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SHORT COMMUNICATION

Validation of an immunochromatographic D-dimer test to presumptively identify menstrual fluid in forensic exhibits

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Abstract Identifying the biological source of a crime scene stain can be crucial for police investigations in many scenarios. Blood is one of the most common fluids found, and accurate differentiation between peripheral blood and menstrual fluid could provide valuable information regarding the issue of consent in sexual assault cases. For the detection of menstrual fluid, no easy-to-use presumptive test is available to date. Therefore, this study aimed to validate a simple immunochromatographic test for the indication of menstrual fluid, focusing on a D-dimer assay. The *Clearview*® rapid D-dimer test provides a diagnostic assay for the detection of fibrin degradation products. We validated the sensitivity and robustness of the assay using fresh and dried menstrual fluid samples, body fluid mixtures, diluted samples, and casework swabs. Cross reactivity was tested for saliva, semen, vaginal fluid, and blood. No false positive results were obtained; it was possible to successfully analyze mixtures, highly diluted samples, and casework swabs. The results of this study indicate that the D-dimer assay reliably detects menstrual fluid in forensic exhibits and is easy to implement into the current workflow of body fluid identification.

Keywords Body fluid identification · Forensic science · Menstrual fluid · Presumptive test · D-dimer

Introduction

For the investigation of a crime, it can be crucial to determine the origin and type of body fluid found at the scene of crime [1]. Blood is one of the most common body fluids found at crime scenes and may indicate a violent assault. In cases where blood is present upon a surface such as fabric, an accurate differentiation of peripheral blood (indicating a traumatic cause) from menstrual fluid is providing potentially valuable information regarding, i.e., the issue of consent in alleged sexual assaults [2]. Thus, a method to reliably distinguish between menstrual and peripheral blood would make a substantial progress in analyzing and interpreting evidence from such cases.

Several innovative methods for menstrual fluid identification have been described recently, such as profiling of messenger RNA (mRNA) [3–5] or microRNA [6–8]. Research in mRNA profiling revealed a number of specific and sensitive mRNA assays for the identification of all forensically relevant body fluids [9, 10]. However, the markers commonly reported as specific for menstrual fluid, such as matrix metalloproteinase 7, 10, and 11 [3, 5, 11, 12], have also been detected in semen, vaginal fluid, and saliva which may lead to false positive results [2]. Even though mRNA profiling is a promising tool in forensic science, the technique is highly time consuming and requires a high level of expertise to ensure correct data interpretation.

Although several presumptive blood tests, such as Kastle-Meyer, Luminol, or Leukomalachite Green, are available for the detection of blood, there is currently no test that is specific to menstrual blood. Thus, the aim of this study was to evaluate and validate a simple presumptive test for the indication of menstrual fluid in forensic stains. The study focused on the detection of degradation products of fibrinolysis, namely D-dimers. D-dimer testing was initially developed as diagnostic assay for intravascular coagulation, deep vein thrombosis, and pulmonary embolism. Cross-linked fibrin degradation products (FDP) contain D-dimers and are produced during fibrinolysis, the endogenous degradation of fibrin after blood coagulation. Thus, fibrin derivatives in human blood

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containing D-dimers are specific markers for fibrinolysis [13, 14]. During menstruation, fibrinolysis is an important step to block blood coagulation and enable the menstrual fluid to easily pour out. The use of FDPs such as D-dimers to identify menstrual blood has been initially described by Miyaishi and colleagues in 1996 [15] and the idea has been picked up recently by Baker et al. [2]. We now report the first forensic validation of an immunochromatographic D-dimer test, the Clearview rapid test [14]. The test has been developed for diagnostics purposes to detect D-dimers in peripheral blood as a marker for venous thromboembolism. Here, we describe the simple implementation into current forensic workflows.

Materials and methods

Validation samples

Menstrual fluid was collected by vaginal swabs from three healthy female donors at days 1, 2, and 3 of the menses. Swabs were stored at room temperature for 2–3 months before testing.

Liquid menstrual fluid was collected using the Moon Cup menstrual cup (Mooncup Ltd, Brighton, UK). This is a soft silicone menstrual cup that is used as an alternative to sanitary towels or tampons. It is inserted into the vagina and collects menstrual fluid in its cavity. Liquid menstrual fluid was tested in a serial dilution (neat 1:25, 1:50, and 1:100). Furthermore, a serial dilution of menstrual blood was prepared (neat 1:1, 1:10, 1:20, 1:30, 1:40, and 1:50), transferred to cotton fabric, and left to dry at room temperature for 4 weeks. Squares of 0.3–0.5 mm^2 were cut out for testing.

Sanitary towels and tampons (nine each) from anonymous women were analyzed to determine inter-individual differences.

Mixtures of menstrual fluid (1:10) were prepared with saliva, blood, and semen, transferred to cotton fabric, and allowed to dry at room temperature over night.

Cross reactivity with other body fluids was tested with fresh and dried saliva, blood, vaginal fluid, urine, and semen.

Postmortem blood samples

Postmortem blood samples were tested for false positive test results due to postmortem fibrinolysis. Dried peripheral blood samples from nine deceased donors with various causes of death (see Table 2 for details) were obtained during medico-legal autopsy and transferred to the laboratory for molecular identification.

