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Body fluid identification in forensics

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Determination of the type and origin of the body fluids found at a crime scene can give important insights into crime scene reconstruction by supporting a link between sample donors and actual criminal acts. For more than a century, numerous types of body fluid identification methods have been developed, such as chemical tests, immunological tests, protein catalytic activity tests, spectroscopic methods and microscopy. However, these conventional body fluid identification methods are mostly presumptive, and are carried out for only one body fluid at a time. Therefore, the use of a molecular genetics-based approach using RNA profiling or DNA methylation detection has been recently proposed to supplant conventional body fluid identification methods. Several RNA markers and tDMRs (tissue-specific differentially methylated regions) which are specific to forensically relevant body fluids have been identified, and their specificities and sensitivities have been tested using various samples. In this review, we provide an overview of the present knowledge and the most recent developments in forensic body fluid identification and discuss its possible practical application to forensic casework. [BMB Reports 2012; 45(10): 545-553]

INTRODUCTION

Biological samples found at the crime scenes play pivotal roles in forensic investigations by providing valuable evidence (1). Since the DNA profile of every individual is considered to be unique (except in the case of identical twins), DNA typing of biological samples can prove whether a suspect was involved in a crime, and even can exonerate innocent persons who have been wrongfully convicted (2). Besides, determination of the type and origin of biological samples found at crime scenes can provide important clues for crime scene reconstructions by supporting a link between sample donors and actual criminal acts. The presence of certain body fluids can be used as excellent indicators of the sequence of events which occurred. For example, blood stains can indicate some form of physical struggle, assault or murder, and detection of semen or vaginal fluid can indicate the involvement of some form of sexual encounter or assault. The common body fluids found at crime scenes are blood, semen, saliva, vaginal fluid, urine, and sweat.

When a potential body fluid is discovered at a crime scene, a particular form of light or chemical addition may be required to visualize the stain (3). Presumptive tests are initially used to give some indication as to the identity of the substance, and further confirmatory tests are then conducted to confirm the origin of the sample (2). For more than a century, numerous types of analysis methods have been developed for investigation of forensically relevant body fluids. Techniques which are currently used for forensic body fluid identification include chemical tests, immunological tests, protein catalytic activity tests, spectroscopic methods, and microscopy. Some of these methods are presumptive tests which are used as screening tests, such as the luminol and Kastle-Meyer test for blood. Others are confirmatory test that will conclusively identify the presence of certain body fluid, such as the microscopic identification of sperm cells (4, 5). Most catalytic, enzymatic and immunologic tests which have been used for presumptive or confirmatory methods suffer from several limitations, such as low specificity, lack of sensitivity, sample destruction, instability of biomolecule assayed, or incompatibility with downstream individual identification assays although they have certain advantages (2).

Recent advances in forensic genetics have led to the development of several new methods, and the majority of these methods involve the detection of specific messenger RNA (mRNA) and micro RNA (miRNA) expressions, as well as differential DNA methylation patterns. Especially, recent approaches based on tissue-specific mRNA or miRNA expression have been proved to be useful because of their high tissue specificity to forensically relevant body fluids. However, ubiquitously present ribonucleases are detrimental to mRNA stability, and RNA typing requires additional sample material, unless the RNA is co-extracted with DNA. More recently, DNA-methylation based assays, which identify differential DNA methylation profiles of different cell or tissue types, have been proposed as a promising new method for distinguishing

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between different types of body fluids because of their high specificity and fit with current forensic casework application.

This review briefly describes current and previous techniques of body fluid identification that are being used in forensic laboratories, and evaluates the advantages and disadvantages of each method. We will also focus on new methods of identification that have been developed in the past decade, specifically mRNA based assays, miRNA based assays and DNA methylation based assays. The review concludes with a discussion of the potential application of these new methods for forensic case works.

CURRENT TECHNIQUES

Alternate light source methods

The simplest way to detect body fluid stains that are difficult to see with the naked eye is to use an alternate light source (ALS) such as ultraviolet light. Because it is a routine procedure to search a crime scene for latent stains of body fluids using ALS, there are various commercial ALS devices, such as Wood's Lamp (WL), specific for semen detection. WL which emits wavelengths ranging about 320-400 nm is safe and handy, but the specificity is somewhat low, thereby carrying a high risk of false positive results for other fluids (6). Another ALS device, Bluemaxx™ BM 500 demonstrated 100% sensitivity to semen stains (7), and Polilight® can detect several body fluids, including semen (8). A newly developed device, Lumatec® Superlight 400 emits light from 320 to 700 nm and is able to detect stains both in darkness and in daylight (9). Using this device, semen was best detected using a range of 415-490 nm, and saliva was also detectable in 60% of cases. However, poor results were obtained when it was applied to dark fabrics and to fabrics which had been washed, although it had been reported that different types of fabrics showed similar results (9, 10).

Chemical tests

Chemical tests are usually based on the color change or chemiluminescence of a particular reagent when it comes into contact with a particular body fluid. Luminol is frequently used in identifying blood stains, particularly when the perpetrator has attempted to clean up the blood, thus rendering it invisible to the naked eye (11, 12). Due to the emission of light as a result of an oxidation of luminol enhanced by iron in hemoglobin and its derivatives in blood, trace amounts of blood can be detected by chemiluminescence of a blue-green color (13, 14). However luminol has been known to react with other substances, including copper containing chemical compounds, certain bleaches, saliva, and various animal and vegetable proteins. The luminol test remains popular due to the relatively high sensitivity and specificity in comparison with other screening tests. However, it is limited to use in dark environments and also has disadvantages regarding the duration of illumination (about 30 seconds) and sometimes has detrimental effects on subsequent DNA analysis (15-17). Bluestar® Forensic is another latent blood stain reagent based on chemiluminescence, and it gives more sensitive and stable results without damaging DNA and thereby allows for subsequent genotyping of stains (16, 18).

