

In comparison with the MSRE-PCR reported by An et al. [8], this multiplex PCR system facilitates definite identification of semen including sperm cells. Even with semen samples showing a low methylation signal at the DACT1 and USP49 tDMRs, semen identification could be carried out by amplification of the semen-specific methylation marker, L81528. In addition, this newly developed multiplex PCR system is different from previously reported bacterial DNA-based methods [12–17] because of the use of *V. atypica* instead of *Streptococcus mutans* as saliva-specific bacteria. In fact, *Streptococcus mutans* DNA is generally obtained using a Gram-positive bacterial DNA extraction method involving dithiothreitol, and the detection of *Streptococcus mutans* DNA is almost impossible using DNA extraction methods that are commonly used in forensic laboratories (data not shown). Therefore, *V. atypica* was substituted for *Streptococcus mutans* for the identification of saliva because its DNA can be easily extracted with commonly used genomic DNA extraction methods [24].

Body fluid identification with the developed multiplex PCR

The developed multiplex PCR was successfully used to differentiate between blood, saliva, semen and vaginal fluid–menstrual blood obtained from 20 males and 14 females. Through DNA methylation analysis, this multiplex PCR system could classify samples as blood–saliva, semen or vaginal fluid–menstrual blood. Bacterial DNA analysis was used to further distinguish saliva and vaginal fluid–menstrual blood (Fig. 2). DNA obtained from blood and saliva produced amplicons for three tDMRs, USP49, DACT1 and PFN3, and then saliva was further differentiated by amplification of saliva-specific bacterial DNA from *Streptococcus salivarius* and/or *V. atypica*. DNA extracted from vaginal fluid and

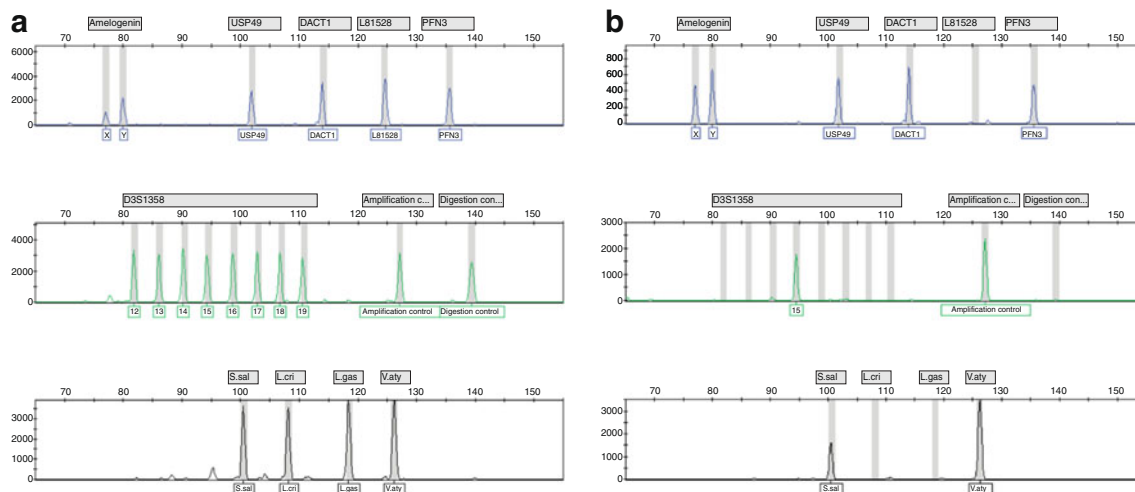


Fig. 1 Scheme of the multiplex PCR system for body fluid identification. **a** The ladder of the multiplex PCR system and **b** multiplex PCR results of MSRE-treated saliva DNA

