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Overview of Methods for Vaginal Fluid Identification

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Abstract

Biological evidence can connect suspects and victims to crime scenes and provide clues for reconstructing events. For example, vaginal fluid is a crucial indicator of sexual assault, especially in cases where semen stains are not found. Identifying the vaginal fluids on the fingers of perpetrators or on the objects used to violate victims can help validate victims' claims. However, unlike for semen, there are still no standard protocols for vaginal fluid identification. Forensic scientists have developed various methods to distinguish vaginal fluids from other body fluids, such as detecting vaginal cells, female hormones, specific proteins, microbes, and nucleic acids in the sample. This article provides an overview of the methods, from traditional to molecular, that has been studied in this regard and concludes all the identified targets and markers that are useful.

Keywords: forensic science, forensic biology, body fluid identification, vaginal fluids, sexual assault

Introduction

Body fluid identification has been a major issue for years. While the identity of the people leaving biological evidence at crime scenes can help forensic scientists reconstruct the events, the information on the type of biofluids is also important. Vaginal fluid is commonly thought of as semen at a crime scene of sexual assault. However, few methods can be used to identify vaginal fluids accurately, likely due to the complex composition of vaginal fluid [1]. In some sexual assault cases, perpetrators violate victims with fingers or objects; thus, semen cannot be found. In such cases, identifying the vaginal fluid is crucial for proving the occurrence of the crime. This review briefly summarizes the methods, from conventional to molecular, that have been studied to identify the presence of vaginal fluids and discusses the use of these methods to investigate future criminal cases.

Detection of Vaginal Cells

Lugol's Iodine Staining Method

Lugol's solution contains iodine, which can be used to indicate the presence of glycogen [2,3]. As vaginal epith-

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elial cells have the highest level of glycogen among all types of epithelial cells, Lugol's method is considered an effective way of identifying vaginal epithelial cells. However, Hausmann et al. found that glycogenated epithelial cells were also detected in smears made from penile and oral swabs and urinary sediment [4]. These epithelial cells, positively stained by Lugol's solution, cannot be distinguished from vaginal epithelial cells by cytomorphology or staining. Therefore, Lugol's staining method can no longer be used to identify vaginal epithelial cells.

Immunohistochemical Detection of Estrogen Receptors

Estrogen receptors, first found in the vaginal mucosa by Wiegenrick et al., are distributed in freshly frozen vaginal sections [5,6]. Based on these findings, Hausmann et al. investigated whether vaginal epithelial cells could be identified in smears by immunohistochemical detection [7]. They used monoclonal antibodies to detect estrogen receptors in paraffin-embedded female and male epithelia. They found a high density of the receptors in the basal and parabasal cell layers of the vaginal mucosa. In contrast, the superficial cell layer of the vaginal mucosa was receptor-free. In addition, estrogen receptors were also found in the basal cell layer of the male epithelium. Since vaginal smears mostly comprise the receptor-free superficial cell layer of the upper epithelium, immunohistochemical detection appears unsuitable for vaginal cell identification.

Analysis of Specific Carboxylic Acids

Vaginal secretions contain a high level of lactic acid. Meanwhile, citric acid is one of the characteristic components of semen [8,9]. Therefore, Martin et al. used capillary isotachophoresis to quantify lactic acid and citric acid in vaginal secretions and semen, respectively, to identify body fluids [10]. They found that the level of citrate was much higher than that of lactate in semen; such a pattern was reversed in vaginal secretions. Although lactate and citrate were also found in extracts from oral swabs and urine, their levels were much lower than those in vaginal secretions and semen, respectively. Therefore, although lactate and citrate are not specific for vaginal secretions and semen, respectively, quantifying these carboxylic acids can help identify them.