Catalytic tests

Catalytic methods are based on the enzyme activity which catalyzes the reaction of a variety of substrates to produce visible color changes. There are several different catalytic tests which are commonly used to presumptively identify blood based on the peroxidase-like activity of heme group (13, 14). The heme group of hemoglobin possesses a peroxidase-like activity which catalyzes the breakdown of hydrogen peroxide, and the oxidizing species formed in this reaction can then react with a variety of substrates to produce a color change (19). Among the substrates in common use are benzidine and various substituted benzidines, ortho-tolidine, leucomalachite green, leucocrystal violet and phenolphthalein, also known as the Kastle-Meyer test (20, 21).

The most commonly utilized of these tests is the benzidine test, where the presence of blood is indicated by blue colored products. However, there are several substances which can generate false positives for this test, such as chemical oxidants and fruit and vegetable peroxidases (22). Moreover, benzidine is known as a carcinogen (23), and accordingly this has largely been replaced by tests using phenolphthalein/hydrogen peroxide. The test using phenolphthalein is also known as the Kastle-Meyer test, which is a very popular presumptive test for blood. The stain in question is collected with a swab or filter paper, and phenolphthalin reagent and hydrogen peroxide are then applied to the sample. If the sample contains hemoglobin, a result produces a pink coloring only after the addition of hydrogen peroxide, by oxidation of phenolphthalin into phenolphthalein. Although false-positives are reported in the presence of chemical oxidants and vegetable peroxidases, the test can detect blood as dilute as 1 part in 10,000. In addition, this test is nondestructive to the sample, which can then be kept and used in further tests, including DNA analysis (20, 21, 24, 25).

The acid phosphatase (AP) test is one of the most common tests for semen detection. AP is an enzyme secreted by the prostate gland that is present in large amounts in seminal fluid (26). The level of AP activity is 500 to 1,000 times higher in human semen than in any other body fluid. AP can catalyze the hydrolysis of phosphates, which results in the formation of a product that will react with the color developer (27). Therefore, in the presence of Alpha-Naphthyl acid phosphate and Brentamine Fast Blue, AP will produce a dark purple color (27). However, the test for AP is highly presumptive because vaginal secretions and other body fluids also contain detectable levels of this enzyme (13).

A catalytic test for the detection of saliva is based on the enzymatic activity of alpha-amylase. Saliva is rich in alpha-amylase, an enzyme that hydrolyses polysaccharides into smaller

sugar molecules (28). The starch-iodine detects the alpha-amylase activity owing to the fact that iodine reacts with the starch producing a purple black color. In the presence of the amylase, the intensity of color decreases as starch is broken down by any amylase present (2). Another method for the detection of alpha-amylase is the Phadebas® test, which includes amylopectin-procion red instead of starch-iodine (29, 30). This test is relatively cheap, quick, and highly sensitive, but false positive results were also observed in hand cream, face lotion, urine, and feces (29, 30). Additionally, there are two different forms of alpha-amylase in the human body; AMY1 found in saliva, breast milk and perspiration and AMY2 found in the pancreas, semen and vaginal secretions. Although AMY1 is found predominantly in saliva compared with other types of body fluids, the two variants are almost indistinguishable in terms of their enzyme activity. Thus, detection of the activity of alpha-amylase can only give presumptive information because it is not exclusive to saliva (2, 27).

Immunological tests

Many traditional tests used in forensic science are immunological tests that are based on specific antibody-antigen reaction.

The OneStep ABAcard® HemaTrace test strip is an immunochromatographic test for the detection of human blood (2, 14, 25). If human hemoglobin is present in the sample, it will combine with a mobile monoclonal anti-human hemoglobin antibody in the test strip. Any antibody-antigen complex formed then migrates through an absorbent membrane to the test area. When the human hemoglobin concentration is above a certain minimum detection limit (0.05 μg/ml), the pink dye becomes visible in the test region. The results have shown the OneStep ABAcard® HemaTrace to be a highly sensitive, convenient, and rapid test for the identification of human blood both in the laboratory and at crime scenes.

The most commonly used confirmatory test for semen detection beyond microscopic identification of the sperm cells is a test based on the detection of prostate specific antigen (PSA) (31). PSA is produced in high amounts by the male prostate gland, and is even detected in the semen from azoospermic males (27, 32). Commercial test kits that depend on antibody-antigen reaction are currently widely used for PSA detection. One of the most popular commercial kits, the OneStep ABAcard® PSA, also uses the technique of a mobile monoclonal anti-human PSA antibody which binds to human PSA and migrates along the strip, forming a visible line in its path (33, 34). However, studies have shown that PSA can also be found in very low levels in other body fluids, such as female urine and breast milk (35, 36).

EMERGING TECHNIQUES

Messenger RNA profiling methods

During the last decade, RNA analysis has emerged as a promising new tool for forensic investigation applications, such as inferences about post-mortem intervals and wound age (37). Additionally, recent development in forensic genetics revealed that tissue-specific RNA expression can be used for human body fluid identification.

RNA is notorious for its instability because of the ubiquitously present ribonucleases. However, recent studies have reported that RNA isolated from some forensic stains showed unexpectedly high stability. Using whole-genome gene expression on aged blood and saliva stains, Zubakov *et al.* (38) identified blood and saliva specific messenger RNA (mRNA) markers that showed stable expression patterns in stains after up to 180 days of storage, and some of these markers showed successful and reliable amplification in much older stains, such as 16 year-old blood stains (39). Setzer *et al.* (40) conducted a more comprehensive study on mRNA stability in forensic samples. They exposed biological stains to a range of environmental conditions and performed mRNA profiling analysis using eight different mRNA transcripts of selected housekeeping and tissue-specific genes. The results demonstrated that RNA is detectable in some samples stored at room temperature, even after 547 days, but heat and humidity appear to be detrimental to RNA stability.