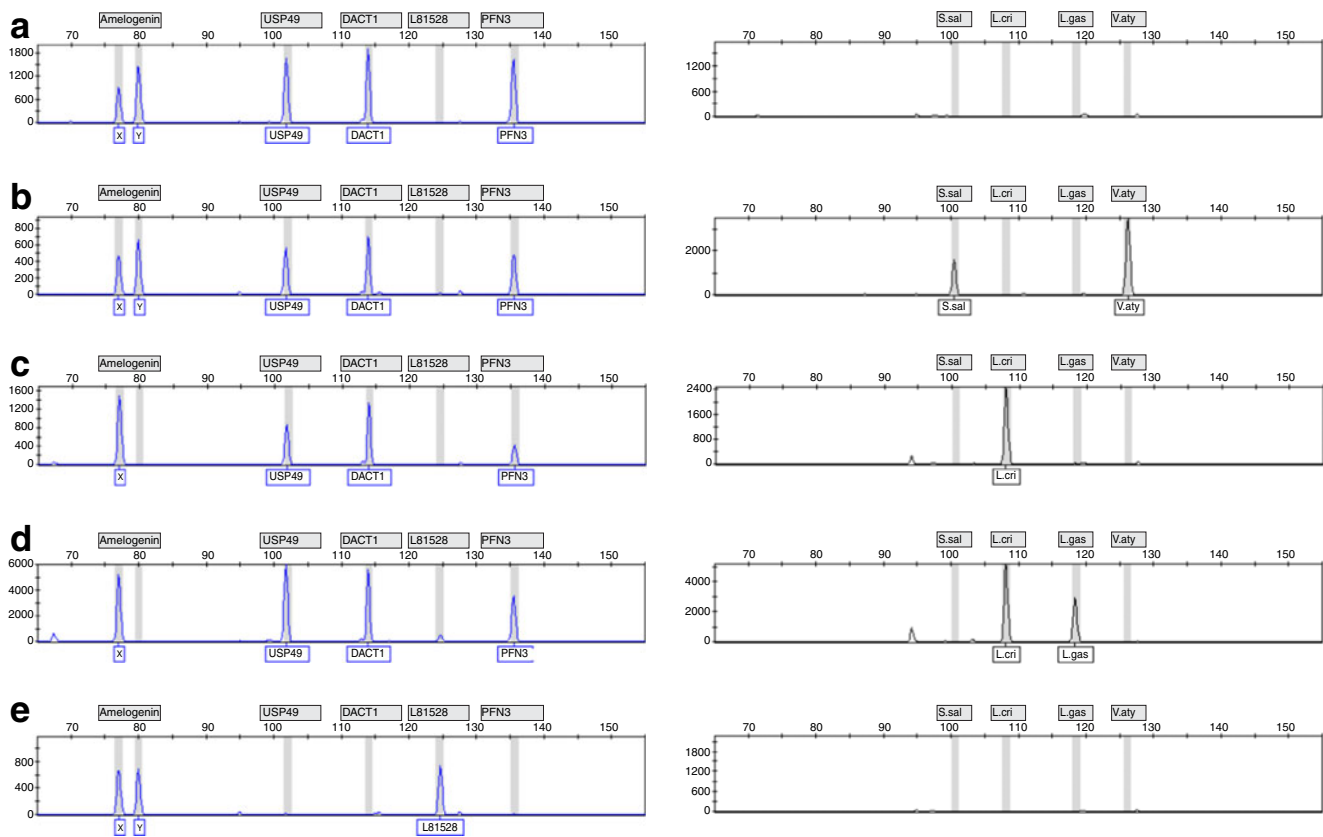


Fig. 2 Typical electropherograms of the multiplex PCR for various body fluids. Samples from **a** blood; **b** saliva; **c** vaginal fluids; **d** menstrual blood and **e** semen using the developed multiplex PCR system. DNA methylation profiles are shown on the *left* and bacterial DNA profiles are shown on the *right*

menstrual blood also produced amplicons for the USP49, DACT1 and PFN3 tDMRs, but with much lower yield at the PFN3 tDMR. Moreover, bacterial DNA amplification of *L. crispatus* and/or *L. gasseri* confirmed the presence of vaginal fluid or menstrual blood. None of the semen samples showed an amplicon for bacterial DNA, but unlike the other body fluids, the representative results of the DNA methylation profile of semen appeared in three patterns (Fig. S1). Ten of the eighteen samples from non-vasectomized males produced an amplicon only at the L81528 tDMR. The other eight semen samples gave very low yields but detectable amplicons at the three semen-specific unmethylation markers, USP49, DACT1 and PFN3 tDMRs in addition to the L81528 amplicon. Nonetheless, semen identification was not problematic because of the high amplification yield of L81528 in all 18 semen samples with spermatozoa. The simultaneous analysis of methylation markers and unmethylation marker facilitated accurate semen identification even with the samples that were difficult to analyse using the MSRE-PCR method developed previously by An et al. [8]. On the other hand, the semen samples from two vasectomized males showed DNA methylation profiles similar to those of vaginal fluids or menstrual blood with no amplicon for bacterial DNA. Taken together, these findings indicate that this multiplex PCR system was very efficient in

the differentiation of blood, saliva, semen and vaginal fluid–menstrual blood.