Casework samples

In a case of alleged sexual assault, low, mid, and high vaginal swabs were taken during medical examination and transferred to the laboratory for body fluid identification and DNA analysis. All three swabs were stained in a reddish-brownish coloring, and blood presumptive testing (Kastle-Meyer, [16]) was positive.

D-dimer testing

The Clearview Simplify D-dimer kit (Alere, Cheshire, UK) was used following the manufacturer's recommendations. The kit uses the D-dimer-specific murine monoclonal antibody DD3B6/2 conjugated to colloidal gold particles to detect D-dimer-containing molecules. This first antibody binds specifically to molecules containing D-dimer. The complex migrates through the test window's membrane until it is captured and concentrated on a zone to which a second D-dimer-specific antibody has been bound. The capture of the complexes at the test zone (T) causes a pink line to appear in the test window. The test also has a procedural control (PC) line where uncaptured first antibodies bind to an anti-murine antibody. The PC line acts as an internal procedural control to indicate that the test is working as designed.

Prior to testing, dried samples (approx. 3–4 mm^2 of cotton fabric or swabs) were submerged in 70 μL of Universal Buffer from the Rapid Stain Identification kit (RSID™, Galantos Genetics) and incubated for 1 h at room temperature.

For each test, 35-μL sample or incubation solution was applied to the sample well followed by two drops of the running buffer provided in the kit. Results were read after a development period of 20 min.

Results

Each test strip worked correctly as shown by the test's clearly visible PC line. Positive test results show a pink line in the "T" field (Fig. 1). Figure 2 shows the interpretation guidelines applied in this work. Depending on the intensity and shape of the pink "T" line, results are categorized from "++++" to "+". The category "(+)" is applied to a very light pink line, which is too faint to be captured by a camera under normal conditions, while category "−" represents a clear test window. This differentiation was used for this validation study to indicate differences in the intensity of the positive line, which changed with changing amounts of starting material. However, the test is not quantitative in nature, and the scoring is highly subjective. Thus, we do not recommend using a scoring system in casework samples.

All test results from validation and casework samples are summarized in Table 1. The liquid menstrual fluid dilution series resulted in positive signals down to a dilution of 1:50. Liquid samples underwent several freeze-thaw cycles, which did not interfere with the test. Dried and stored dilution series

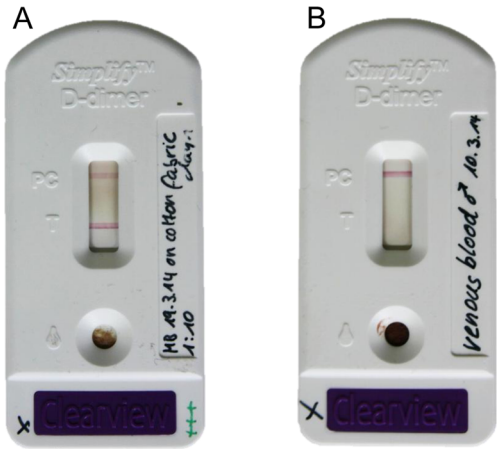

Fig. 1 Examples for a positive D-dimer test from menstrual blood (**a**) and a negative D-dimer test from peripheral blood (**b**)

gave positive results down to a dilution of 1:40 for day 1 of the menses and 1:30 for day 2 of the menses (Table 1). Vaginal swabs from two different donors gave positive test results on all 3 days of menstruation. Sanitary towels and tampons from 18 women all resulted in positive D-dimer test results with only slight inter-individual differences.

Excess of other body fluids (saliva, semen, blood) did not hamper the test (Table 1), and all mixtures containing menstrual fluid resulted in a clearly visible positive test line. No false positive signals were observed from any other body fluid: semen, saliva, vaginal fluid, and peripheral blood from

Label	Description	Example
++++	Strong pink bar with sharp edges	PC T
+++	Pink bar with sharp edges	PC T
++	Light pink bar with one sharp and one blurry edge	PC T
+	Faint pink line	PC T
(+)	Very faint pink line, difficult to catch by camera	PC T
-	Clear result window	PC T

Fig. 2 Interpretation guidelines for D-dimer results are based on the intensity of the *pink line* in the test window (T). The *line* in the performance control (PC) *window* indicates that the test works correctly

living donors showed negative test results. The three casework swabs also all gave positive D-dimer test results leading to the conclusion that menstrual blood was present on the swabs.

Eight of the nine postmortem blood samples from individuals with varying causes of death showed positive results for the detection of D-dimers. Test results and causes of death are given in Table 2.

Discussion

The D-dimer test proved to show positive results in all samples known to contain menstrual fluid with no obvious inter-individual differences. No positive result was achieved with peripheral blood from living individuals. Our results show that the test reliably identifies menstrual fluid in both fresh and dried samples down to dilution of 1:30. The sensitivity was tested by a dilution series and was found to be within the range of other immunochromatographic presumptive tests used in forensic science, such as the RSID-blood kit [17] or the Hexagon OBTI kit [18].