One of major advantages of body fluid identification by mRNA profiling is the possibility of simultaneous extraction of mRNA and DNA from the same stain, posing a major advantage in forensic investigations, where sample material is often limited. Actually, several optimized methods have been developed for simultaneously isolating mRNA and DNA from same physical stains (41, 42), and successful identification of the stain could be performed from the same stain where the donor's identity was confirmed (43, 44). Another important advantage of mRNA profiling is the possibility of detecting several body fluids in one multiplex reaction, providing data on the expression of multiple genes simultaneously.

Various multiplexes have been reported, using reverse transcription endpoint polymerase chain reaction (RT-PCR) methods and real-time quantitative reverse transcriptase-PCR (qRT-PCR) assay, including body fluid-specific markers (43-48). Since many transcripts are not completely tissue specific but usually show differences in expression level, qRT-PCR might be more appropriate for the detection of relative gene expression levels in different samples, and endpoint PCR might be suitable for the detection of certain transcripts with highly tissue-specific expression (48, 49).

In 2005, Juusola and Ballantyne (45) proposed a multiplex RT-PCR method for the identification of body fluids that are commonly encountered in forensic casework. Using eight selected body fluid-specific genes, i.e., β-spectrin (SPTB) and porphobilinogen deaminase (PBGD) for blood, statherin (STATH) and histatin 3 (HTN3) for saliva, protamine 1 (PRM1) and protamine 2 (PRM2) for semen, and human beta-defensin 1 (HBD-1) and mucin 4 (MUC4) for vaginal secretions, they were able to detect each of the four body fluids as single or

mixed stains. The sensitivity of the system was suitable for forensic casework because successful identification of body fluids was possible with 200 pg-12 ng of input RNA. In the updated experiment in 2007, Juusola and Ballantyne (46) reported multiplex qRT-PCR assays for the identification of blood, saliva, semen, and menstrual blood. In this work, previously identified body fluid-specific genes were used for saliva and semen, but blood specific marker PBGD was replaced with δ-aminolevulinate synthase 2 (ALAS2). In addition, matrix metalloproteinase 7 (MMP7) and matrix metalloproteinase 10 (MMP10) were added for the detection of menstrual blood, and housekeeping gene GAPDH was introduced for the normalization of the expression of body fluid-specific genes. Nussbaumer *et al*. (47) also reported multiplex qRT-PCR assays, which include hemoglobin A (HBA) for blood, kallikrein (KLK) for semen and mucin 4 (MUC4) for vaginal fluid or saliva. In the results, all semen samples showed a high expression of KLK mRNA, while none of other body fluids showed any expression of KLK. On the other hand, HBA mRNA was highly expressed in all blood samples screened, but some of the saliva samples also showed a weak reaction, while still being discriminative in expression level. Haas *et al*. (48) reported a multiplex RT-PCR and a multiplex qRT-PCR for the identification of blood, saliva, semen, menstrual blood and vaginal fluid using previously reported mRNA markers; SPTB, PBGD, STATH, HTN3, PRM1, PRM2, HBD1, MUC4, MMP7 and MMP11 (Fig. 1). The two developed multiplex RT-PCRs showed high specificity, sensitivity and suitability for forensic body fluid analysis, and mRNA stability was demonstrated for up to 2-year-old stains. Recently, a collaborative exercise on mRNA profiling for the identification of blood was organized by the European DNA Profiling Group (EDNAP) (43), and the results demonstrated that all but one of the 16 participating laboratories were able to successfully isolate and detect blood specific-mRNA from the dried blood stains.

Incidentally, several mRNA markers proposed for the identification of vaginal secretions show controversial results leading to false positives in saliva samples (45, 47, 50). Hence, Fleming and Harbison (51) suggested improving the mRNA multiplex system for body fluid identification by applying microbial markers for vaginal fluids. They have incorporated 16S-23S rRNA intergenic spacer region (ISR) of *Lactobacillus crispatus* and *Lactobacillus gasseri*, reportedly predominant in the vagina of women, into previously described mRNA multiplex. From their results, the 16S-23S ISR of *L. crispatus* and *L. gasseri* were detected in vaginal secretions and in some menstrual blood samples but not in any other fluids, including blood, semen, and saliva. Although further works are needed for samples of various age ranges, this study provided the potential use of vaginal bacteria as a tool for the identification of vaginal fluids.