Further examination revealed that checking for the presence of bacterial DNA complements DNA methylation analysis for body fluid identification. While most of the saliva samples had a very high yield for the PFN3 tDMR, two of the samples produced low amplicon yields for the PFN3 tDMR (<70 % of the yield without enzyme treatment). However, detecting saliva-specific bacteria enabled these samples to be accurately identified as saliva. Saliva-specific bacteria were detected in all but two saliva samples; *Streptococcus salivarius* was discovered in 18 saliva samples and *V. atypica* was identified in 12 saliva samples (Table 2). Therefore, integration of DNA methylation analysis and bacterial DNA detection facilitated exact identification of saliva. However, the vaginal fluid-specific bacterial species, *L. crispatus*, was detected in the saliva of one donor in addition to saliva-specific bacteria. However, the sample showed a much higher amplicon yield for *Streptococcus salivarius* and *V. atypica* than for *L. crispatus*, and thereby was predicted to be saliva rather than vaginal fluid or menstrual blood. Giampaoli et al. [17] reported that *Lactobacillus* species could be detected in postprandial oral cavity fluid and in yogurt, suggesting that the donor ate prior to donating the sample. Vaginal fluid–

Table 2 Bacterial profiles for each body fluid

Body fluid	No. of total samples	No. of positive samples				No. of negative samples
		<i>L. crispatus</i>	<i>L. gasseri</i>	<i>S. salivarius</i>	<i>V. atypica</i>	
Blood	20	0	0	0	0	–
Saliva	20	1	0	18	12	2
Semen	20	0	0	0	0	–
Vaginal fluid	14	8	9	0	0	1
Menstrual blood	14	8	8	0	0	3

specific bacteria facilitated discrimination of vaginal fluid and menstrual blood from other body fluids by amplifying sequences from one or both of the two vaginal fluid-specific bacteria. *L. crispatus* was detected in eight vaginal fluid and eight menstrual blood samples, and *L. gasseri* was detected in nine vaginal fluid and eight menstrual blood samples. Vaginal fluid and menstrual blood from the same donor did not always contain the same bacteria, but the predominance of one *Lactobacillus* species almost always coincided between samples. Since the population of *Lactobacillus* species can change during the course of the menstrual cycle [27–29], different types of vaginal fluid-specific bacteria may be found in vaginal fluid or menstrual blood from the same donor. There was one vaginal fluid and three menstrual blood samples with no detectable bacterial DNA. A bacterial DNA amplicon was also not detected in the menstrual blood sample from the donor who had no detectable bacterial DNA in her vaginal fluid. We speculate that the reason for this may be either the presence of other predominant species of bacteria such as *L. jensenii* and *L. iners* or the intake of antibiotics could cause this result. Our results showing more menstrual blood with no detection of target bacteria compared with vaginal fluid was similar to the result of a previous report [30].

Overall, the results indicate that this multiplex PCR system enables discrimination of more types of body fluid in one multiplex PCR reaction than hitherto reported body fluid identification methods using DNA. Still, there is yet much to be solved for the routine use of the developed methods in common forensic laboratories. In particular, with regard to blood and semen identification, addition or subtraction of markers might improve the analysis. Because blood and semen do not have any detectable bacteria, their identification quite relies on the DNA methylation pattern at tDMRs. However, in the developed multiplex PCR system, blood has no specific tDMR, and semen is identified by the presence of a single tDMR signal with very low or no methylation at the rest of three tDMRs. This may be mitigated by adding a second semen-specific marker because relying on a single positive signal or on the negative signals only can lead to false

negatives or false positives. Moreover, bacterial DNA analysis also showed false negatives and a false positive in some samples. Therefore, identification and selection of more appropriate markers will be necessary for the future body fluid identification using DNA.