The simple implementation of the test into current forensic workflows is demonstrated by the use of this D-dimer assay in a real casework sample: A heavily stained vaginal swab obtained shortly after an incident of alleged sexual assault showed a positive D-dimer test and thus, was identified as menstrual fluid. This confirmed the medical examiner's finding of intact vaginal mucosa.

For this work, the RSID universal buffer was used to lyse dried stains of suspected menstrual fluid. This enables a two-step procedure in the analysis of crime scene exhibits: In the first step, RSID-blood kit (Galantos) could be used to verify the presence of human blood followed by a D-dimer test from the same lysate to distinguish whether the stain is of peripheral blood (probably of a traumatic cause) or blood as part of the menstrual fluid.

No cross reaction with blood, semen, saliva, or vaginal fluid was observed. This proves the high specificity of the D-dimer kit for menstrual fluid in healthy living individuals. The kit was, however, developed to diagnose FDPs in patients suffering from acute or recurrent venous thromboembolism (e.g., [19]). D-dimers are normally not detected in blood of healthy individuals. But advanced age, active malignancy, untreated prior venous thromboembolism, pregnancy, or surgery can lead to a positive test result [20]. This is the only currently known limitation of the forensic use of the D-dimer test in living individuals and should be considered during interpretation of test results. If in doubt, a reference sample (e.g., blood prick) of the victim should be tested to rule out a false positive detection of menstrual fluid.

A D-dimer test has previously been suggested to enable the differentiation between ante- and postmortem blood stains [21] and wounds [22]. Consequently, all dried postmortem

Table 1 Summary of all results. Results are interpreted according to Fig. 2 taking into account the intensity of the pink line in the test window

Validation samples	Dilution (or mixture ratio)	Results
Liquid menstrual fluid (day 1 of menstruation)	Undiluted	+++
	1:25	++
	1:50	(+)
	1:100	−
Dried menstrual fluid on cotton fabric (day 1 of menstruation)	Undiluted	+++
	1:1	+++
	1:10	+++
	1:20	++
	1:30	++
	1:40	(+)
	1:50	−
Dried menstrual fluid on cotton fabric (day 2 of menstruation)	Undiluted	++++
	1:1	++++
	1:10	+++
	1:20	++
	1:30	+
	1:40	−
	1:50	−
Dried vaginal swab (donor A):	Undiluted	
Day 1 of menstruation		+++
Day 2 of menstruation		+++
Day 3 of menstruation		++++
Dried vaginal swab (donor B):	Undiluted	
Day 1 of menstruation		++++
Day 2 of menstruation		++++
Day 3 of menstruation		++++
Sanitary towels (n=9)	Undiluted	+++ to ++++
Sanitary tampons (n=9)	Undiluted	+ to ++++
Menstrual fluid and peripheral blood	1:10	++++
Menstrual fluid and saliva (1:10)	1:10	++++
Menstrual fluid and semen (1:10)	1:10	++++
Fresh semen	Undiluted	−
Semen dried on cotton	Undiluted	−
Fresh saliva	Undiluted	−
Saliva dried on cotton	Undiluted	−
Fresh Peripheral blood	Undiluted	−
Peripheral blood dried on cotton	Undiluted	−
Fresh vaginal secretion on swab	Undiluted	−
Vaginal secretion dried on swab	Undiluted	−
Fresh urine	Undiluted	−
Urine dried on cotton	Undiluted	−

blood samples except one showed positive D-dimer test results due to postmortem fibrinolysis. The formation of FDPs such as D-dimers in postmortem blood is well known [23] and was confirmed in our study. Thus, postmortem blood stains might be mistaken for menstrual fluid after D-dimer testing, and this possible limitation must certainly be kept in mind.

With its high sensitivity and specificity in living individuals, the D-dimer test is a valuable extension to the current forensic toolbox of body fluid identification. Limitations of this method must be kept in mind to avoid false positive detection of menstrual fluid in blood samples from deceased individuals or from patients suffering from venous thromboembolism.

Table 2 Summary of results obtained from postmortem blood samples with the donors' causes of death. Positive results were obtained from all except one sample according to postmortem fibrinolysis

Cause of death	Result
Hypothermia	+++
Unascertained cause of death (advanced decomposition)	–
Cerebral hemorrhage (advanced decomposition)	(+)
Peritonitis	++
Burning	++++
Drowning (advanced decomposition)	++++
Polytrauma	++++
Hyperglycemia	+++
Hanging	++

With such few limitations, the D-dimer kit is superior in its performance to other commonly used presumptive tests such as acid phosphatase for the presence of seminal fluid or Kastle-Meyer test for the presence of blood. Immunochromatographic tests have several advantages over conventional presumptive tests such as catalytic or enzymatic tests: Firstly, they are very easy to use and show immediate results. Secondly, their performance is standardized and user-independent so that objective interpretation of test results is guaranteed. Finally, no special instrumentation is needed so that such tests can be performed in the forensic lab but also directly at the crime scene or in the mortuary.

Overall the D-dimer kit is a reliable method that expresses high sensitivity and specificity, requires minimal training of the analyst, and is a cheap and fast method to presumptively identify seminal fluid.

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