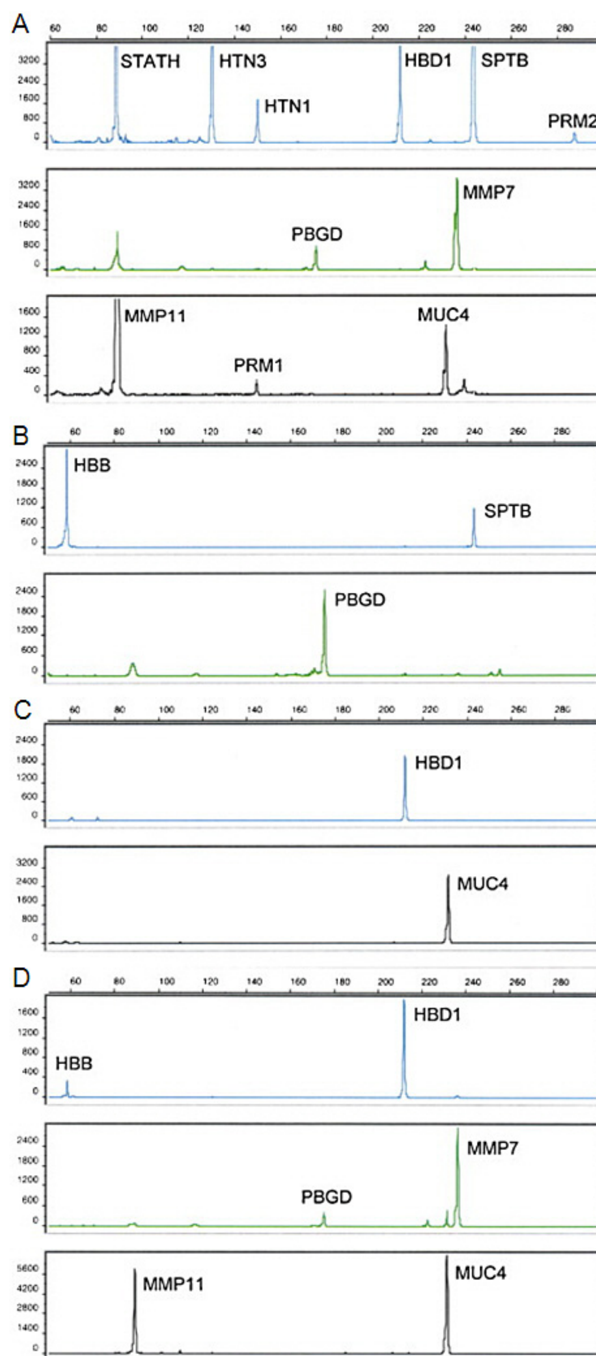

Fig. 1. (A) Endpoint PCR multiplex 1 result of a blood-saliva-semen-vaginal secretion-menstrual blood mixture, revealing two peaks for each body fluid. Electropherograms of blood (B), vaginal secretion (C) and menstrual blood (D) samples detected with endpoint PCR multiplex 2. Reprinted from Haas *et al*. (48) with kind permission from Elsevier.

MicroRNA profiling methods

Although a number of mRNA markers have been proposed as

sensitive and specific methods for forensic body fluid determination, and have also proven their stability successfully in samples over long periods of time, additional environmental conditions such as humidity and temperature are expected to influence mRNA stability (39, 40). Therefore, recently, microRNA (miRNA) markers have started to be explored as an alternative tool for forensic body fluid identification (52-54). MiRNAs are non-coding RNA molecules of 18 to 22 nucleotides in length, and regulate gene expression at the post-transcriptional level (55-57). Several recent studies have demonstrated that many miRNAs showed tissue-specific expression patterns (58, 59). In addition, the intrinsically small size of miRNAs makes them less prone to degradation by environmental factors, thus offering an obvious advantage as a useful biomarker for body fluid identification (52). In 2009, Hanson *et al*. (52) provided the first introduction of miRNA profiling to forensic science, and evaluated the miRNA expression in forensically relevant biological fluids. In the study, they demonstrated that miRNA can be extracted from forensic samples and examined the expression of 452 human miRNAs in blood, saliva, semen, vaginal secretions and menstrual blood using qRT-PCR analysis (miScript SYBR Green PCR kit, Qiagen). They have identified nine miRNAs-miR451, miR16, miR135b, miR10b, miR658, miR205, miR124a, miR372, and miR412- that are differentially expressed in forensic biological samples, and have proven their abilities to identify body fluid using as little as 50 pg of total RNA. Zubakov *et al*. (53) also profiled the expression levels of 718 miRNAs in forensically relevant body fluids using genome-wide microarrays and identified 14 differentially expressed candidates for potential body-fluid identification. In the subsequent validation test using qRT-PCR, only blood and semen specific miRNA candidates showed comparable expression levels in microarray and qRT-PCR data, whereas less concordance was found for saliva, menstrual blood and vaginal secretions. Although these two initial studies showed the potential of miRNA profiling for forensic investigation, their results were inconsistent when different technology platforms and statistical methods were applied. Therefore, it has been suggested that rigorous methodological validation and accurate models for data analysis are needed for accurate miRNA quantification in forensic applications (54).

DNA methylation profiling methods

Since recent whole-epigenome analyses indicated that DNA carries tissue-specific methylation patterns, the potential of tissue-specific differential DNA methylation for body fluid identification has been examined in the forensic field (60-65). DNA methylation, which occurs at the 5'-position of the pyrimidine ring of cytosine in CpG dinucleotides, is generally believed to inhibit gene expression by affecting chromatin structure (66, 67). Different cell types have different methylation patterns (68), and chromosome segments called tissue-specific differentially methylated regions (tDMRs) are known to show different DNA methylation profiles according to cell or tissue type (69, 70). Thus, detection of DNA methylation status at a certain CpG site of tDMR would allow for identification of the tissue or cell type of DNA samples.

In 2011, Frumkin *et al*. (60) reported 15 genomic loci which are differentially methylated among blood (venous/menstrual), saliva, semen, skin epidermis, urine and vaginal secretion. In this study, they implemented an assay for selected markers using methylation-sensitive restriction enzyme-PCR (MSRE-PCR) made up of methylation-sensitive restriction enzyme digestion of sample DNA followed by multiplex PCR of specific genomic loci with fluorescence-labeled primers, capillary electrophoresis of amplification products and automatic signal detection. The assay can be easily integrated into standardized procedures of forensic laboratories like DNA typing using short tandem repeat (STR), and could successfully identify source tissues in 50 DNA samples from blood, saliva, semen, and skin epidermis. Later, Wasserstrom *et al*. (63) advanced the previously reported approach by developing a kit, DNA source identifier (DSI)-Semen^{TM}, which aims to replace microscopic examination of sperm cells for forensic semen identification in casework samples. This assay is based on the detection of semen-specific DNA methylation patterns in five genomic loci using MSRE-PCR. The kit was validated with 135 samples of various body fluids and 33 actual casework samples from the forensic biological laboratory, and was proven to be robust and reliable by showing a positive result for semen given as little as 31 pg of template DNA input (63, 64).