Sensitivity test

Sensitivity was tested to verify the detection limit of the developed multiplex PCR method. Among the five types of body fluid, saliva, semen and vaginal fluid were subjected to the test because of their different DNA methylation and body fluid-specific bacteria profiles (Fig. S2). Saliva and semen were successfully identified with 500 pg of DNA or more, and vaginal fluids could be identified with 250 pg of DNA or more without any drop-in or drop-out. Allele drop-in or drop-out was observed at some tDMRs with 125 pg of DNA, but appropriate body fluid-specific bacteria were detected in most saliva and vaginal fluid samples. Although the type and amount of bacteria in body fluids would vary from person to person, bacterial DNA analysis seems to be more sensitive than DNA methylation analysis. Therefore, the developed multiplex PCR system, which detects DNA methylation and bacterial presence simultaneously, not only allows accurate body fluid identification but also surpasses the previous MSRE-PCR in terms of sensitivity.

Aged sample test

To test the efficacy of the developed multiplex system with aged samples, DNA extracted from samples exposed to the environment for 75 days were analysed (Fig. S3). Similar to a previous publication [8], DNA methylation analysis was possible for all aged samples except saliva. However, the presence of saliva was indicated by detection of *Streptococcus salivarius* DNA. The same result had also been observed for low amounts of templates in sensitivity test (Fig. S2b), which suggested the possibility that bacterial DNA analysis can be more sensitive than DNA methylation analysis. Therefore, inferring the type of body fluids first based on the bacterial DNA profile should facilitate more rapid identification of body fluids, particularly with saliva. In this case, DNA methylation profile will, nevertheless, be very useful to confirm the identification results and to check for the presence of other body fluid types. On the other hand, an aged menstrual blood sample did not show *Lactobacillus* amplicons, consistent with the very low signal for bacterial DNA in the same fresh sample. Thus, this multiplex PCR system could be used to successfully analyse most aged body fluid samples with improved accuracy, suggesting the potential for its application to forensic casework.

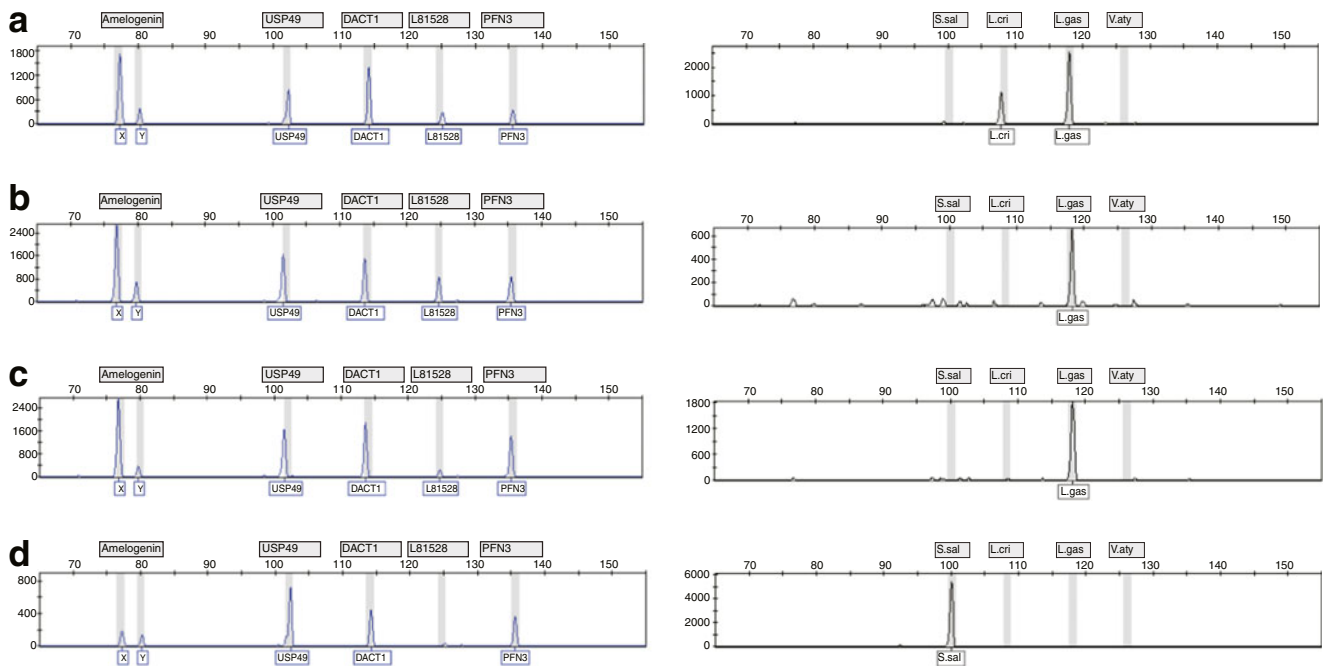


Fig. 3 Artificial casework sample test using the multiplex PCR system. Sources were **a** a post-coital vaginal swab; **b** a post-coital vaginal sample; **c** a post-coital penile sample obtained with clean tissue papers and **d** a skin swab from a kissing site