Detection of 17β-Estradiol (E2-17β)

E2-17β is a form of the female hormone estrogen, which is synthesized from testosterone by aromatase [11]. Vaginal secretions are expected to contain high concentrations of E2-17β, as it is primarily produced in the ovaries and placenta. Therefore, Sakurada et al. determined whether E2-17β could be a marker of vaginal secretions by measuring the concentration of E2-17β in vaginal secretions and other body fluids using gas chromatography-mass spectrometry (GC-MS) [12]. They found that, although E2-17β existed in other body fluids, it was much more highly concentrated in vaginal secretions. However, the authors only tested a few samples; thus, the threshold of E2-17β for identifying vaginal secretions could not be determined. In addition, this study conducted the experiments using a commercial E2-17β ELISA kit (Japan Enbilochemicals, Tokyo, Japan) for environmental pollution testing. While the ELISA results were consistent with those from GC-MS, they could not quantify E2-17β accurately. Meanwhile, the authors observed that testosterone was not converted to E2-17β in body fluid stains, indicating that the amount of E2-17β detected in the stains was unchanged.

Detection of Peptidase Isozyme or Other Specific Proteins

Divall et al. found that an enzyme, termed vaginal peptidase, is only collected from vaginal swabs [13]. Vaginal peptidase can be identified by its high mobility toward the anode in starch gel electrophoresis and its ability to hydrolyze L-valyl-L-leucine. In their study[13], extracts of semen-free or blood-free vaginal swabs contained only vaginal peptidase, whereas samples containing semen or blood contained both vaginal peptidase and peptidase A [14,15]. Moreover, vaginal peptidase was not detected in other body fluids commonly encountered in crime scenes.

In addition to peptidases, other specific proteins have been identified as useful biomarkers of vaginal secretion. For example, acid phosphatase is present in

both seminal and vaginal fluids [16], furthermore, Anzai et al. found that seminal and vaginal acid phosphatases could be differentiated by agarose gel electrophoresis [17]. Therefore, this method can be used to distinguish vaginal fluids from semen.

Meanwhile, Steendam et al. used mass spectrometry (MS) to detect protein biomarkers in body fluids [18] and found that cornulin, cornifin, and involucrin could be biomarkers of vaginal fluids. MS is highly sensitive. The stability of proteins also makes it possible to identify these markers in old samples. Another advantage of this approach is that it is non-destructive; thus, it can help identify species and body fluid types in a single sample. However, it is more time-consuming than other biochemical approaches. Nevertheless, Legg et al. discovered several protein candidate biomarkers of vaginal fluids using high-performance liquid chromatography, followed by MS [19], further validating the specificity of these candidate biomarkers using quadrupole time-of-flight mass spectrometry [20]. In addition to cornulin, IgGFc-binding protein, neutrophil gelatinase-associated lipocalin, Ly6/PLAUR containing protein 3, suprabasin, and matrix metalloproteinase-9 are highly specific for vaginal fluids. Therefore, the authors concluded that detection of cornulin, neutrophil gelatinase-associated lipocalin, and Ly6/PLAUR containing protein 3 could confirm the presence of vaginal fluids in an unknown sample [20].

Detection of Specific Microbes

The detection of specific bacteria in target body fluids is another method of body fluid identification. For example, Lactobacillus species are the predominant vaginal bacteria [21,22]; thus, they have been used as targets for vaginal secretions. Fleming et al. used the 16S-23S rRNA intergenic spacer regions (ISRs) in *Lactobacillus crispatus (L. crispatus)* and *Lactobacillus gasseri (L. gasseri)* genomes to identify vaginal secretions [23]. They found that blood, saliva, and semen samples were all negative for *L. crispatus* and *L. gasseri*, and menstrual blood samples have one or both species. Therefore, the authors concluded that the 16S-23S rRNA ISRs of *L. crispatus* and *L. gasseri* were new markers for vaginal fluid identification. These markers could even be detected in samples left at room temperature for 6 weeks.

Meanwhile, Giampaoli et al. developed a multiplex real-time PCR system using microflora DNA to identify vaginal fluids, saliva, and fecal samples [24]. Using *L. crispatus* and *L. gasseri* as targets for vaginal secretions, the authors successfully differentiated vaginal fluids from saliva and fecal samples using this system. Furthermore, the authors found that *Lactobacillus* species could be detected in the vaginal fluids of a woman older than 70. Moreover, Doi et al. designed a pair of primers for a conserved region in the *Lactobacillus* genome, i.e., the 16S rRNA gene sequences of four major vaginal *Lactobacillus* species, *L. crispatus, L. gasseri, L. iners*, and *L. jensenii* [25]. This way, *Lactobacillus* species could be detected using real-time PCR. The results indicated that this method could distinguish vaginal fluids from other body fluids with high sensitivity. However, the authors found a vaginal fluid sample in which Lactobacillus species were not the predominant bacteria. Therefore, it must be noted that a negative result of *Lactobacillus* species does not necessarily indicate the absence of vaginal secretions.