Lee *et al*. (61) also examined the potential of tDMRs for forensic body fluid identification using a bisulfite sequencing method. Bisulfite sequencing determines the DNA methylation status by detection of nucleotide base change due to sodium bisulfite treatment. Bisulfite treatment has no influence on methylated cytosine, but converts free or unmethylated cytosine of CpG to uracil, which becomes thymine during subsequent PCR. Using this method, they produced methylation profiles for five tDMRs in pooled DNA samples from blood, saliva, semen, menstrual blood, and vaginal fluid. The tDMRs for DACT1 and USP49 were selected as a semen-specific marker by showing semen-specific hypomethylation, and the PFN3 tDMR was suggested to be used for vaginal fluid identification. In a recent paper, An *et al*. (62) further investigated age-related methylation changes in semen-specific tDMRs using body fluids from young and elderly man, since DNA methylation patterns are known to be susceptible to change by aging (71,72). After confirming the stability of the body fluid-specific DNA methylation profile, they proposed two multiplex systems to analyze the methylation status of the USP49, DACT1, PRMT2 and PFN3 tDMRs. The two multiplex systems were constructed using MSRE-PCR and methylation SNaPshot, and both could successfully identify semen with sperm cells and could distinguish menstrual blood and vaginal fluids from other body fluids in a test with 144 DNA samples (Fig. 2). Unlike MSRE-PCR, which shows only the amount of methylated CpGs, methylation SNaPshot has a merit that can measure the pro-

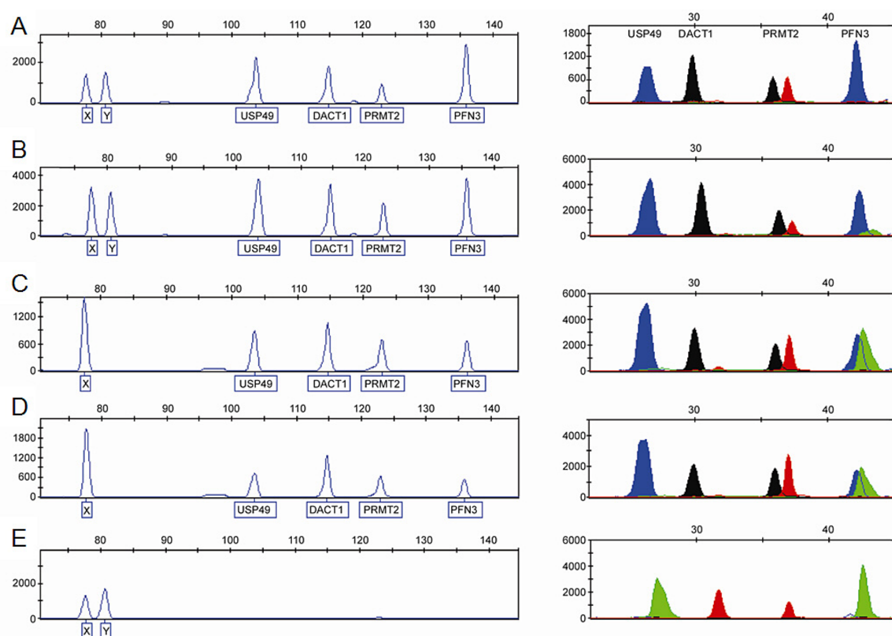

Fig. 2. Typical DNA methylation profiles for samples from blood (A), saliva (B), menstrual blood (C), vaginal fluids (D) and semen (E) using MSRE-PCR (left) and methylation SNaPshot (right). Reprinted from An *et al.* (62) with kind permission from Springer Science + Business media.

portion of the methylated and/or unmethylated cytosine of the target CpG site simultaneously, because this assay is carried out by the amplification of bisulfite-converted DNA and subsequent single base extension reaction. On the other hand, since genomic DNA can be degraded during bisulfite treatment, a bisulfite-based methylation SNaPshot assay may consume more samples than MSRE-PCR. Therefore, a sensitivity test was performed for the multiplex methylation SNaPshot, and the result showed that a minimum of 500 pg of starting genomic DNA, or 125 pg of bisulfite-converted DNA, was sufficient for successful DNA methylation profiling of the selected tDMRs, which demonstrates the possible practical application of the multiplex system to forensic casework.

A recent paper by Madi *et al*. (65) also reported tissue-specific DNA methylation in forensically relevant biological samples including blood, saliva, semen and epithelial cells. They examined a few genomic loci using bisulfite modification and pyrosequencing to find that the methylation patterns at the ZC3H12D and FGF7 loci can differentiate sperm from other biological samples while the C20orf117 locus and the BCAS4 locus can differentiate blood and saliva from other samples, respectively. These results also indicate that the DNA methylation-based methods could be a valuable analysis tools for the characterization of forensically relevant biological fluids, but further validation studies including more markers will be required for actual casework applications.

