Forensic Body Fluid Identification by Simultaneous Detection of DNA Methylation Changes and the Presence of Bacterial DNA

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Introduction

The identification of body fluids found at crime scenes can contribute to solving crimes by providing information that reveals important insights into crime scene reconstruction. DNA methylation profiling has been in the spotlight as a promising new tool for distinguishing between different types of body fluids because of the high specificity, compatibility with existing STR typing protocols, and fit with current forensic casework application. Another method to identify body fluids is the detection of bacterial DNA that are present in a specific body fluid by amplifying bacterial 16S ribosomal RNA (rRNA). Here, we developed a multiplex methylation SNaPshot which uses bisulfite-converted DNA and a multiplex PCR which incorporates methylation-sensitive restriction enzyme(MSRE)-PCR and amplification of bacterial 16S rRNA. The CpG sites included in the multiplexes had been identified from genome-wide epigenetic analysis of 24 body fluid samples using the Illumina HumanMethylation450 BeadChip array.

Materials and Methods

Samples Body fluid samples (venous blood, saliva, semen, menstrual blood and vaginal fluid) were collected from 50 volunteers (34 males and 16 females). DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and quantified using a Quantifiler® Duo DNA Quantification Kit (Applied Biosystems, Foster City, CA, USA). Bisulfite-converted DNA was obtained by modification of 5-200 ng genomic DNA using the Imprint™ DNA Modification Kit (Sigma-Aldrich Inc., St. Louis, MO, USA) according to the manufacturer's protocol. One nanogram of genomic DNA was digested with *Hha* I at 37 ˚C for 30 min, and then the enzyme was heat inactivated by incubation at 65 ˚C for 20 min.

HumanMethylation450 BeadChip Array We analyzed DNA methylation profile of 485,000 CpG loci in various body fluids DNA from a group of volunteers using HumanMethylation450 BeadChip array (Illumina, San Diego, CA, USA). We assayed 24 samples, including each 6 samples of blood, saliva, semen and each 3 samples of menstrual blood and vaginal fluid. Methylation values are reported as betas (fraction between 0 and 1), which represent the methylation level of each CpG site. Based on delta mean value (difference in average beta for each body fluid), we selected body fluid-specific candidate methylation CpG markers. Clustering analysis of selected candidate CpG markers was performed with BDPC program (http://services.ibc.uni-stuttgart.de/BDPC/).

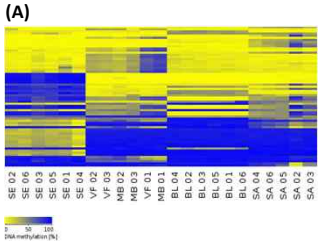
Methylation SNaPshot One to ten nanograms of bisulfite-converted DNA was amplified in a 20 μL final volume containing 3.0 U of AmpliTaq Gold® DNA Polymerase (Applied Biosystems), 2 μL of Gold ST*R 10× Buffer (Promega, Madison, WI, USA), and appropriate concentrations of each primer. Thermal cycling was conducted under the following conditions: 95˚C for 11 min; 34 cycles of 94˚C for 20 sec, 56˚C for 1 min, 72˚C for 30 sec; and a final extension at 72˚C for 7 min. For the following single-base extension (SBE), PCR product was purified and SBE reaction was carried out with a SNaPshot™ Multiplex Kit (Applied Biosystems) according to the manufacturer's instructions. Multiplex methylation SNaPshot system was constructed to analyze final selected 8 CpG markers simultaneously.

Multiplex PCR incorporating MSRE-PCR and bacterial DNA amplification In multiplex MSRE-PCR system, five CpG site were included to identify body fluid by detection methlyation status. For body fluid identification based on the detection of bacterial DNA, *Streptococcus salivarius* and *Veillonella atypica* were selected as saliva-specific bacteria and *Lactobacillus crispatus* and *Lactobacillus gasseri* were selected as vaginal fluid-specific bacteria. 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, 1 μL of Gold ST*R 10×Buffer, 5% dimethyl sulphoxide and appropriate concentrations of each primer. PCR cycling was conducted 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.