Samples at crime scenes may not be of good quality, increasing the difficulty of identification. Therefore, forensic scientists are trying to develop new methods to reduce the number of inconclusive results. Giampaoli et al. analyzed the DNA in vaginal, oral, fecal, and yogurt samples using real-time PCR and next-generation sequencing (NGS) and compared the results [26]. They found that NGS could enhance the detection of some key bacteria to increase the credibility of the results. Therefore, NGS can be used to identify more challenging samples.

Analysis of Messenger RNA, MicroRNA, and Circular RNA Markers

Conventionally, forensic scientists detect human body fluids using immunochemical or enzymatic methods. Because of the development of molecular techniques, RNA has become a new biomarker for body fluid identification. Juusola et al. used reverse-transcription PCR (RT-PCR) and capillary electrophoresis (CE) to identify body fluids [27]. They found that the messenger RNAs (mRNAs) of human beta-defensin 1 *(HBD-1)* and mucin 4 *(MUC4)* genes were specific for vaginal secretions. According to their study, the *HBD-1* and *MUC4* mRNA markers were highly sensitive and

specific; *HBD-1* and *MUC4* mRNA were detected in only 12 ng and less than 2 ng of total RNA, respectively. However, Nussbaumer et al. used real-time PCR to test the sensitivity and specificity of *MUC4* for vaginal fluids [28] and found that *MUC4* expression levels in some saliva samples were similar to those in vaginal fluids; thus, *MUC4* could not be used as the only marker to differentiate between saliva and vaginal fluids. They also investigated the stability of *MUC4* mRNA; after a 10-day storage, the originally positive saliva samples became negative, whereas the originally positive vaginal fluid samples remained the same.

In addition, Hanson et al. used whole-transcriptome sequencing (RNA-seq) to identify candidate biomarkers for vaginal secretions [29]. Six markers were identified, including surfactant associated 2 *(SFTA2)*, fucosyltransferase 6 *(FUT6)*, Dickkopf homolog 4 *(DKK4)*, interleukin-19 *(IL19)*, myozenin-1 *(MYOZ1)*, and cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene 1 *(CYP2B7P1)*. Then, the authors verified the specificity of these candidates by detecting them in other body fluids using CE. They found that *MYOZ1* and *CYP2B7P1* RNA had high specificity for vaginal secretions without any cross-reaction with other body fluids. However, *SFTA2*, *FUT6*, *DKK4*, and *IL19* RNA were also present in saliva, although at lower levels than in vaginal secretions. Therefore, the authors recommended using the last four markers in quantitative RT-PCR-based assays.

Moreover, Akutsu et al. analyzed *MUC4, CYP2B7P1*, and the genes encoding estrogen receptor 1 *(ESR1)*, serpin family B member 13 *(SERPINB13)*, and kallikrein-related peptidase 13 *(KLK13)* as candidate genes for vaginal fluids using multiplex RT-PCR [30]. The amplicons were separated using chip electrophoresis. They boost the multiplex RT-PCR results statistically by calculating the likelihood ratio (LR). When all five markers were positive, the LR was high. Therefore, this procedure provides a tool for objective identification.

Additionally, microRNAs have been explored as biomarkers for vaginal fluids. MicroRNA is non-coding RNA molecules. They offer an advantage as biomarkers because they are less degradable owing to their small size, at 18 to 22 nucleotides. For example, Sirker et al. selected miR1280 and miR4286 as candidate biomarkers for vaginal fluids by literature research [31] and tested them using reverse transcription quantitative real-time PCR. However, although the median expression levels for both marker genes were the highest in vaginal fluids, there was no significant difference in expression levels for clear distinction.