CONCLUDING REMARKS

While current forensic DNA profiling based on STRs allows personal identification of biological sample donors, recent advances in forensics has suggested using other types of markers in order to add more informative layers to the evidence. Especially, various kinds of markers have been proposed for forensic body fluid identification. Among emerging techniques we have discussed, mRNA markers have been most rigorously investigated and the number of specific markers is sufficient for the identification of forensically relevant body fluids. Some of the other currently used methods have potential for the rapid and non-destructive identification of body fluids, but in most cases, tissue specificity is problematic due to cross reaction with biological or non-biological material present in samples at crimes scene. On the other hand, mRNA markers show high tissue-specificity and adequate sensitivity for forensic analysis, thereby being considered as a valuable new approach to overcome the limitations of conventional methods. However, mRNA profiling is not yet widely used in forensic laboratories probably owing to the fact that it does not allow for the simultaneous analysis of DNA profiling and body fluid identification. In addition, heat and humidity remain threats to the stability of mRNA markers. Recently, DNA methylation profiling was proposed as a promising new tool for forensic body fluid identification, which uses the same DNA samples which are used for DNA typing. Like mRNA profiling methods, DNA methylation profiling showed high specificity and sensitivity, and also allowed for the simultaneous analysis of multiple markers specific for various tissues in a single multiplex system. Moreover, DNA methylation profiling methods fit well with current forensic applications, and accordingly, can be easily integrated into forensic standardized procedures. However, excepting semen applications, DNA methylation still needs to identify more markers for future practical applica-

tion to casework. We believe that it is now possible to identify more informative markers for body fluid identification using various high-throughput screening technologies. In the near future, forensic investigations should improve a great deal because of the continued advances in genetics, epigenetics and molecular biology, and hence, the extraction of more information from forensically relevant biological samples will be possible.

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REFERENCES

1. Kayser, M. and de Knijff, P. (2011) Improving human forensics through advances in genetics, genomics and molecular biology. *Nat. Rev. Genet.* **12**, 179-192.
2. Virkler, K. and Lednev, I. K. (2009) Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene. *Forensic Sci. Int.* **188**, 1-17.
3. Shaler, R. C. (2002) Modern forensic biology; in Forensic Science Handbook; Saferstein, R. (ed.), pp. 529-546, Prentice Hall, Upper Saddle River, USA.
4. Allery, J. P., Telmon, N., Mieusset, R., Blanc, A. and Rouge, D. (2001) Cytological detection of spermatozoa: comparison of three staining methods. *J. Forensic Sci.* **46**, 349-351.
5. Romero-Montoya, L., Martínez-Rodríguez, H., Pérez, M. A. and Argüello-García, R. (2011) Relationship of spermatoscopy, prostatic acid phosphatase activity and prostate-specific antigen (p30) assays with further DNA typing in forensic samples from rape cases. *Forensic Sci. Int.* **206**, 111-118.
6. Santucci, K. A., Nelson, D. G., McQuillen, K. K., Duffy, S. J. and Linakis, J. G. (1999) Wood's lamp utility in the identification of semen. *Pediatrics* **104**, 1342-1344.
7. Nelson, D. G. and Santucci, K. A. (2002) An alternate light source to detect semen. *Acad. Emerg. Med.* **9**, 1045-1048.
8. Vandenberg, N. and van Oorschot, R. A. (2006) The use of Polilight in the detection of seminal fluid, saliva, and bloodstains and comparison with conventional chemical-based screening tests. *J. Forensic Sci.* **51**, 361-370.
9. Fielder, A., Rehdorf, J., Hilbers, F., Johrdan, L., Stribl, C. and Benecke, M. (2008) Detection of semen (human and boar) and saliva on fabrics by a very high powered UV-/VIS- light source. *Open Forensic Sci. J.* **1**, 12-15.
10. Seidl, S., Hausmann, R. and Betz, P. (2008) Comparison of laser and mercury-arc lamp for the detection of body fluids on different substrates. *Int. J. Legal Med.* **122**, 241-244.
11. Weber, K. (1966) The use of chemiluminescence of Luminol in forensic medicine and toxicology. I. Identification of blood stains. *Dtsch. Z. Gesamte Gerichtl. Med.* **57**, 410-423.
12. Barni, F., Lewis, S. W., Berti, A., Miskelly, G. M. and Lago, G. (2007) Forensic application of the luminol reaction as a preumptive test for latent blood detection. *Talanta* **72**, 896-913.
13. Gaensslen, R. E. (1983) Sourcebook in Forensic Serology, Immunology, and Biochemistry, U.S. Department of Justice, Washington, USA.
14. Spalding, R. P. (2003) Identification and characterization of blood and bloodstains; in Forensic Science: an Introduction to Scientific and Investigative Techniques; James, S. H. and Nordby, J. J. (eds.), pp. 181-201, CRC Press, Boca Raton, USA.
15. Quinones, I., Sheppard, D., Harbison, S. and Elliot, D. (2006) Comparative analysis of luminal formulations. *Can. Soc. Forensic Sci. J.* **40**, 53-63.
16. łuczak, S., Woźniak, M., Papuga, M., Stopińiska, K. and Sliwka, K. (2002) A comparison of the Bluestar and luminol effectiveness in bloodstain detection. *Arch. Med. Sadowej Kryminol.* **56**, 239-245.
17. Castello, A., Alvarez, M. and Verdu, F. (2002) Accuracy, reliability, and safety of luminol in bloodstain investigation. *Can. Soc. Forensic Sci. J.* **35**, 113-121.
18. Blum, L. J., Esperanca, P. and Rocquefelte, S. (2006) A new high-performance reagent and procedure for latent bloodstain detection based on luminol chemiluminescence. *Can. Soc. Forensic Sci. J.* **39**, 81-100.
19. Ahlquist, D. A. and Schwartz, S. (1975) Use of leuco-dyes in the quantitative colorimetric microdetermination of hemoglobin and other heme compounds. *Clin. Chem.* **21**, 362-369.
20. Hunt, A. C., Corby, C., Dodd, B. E. and Camps, F. E. (1960) The identification of human blood stains: a critical survey. *J. Forensic Med.* **7**, 112-130.
21. Cox, M. (1991) A study of the sensitivity and specificity of four presumptive tests for blood. *J. Forensic Sci.* **36**, 1503-1511.
22. Culliford, B. J. and Nickolls, L. C. (1964) The benzidine test: a critical review. *J. Forensic Sci.* **9**, 175-191.
23. Holland, V. R. and Saunders, B. C. (1974) A safer substitute for benzidine in the detection of blood. *Tetrahedron* **20**, 3299-3302.
24. Webb, J. L., Creamer, J. I. and Quickenden, T. I. (2006) A comparison of the presumptive luminol test for blood with four non-chemiluminescent forensic techniques. *Luminescence* **21**, 214-220.
25. Johnston, E., Ames, C. E., Dagnall, K. E., Foster, J. and Daniel, B. E. (2008) Comparison of presumptive blood test kits including Hexagon OBTI. *J. Forensic Sci.* **53**, 687-689.
26. Kaye, S. (1949) Acid phosphatase test for identification of seminal stains. *J. Lab. Clin. Med.* **34**, 728-733.
27. Greenfield, A. and Sloan, M. A. (2003) Identification of biological fluids and stains; in Forensic Science: an Introduction to Scientific and Investigative Techniques; James, S. H. and Nordby J. J. (eds.), pp. 203-220, CRC Press, Boca Raton, USA.
28. Whitehead, P. H. and Kipps, A. E. (1975) The significance