Mixture sample test

Body fluids are often found at a crime scene as mixtures. Therefore, mixed samples including saliva–semen, saliva–vaginal fluid and semen–vaginal fluid were tested using the newly developed multiplex PCR method (Fig. S4). Saliva–semen mixtures in 1:1 and 1:2 ratios could be clearly identified by amplifying L81528 and *Streptococcus salivarius* DNA. Saliva mixed with vaginal fluid swabs produced both of the *Lactobacillus* species and *Streptococcus salivarius* amplicons, but the yield of the *Streptococcus salivarius* amplicon was very low. Similarly, semen mixed with vaginal fluid swabs showed a profile consistent with vaginal fluid when DNA was extracted using general DNA extraction methods (data not shown). However, differential DNA extraction, which can be used in sexual assault cases, enabled identification of semen and vaginal fluid from the resultant pellet and supernatant, respectively. Therefore, the multiplex PCR method developed in this study is useful in mixture analysis as well as analysis of single source samples.

Artificial casework sample test

Since the vaginal swab spotted with semen showed a profile consistent with vaginal fluids in a mixture sample test using general DNA extraction methods, three post-coital vaginal samples and a penile sample were collected for an artificial sexual assault casework sample test. When DNA was extracted using general DNA extraction methods, two of three post-coital

vaginal samples and a penile sample showed a mixed sample profile by producing semen-specific L81528 amplicon in addition to the vaginal fluid-specific bacterial signal and a low peak for PFN3 tDMR (Fig. 3). Amelogenin and D3S1358 also displayed a tendency of mixed sample with low AMELY amplicon signal and three or more peaks at the STR locus (Fig. S5). However, another post-coital vaginal sample obtained with tissue paper showed a profile that is consistent with vaginal fluids. In this case, differential DNA extraction enabled identification of semen and vaginal fluid from the resultant pellet and supernatant, respectively. In artificial casework samples, the ratio of male to female DNA varied from case to case. Only when male DNA was less than 10 % of total DNA in DNA quantification with a Quantifiler® Duo DNA Quantification Kit (Applied Biosystems), the developed multiplex PCR system showed a profile consistent with vaginal fluid.

A skin swab from kissing site successfully confirmed the presence of saliva by producing saliva-specific bacterial DNA amplicon and methylation profile. The skin swab obtained from clean skin surface did not produce any DNA profile because extracted DNA was scarce. Although additional validation will be needed, the newly developed multiplex PCR system is considered to facilitate actual casework analysis.

Conclusion

Analyses of tDMRs and detection of body fluid-specific bacteria have been proposed as a promising new method for body

fluid identification using DNA. In this study, we presented a highly reliable and sensitive multiplex PCR system that allows simultaneous analysis of tDMRs and bacterial DNA by integrating the two methods. The multiplex PCR method developed in this study can be used to discriminate between four types of body fluid: blood, saliva, semen and vaginal fluid–menstrual blood. Five hundred picogram of DNA from saliva and semen and 250 pg of DNA from vaginal fluid were enough to identify the type of body fluid, while body fluid-specific bacteria were detected in much lower concentrations of DNA (125 pg). In addition, the multiplex PCR allowed successful body fluid identification with artificial casework samples as well as aged or mixed samples.

The multiplex PCR developed in the present study was designed to use a standard capillary electrophoresis platform; thus, special training is not required for these protocols. Moreover, the multiplex system uses the same biological source of DNA for personal identification profiling and simultaneously analyses various body fluids in one PCR reaction. Although it remains to be investigated whether this approach is more sensitive and more practical than RNA- or peptide-based assays, the newly developed multiplex method ensures forensic applicability as well as high specificity, reliability and sensitivity, thereby facilitating more efficient body fluid identification in forensic casework. A few false negatives and a false positive were observed, but this problem could be solved by identification and addition of more appropriate markers.

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Conflicts of interest The authors declare that they have no conflict of interest.

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