Circular RNAs (circRNAs) are known to be co-expressed with linear RNAs and have common exonic sequences [32]. In addition, studies have proved that the transcripts in mRNA profiling would be increased if circRNAs were included [33,34]. Liu et al. evaluated the expression of circRNA biomarker genes in peripheral blood, saliva, semen, urine, and fluid samples from female reproductive organs [35] and identified four biomarkers for vaginal secretions, *SPINK5, SERPINB3, ESR1*, and *CYP2B7P1*. *ESR1* was only expressed in vaginal secretions and menstrual blood, suggesting that it had high specificity to vaginal fluids. *SPINK5* and *SERPINB3* showed cross-reactivity with saliva samples. Later, Liu et al. constructed a multiplex F18plex assay to simultaneously amplify linear and circular RNA amplicons [36]. Linear and circular RNAs of *SPINK5, ESR1*, and *CYP2B7P1* were used as biomarkers of vaginal secretions in this system. The authors concluded that circRNAs were stabler than linear RNAs. Therefore, including circRNAs in an analysis will help the examination of aged or samples in small amounts.

Analysis of DNA Methylation Markers

DNA methylation, an epigenetic mechanism, can regulate a gene's expression without altering its sequence [37]. Numerous tissue-differentially methylated regions (tDMRs) have been identified using high-throughput DNA technologies. They represent different methylation levels in various cell types and tissues, suggesting possible applications of DNA methylation markers in forensic science, such as the identification of body fluids [38]. Lee et al. detected the methylation status of each CpG site in five tDMRs [39], namely, those in Dapper 1 isoform 2 *(DACT1)*, ubiquitin carboxyl-terminal hydrolase 49 *(USP49)*, homeobox protein Hox-A4 *(HOXA4)*, profilin-3 *(PFN3)*, and protein arginine N-methyltransferase 2 *(PRMT2)* using bisulfite sequencing. The authors found that *DACT1, USP49*, and *PRMT2* were hypermethylated in vaginal fluids but hypomethylated in semen. As for *PFN3*, the four CpG sites were unmethylated in vaginal fluids. In conclusion,

the authors suggested that *DACT1, USP49, PRMT2*, and *PFN3* could be used to identify vaginal fluids.

In addition, Park et al. screened for candidate markers using a HumanMethylation 450K Beadchip array (Illumina, CA, USA). They then validated the markers using pyrosequencing [40]. Subsequently, they found that cg01774849 and cg14991487 could successfully distinguish vaginal fluids from other body fluids.

Moreover, Lee et al. developed a multiplex methylation SNapshot system to distinguish the blood, saliva, semen, and body fluid samples from female reproductive organs [41]. In this system, vaginal fluid samples showed high methylation signals, and cg09765089 and cg26079753 were found to be vaginal fluid-specific markers. Meanwhile, Forat et al. developed another system using a methylation-sensitive single-nucleotide primer extension assay [42]. In this study, the authors found that cg14991487 and cg03874199 were good markers of vaginal fluids.

Additionally, Lin et al. constructed two biofluid identification systems, one using methylation-specific PCR combined with single-base extension (SBE) and the other using methylation-sensitive restriction enzyme-PCR (MSRE-PCR) [43,44]. In the SBE system, cg25416153 and cg09765089 were found to be vaginal fluid-specific markers, and cg15402210 was identified as a marker in the MSRE-PCR system. These markers can successfully identify vaginal fluids, and thus could be applied to real forensic cases.

Tian et al. reported a new method for detecting methylation profiles [45]. The authors combined amplification refractory mutation system-PCR and a random forest model to build a multiplex assay. Then, the authors tested the vaginal fluid markers, including cg26285089, cg09765089, and cg25416153 from previous studies [40,42,43,46-55], using this system. They confirmed that these markers could successfully identify vaginal fluids. The methylation levels of cg09765089 and cg25416153 in vaginal fluids were much higher in this study than in previous studies, likely because of the differences in detection methods. Thus, the authors have provided a new and effective method for obtaining methylation profiles for body fluid identification.