of amylase in forensic investigations of body fluids. *Forensic Sci.* **6**, 137-144.
29. Hedman, J., Gustavsson, K. and Ansell, R. (2008) Using the new Phadebas® Forensic Press test to find crime scene saliva stains suitable for DNA analysis. *Forensic Sci. Int. Genet. Suppl. Series* **1**, 430-432.
30. Myers, J. R. and Adkins, W. K. (2008) Comparison of modern techniques for saliva screening. *J. Forensic Sci.* **53**, 862-867.
31. Healy, D. A., Hayes, C. J., Leonard, P., McKenna, L. and O'Kennedy, R. (2007) Biosensor developments: application to prostate-specific antigen detection. *Trends Biotechnol.* **25**, 125-131.
32. Nadler, R. B., Humphrey, P. A., Smith, D. S., Catalona, W. J. and Ratliff, T. L. (1995) Effect of inflammation and benign prostatic hyperplasia on elevated serum prostate specific antigen levels. *J. Urol.* **154**, 407-413.
33. Hochmeister, M. N., Budowle, B., Rudin, O., Gehrig, C., Borer, U., Thali, M. and Dirnhofer, R. (1999) Evaluation of prostate-specific antigen (PSA) membrane test assays for the forensic identification of seminal fluid. *J. Forensic Sci.* **44**, 1057-1060.
34. Kearsey, J., Louie, H. and Poon, H. (2001) Validation study of the "OneStep ABAcard PSA test" kit for RCMP casework. *Can. Soc. Forensic Sci. J.* **34**, 63-72.
35. Schmidt, S., Franke, M., Lehmann, J., Loch, T., Stöckle, M. and Weichert-Jacobsen, K. (2001) Prostate-Specific antigen in female urine: a prospective study involving 217 women. *Urology* **57**, 717-720.
36. Yokota, M., Mitani, T., Tsujita, H., Kobayashi, T., Higuchi, T., Akane, A. and Nasu, M. (2001) Evaluation of prostate-specific antigen (PSA) membrane test for forensic examination of semen. *Legal Med. (Tokyo)* **3**, 171-176.
37. Bauer, M. (2007) RNA in forensic science. *Forensic Sci. Int. Gen.* **1**, 69-74.
38. Zubakov, D., Hanekamp, E., Kokshoorn, M., van IJcken, W. and Kayser, M. (2008) Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples. *Int. J. Legal Med.* **122**, 135-142.
39. Zubakov, D., Kokshoorn, M., Kloosterman, A. and Kayser, M. (2009) New markers for old stains: stable mRNA markers for blood and saliva identification from up to 16-year-old stains. *Int. J. Legal Med.* **123**, 71-74.
40. Setzer, M., Juusola, J. and Ballantyne, J. (2008) Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains. *J. Forensic Sci.* **53**, 296-305.
41. Bauer, M. and Patzelt, D. (2003) Simultaneous RNA and DNA isolation from blood and semen stains. *Forensic Sci. Int.* **136**, 76-78.
42. Alvarez, M., Juusola, J. and Ballantyne, J. (2004) An mRNA and DNA co-isolation method for forensic casework samples. *Anal. Biochem.* **335**, 289-298.
43. Haas, C., Hanson, E., Bär, W., Banemann, R., Bento, A. M., Berti, A., Borges, E., Bouakaze, C., Carracedo, A., Carvalho, M., Choma, A., Dötsch, M., Durianciková, M., Hoff-Olsen, P., Hohoff, C., Johansen, P., Lindenbergh, P. A., Loddenkötter, B., Ludes, B., Maroñas, O., Morling, N., Niederstätter, H., Parson, W., Patel, G., Popielarz, C., Salata, E., Schneider, P. M., Sijen, T., Sviezena, B., Zatkalíková, L. and Ballantyne, J. (2011) mRNA profiling for the identification of blood-results of a collaborative EDNAP exercise. *Forensic Sci. Int. Genet.* **5**, 21-26.
44. Haas, C., Hanson, E., Anjos, M. J., Bar, W., Banemann, R., Berti, A., Borges, E., Bouakaze, C., Carracedo, A., Carvalho, M., Castella, V., Choma, A., De Cock, G., Dotsch, M., Hoff-Olsen, P., Johansen, P., Kohlmeier, F., Lindenbergh, P. A., Ludes, B., Maronas, O., Moore, D., Morerod, M. L., Morling, N., Niederstatter, H., Noel, F., Parson, W. Patel, G., Popielarz, C. Salata, E., Schneider, P. M., Sijen, T., Sviezena, B., Turanska, M., Zatkalikova, L. and Ballantyne, J. (2012) RNA/DNA co-analysis from blood stains—results of a second collaborative EDNAP exercise. *Forensic Sci. Int. Genet.* **6**, 70-80.
45. Juusola, J. and Ballantyne, J. (2005) Multiplex mRNA profiling for the identification of body fluids. *Forensic Sci. Int.* **152**, 1-12.
46. Juusola, J. and Ballantyne, J. (2007) mRNA profiling for body fluid identification by multiplex quantitative RT-PCR. *J. Forensic Sci.* **52**, 1252-1262.
47. Nussbaumer, C., Gharehbaghi-Schnell, E. and Korschineck, I. (2006) Messenger RNA profiling: a novel method for body fluid identification by real-time PCR. *Forensic Sci. Int.* **157**, 181-186.
48. Haas, C., Klesser, B., Maake, C., Bär, W. and Kratzer, A. (2009) mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR. *Forensic Sci. Int. Genet.* **3**, 80-88.
49. Vennemann, M. and Koppelkamm, A. (2010). mRNA profiling in forensic genetics I: Possibilities and limitations. *Forensic Sci. Int.* **203**, 71-75.
50. Liu, B., Lague, J. R., Nunes, D. P., Toselli, P., Oppenheim, F. G., Soares, R. V., Troxler, R. F. and Offner, G. D. (2002) Expression of membrane-associated mucins MUC1 and MUC4 in major human salivary glands. *J. Histochem. Cytochem.* **50**, 811-820.
51. Fleming, R. I. and Harbison, S. (2010) The use of bacteria for the identification of vaginal secretions. *Forensic Sci. Int. Genet.* **4**, 311-315.
52. Hanson, E. K., Lubenow, H. and Ballantyne, J. (2009) Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. *Anal. Biochem.* **387**, 303-314.
53. Zubakov, D., Boersma, A. W., Choi, Y., van Kuijk, P. F., Wiemer, E. A. and Kayser, M. (2010) MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. *Int. J. Legal Med.* **124**, 217-226.
54. Wang, Z., Luo, H. B., Pan, X. F., Liao, M. and Hou, Y. P. (2012) A model for data analysis of microRNA expression in forensic body fluid identification. *Forensic Sci. Int. Genet.* **6**, 419-423.
55. Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281-297.
56. Kim, V. N. (2005) MicroRNA biogenesis: coordinated cropping and dicing. *Nat. Rev. Mol. Cell Biol.* **6**, 376-385.
57. Jost, D., Nowojewski, A. and Levine, E. (2011) Small RNA biology is systems biology. *BMB Rep.* **44**, 11-21.
58. Sood, P., Krek, A., Zavolan, M., Macino, G. and Rajewsky, N. (2006) Cell-type-specific signatures of