Results

Fig. 1. Selection of 66 body fluid-specific CpG marker candidates (A) Heatmap of DNA methylation at 66 CpG sites in 23 body fluid samples from the HumanMethylation450 Bead Chip array. (B) Body fluid-specific CpG sites selected for the construction of a multiplex assay. Mean beta values were obtained from the Human Methylation450 BeadChip analysis of 6 blood, 5 saliva, 3 vaginal fluid, 3 menstrual blood and 6 saliva samples. Body fluid-specific hyper-methylation is indicated in bold.

(A)



(B)

Target ID	Mean beta values				
	Semen	Blood	Vaginal fluid	Menstrual blood	Saliva
cg17610929	**0.94**	0.02	0.02	0.02	0.03
cg26763284	**0.93**	0.02	0.02	0.02	0.02
cg17621389	0.32	**0.96**	**0.96**	**0.96**	**0.96**
cg06379435	0.10	**0.43**	0.05	0.08	0.04
cg01543184	**0.87**	**0.43**	0.07	0.10	0.06
cg09765089	0.08	0.10	**0.44**	**0.47**	0.06
cg26079753	0.06	0.08	**0.47**	**0.47**	0.06
cg09652652	0.02	0.02	0.03	0.02	**0.45**

Fig. 2. Representative electropherograms of body fluid identification using multiplex methylation SNaPshot The peaks represent methylation status at each CpG site.

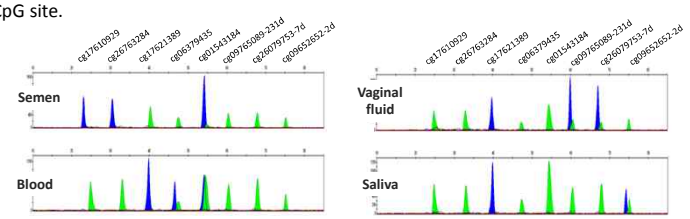


Fig. 3. Construction of multiplex PCR for simultaneous detection of methlyation status at CpG sites and presence of body fluid-specific bacterial DNA The blue peaks at SA, BL, DACT1, VF and SE represent methylation status at cg09652652, cg01543184, DACT1, cg09765089 and cg17610929, respectively. After *Hha I* digestion, the fragments bearing the methlyated CpG site are amplified and detected. The green lane is to confirm complete enzyme digestion and autosomal STR(D3S1358) profile. The black peaks represent the presence of body fluid-specific bacteria in vaginal fluid or saliva.

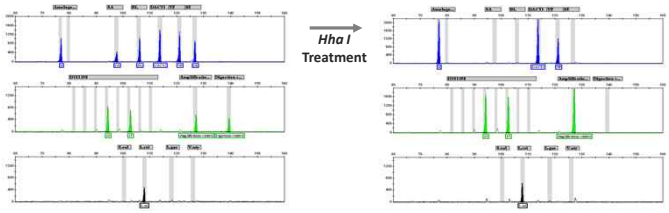
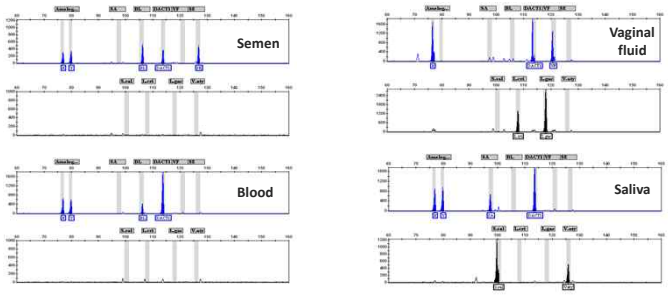


Fig. 4. Representitive electropherograms of multiplex PCR in body fluids



Conclusion

- We analyzed DNA methylation profile of five body fluids including venous blood, saliva, semen, menstrual blood and vaginal fluid using the Illumina Human Methylation450 BeadChip array and selected 66 body fluid-specific candidate CpG markers.
- We selected eight candidate CpG markers specific to certain types of body fluid and constructed multiplex methylation SNaPshot system to analyze methylation patterns of eight CpG markers simultaneously. This multiplex system was able to discriminate four types of body fluids, i.e. venous blood, saliva, semen and vaginal fluid-menstrual blood.
- In addition, we developed mutiplex PCR system that enables simultaneous detection of DNA methylation and presence of saliva- and vaginal fluid-specific bacterial DNA. However, this multiplex PCR provided less accurate results than metylation SNaPshot because of the restriction in selecting target CpG sites.