Non-Destructive Methods

Owing to the limited amount of biological material at some crime scenes, non-destructive methods are under development. Sikirzhytskaya et al. used Raman spectroscopy to identify vaginal fluids [56]. They determined the principal components necessary for identification according to the results of the significant factor analysis and principal component analysis. High goodness-of-fit results were obtained from the statistical analysis, indicating that Raman spectroscopy provided an effective and non-destructive way to identify vaginal fluids. However, the authors did not know whether the components of vaginal fluid would be changed by other factors, such as diseases and pregnancy. Because the authors did not study mixed samples or contaminated samples, further investigation would be needed.

Meanwhile, Sharma et al. detected vaginal fluids using attenuated total reflectance Fourier transform infrared spectroscopy [57]. They first constructed a standard characteristic spectrum of vaginal fluids. Then, they investigated the spectra of vaginal fluids on different substrates, vaginal fluids mixed with other body fluids, and aged vaginal fluids. They found that vaginal fluids on non-porous substrates could generate the same spectrum as that of a pure vaginal sample. However, in the fluid mixtures, spectral peaks of semen, menstrual blood, and peripheral blood overwhelm the peaks of vaginal fluids. Therefore, this method cannot be used for analyzing mixed samples. Finally, the authors found that 7-day-old vaginal fluids on glass slides could still be successfully identified using this method.

Conclusion

In this article, we reviewed the methods that have been investigated for vaginal fluid identification. Some methods are ineffective in identifying vaginal fluids, such as Lugol's staining and detection of estrogen receptors. However, most methods, such as detecting or quantifying specific markers like carboxylic acids, female hormones, microbes, RNA markers, and DNA markers, can distinguish vaginal fluids from other body fluids. As for non-destructive methods, spectroscopic methods are effective. We have listed all the targets and markers considered useful in Table 1, excluding the non-destructive methods. In addition, to help readers find interested topics and papers, all topics and their related references were listed in Table 2. We hope this review helps establish future standard protocols for vaginal fluid identification.

Table 1 Targets and markers that are considered useful in vaginal fluid identification.

Classification	Targets or Markers	References
Carboxylic Acids	Lactic acid and citric acid	[10]
Hormones	17β-estradiol	[12]
Peptidase Isozyme or Other Specific Proteins	Vaginal peptidase	[13]
	Acid phosphatase	[17]
	Cornulin	[18-20]
	Cornifin	[18]
	Involucrin	[18]
	IgGFc-binding protein	[20]
	Neutrophil gelatinase-associated lipocalin	[20]
	Ly6/PLAUR containing protein 3	[20]
	Suprabasin	[20]
	Matrix metalloproteinase-9	[20]
Microbes	*L. crispatus*	[23-25]
	L. gasseri	[23-25]
	L. iners	[25]
	L. jensenii	[25]
RNA	*HBD-1*	[27]
	MUC4	[27,28,30]
	SFTA2	[29]
	FUT6	[29]
	DKK4	[29]
	IL-19	[29]
	MYOZ1	[29]
	CYP2B7P1	[29,30,35,36]
	ESR1	[30,35,36]
	SERPINB13	[30,35]
	KLK13	[30]
	SPINK5	[35,36]
DNA Methylation	*DACT1*	[39]
	USP49	[39]
	PRMT2	[39]
	PFN3	[39]
	cg01774849	[40]
	cg14991487	[40,42]
	cg09765089	[41,43,45]
	cg26079753	[41]
	cg03874199	[42]
	cg25416153	[43]
	cg15402210	[44]
	cg26285089	[45]
	cg25416153	[45]

Table 2 Topics and their related references.

Topics	References
Detection of Vaginal Cells	[2-7]
Analysis of Specific Carboxylic Acids	[8-10]
Detection of E2-17β	[11,12]
Detection of Peptidase Isozyme of Other Specific Proteins	[13-20]
Detection of Specific Microbes	[21-26]
Analysis of Messenger RNA, MicroRNA, and Circular RNA Markers	[27-36]
Analysis of DNA Methylation Markers	[37-55]
Non-Destructive Methods	[56,57]

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