microRNAs on target mRNA expression. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 2746-2751.
59. Liang, Y., Ridzon, D., Wong, L. and Chen, C. (2007) Characterization of microRNA expression profiles in normal human tissues. *BMC Genomics* **8**, 166.
60. Frumkin, D., Wasserstrom, A., Budowle, B. and Davidson, A. (2011) DNA methylation-based forensic tissue identification. *Forensic Sci. Int. Genet.* **5**, 517-524.
61. Lee H. Y., Park, M. J., Choi, A., An, J. H., Yang, W. I. and Shin, K. J. (2012) Potential forensic application of DNA methylation profiling to body fluid identification. *Int. J. Legal Med.* **126**, 55-62.
62. An, J. H., Choi, A., Shin, K. J., Yang, W. I. and Lee, H. Y. (2012) DNA methylation-specific multiplex assays for body fluid identification. *Int. J. Legal Med.* (in press) http://dx.doi.org/10.1007/s00414-012-0719-1.
63. Wasserstrom, A., Frumkin, D., Davidson, A., Shpitzen, M., Herman, Y. and Gafny, R. (2012) Demonstration of DSI-semen-A novel DNA methylation-based forensic semen identification assay. *Forensic Sci. Int. Genet.* (in press) http://dx.doi.org/10.1016/j.fsigen.2012.08.009.
64. Larue, B. L., King, J. L. and Budowle, B. (2012) A validation study of the Nucleix DSI-Semen kit-a methylation-based assay for semen identification. *Int. J. Legal Med.* (in press) http://dx.doi.org/10.1007/s00414-012-0760-0.
65. Madi, T., Balamurugan, K., Bombardi, R., Duncan, G. and McCord, B. (2012) The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing. *Electrophoresis* **33**, 1736-1745.
66. Miranda, T. B. and Jones, P. A. (2007) DNA methylation: the nuts and bolts of repression. *J. Cell Physiol.* **213**, 384-390.
67. Hashimshony, T., Zhang, J., Keshet, I., Bustin, M. and Cedar, H. (2003) The role of DNA methylation in setting up chromatin structure during development. *Nat. Genet.* **34**, 187-192.
68. Byun, H. M., Siegmund, K. D., Pan, F.,Weisenberger, D. J., Kanel, G., Laird, P. W. and Yang, A. S. (2009) Epigenetic profiling of somatic tissues from human autopsy specimens identifies tissue- and individual-specific DNA methylation patterns. *Hum. Mol. Genet.* **18**, 4808-4817.
69. Song, F., Smith, J. F., Kimura, M. T., Morrow, A. D., Matsuyama, T., Nagase, H. and Held, W. A. (2005) Association of tissue-specific differentially methylated regions (TDMs) with differential gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 3336-3341.
70. Ohgane, J., Yagi, S. and Shiota, K. (2008) Epigenetics: the DNA methylation profile of tissue-dependent and differentially methylated regions in cells. *Placenta* **29**, S29-35.
71. Koch, C. M. and Wagner, W. (2011) Epigenetic-aging-signature to determine age in different tissues. *Aging* **3**, 1018-1027.
72. Lee, J. and Ryu, H. (2010) Epigenetic modification is linked to Alzheimer's disease: is it a maker or a marker? *BMB Rep.* **43**, 649-655.