Forensic Science International 212 (2011) 210–214

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

D-dimer assays for the identification of menstrual blood

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ARTICLE INFO

Article history:
Received 14 February 2011
Received in revised form 28 April 2011
Accepted 7 June 2011
Available online 7 July 2011

Keywords:
Fibrinolysis
Sexual offences
Agglutination assay
Exercise
Specificity
Sensitivity

ABSTRACT

A method to reliably distinguish menstrual blood from blood in the normal circulation (peripheral blood) would be of considerable use in the forensic analysis and interpretation of evidence in sexual offence investigations. Previous attempts to address this issue have explored microscopy, lactate dehydrogenase isozyme identification, mRNA and miRNA profiling, and identification of the products of fibrinolysis. Here, four assays for D-dimer, a terminal degradation product of fibrinolysis, are evaluated for their specificity and sensitivity in detection of menstrual blood. In addition the effect of exercise, and sample storage upon D-dimer detection was investigated. Comparison of different assays revealed significant differences in results given. Nevertheless, no positive results for D-dimer were obtained using peripheral blood, mixtures of peripheral blood with semen, or peripheral blood taken from donors after moderate exercise. D-dimer was found to be detectable in 100% of menstrual blood samples after 1 week at room temperature and also in samples stored long-term (>3 years) at −20 °C. D-dimer may be an effective, simple to use tool for the presumptive identification of menstrual blood identification.

1. Introduction

The ability to identify the origin of a body fluid in a sample is of specific interest in the forensic investigation of crime. Evidence that a sample contains blood may prove influential in cases of violent assault, whilst identification of semen in a stain may provide evidence of sexual activity. In sexual offence investigations, evidence for the insertion of an object into the vagina can be made using microscopy to identify the presence of vaginal cells on the object [1]. However, such observations are not evidentially valuable due to epithelial cells from the buccal cavity and male urethra being morphologically indistinguishable from those of the vagina, or if the defence is that consent was given. In cases where blood may be present upon a surface such as clothing, an accurate differentiation of menstrual blood from peripheral blood could be a key finding in distinguishing whether the bloodstain had originated as a result of trauma or from menses, providing potentially valuable information regarding the issue of consent.

Several methods have been proposed for the identification of menstrual blood including: microscopy [2–4], lactate dehydrogenase isozyme identification [5–10], profiling of messenger RNA (mRNA) and micro-RNA (miRNA) [11–21], and detection of the products of fibrinolysis [22–34].

In particular, the use of mRNA and miRNA profiling has been the focus of much effort over the last few years and these methods have been demonstrated to be effective for distinguishing between peripheral and menstrual blood under controlled conditions. However, RNA-based methods are not without their issues in the forensic field. False negatives have been observed even with the increased sensitivity conferred by the application of PCR technology. Furthermore, as an increase in sensitivity has been achieved, the tissue specificity of some chosen mRNA targets has been brought into question: the matrix metalloprotease (MMP) targets MMP-7 and MMP-11, identified as specific to menstrual blood by Juusola and Ballantyne [12] have recently been demonstrated to be present in vaginal fluid outside the period of menses, albeit at low levels of expression [15]. Furthermore, MMP-7 may be absent in samples from donors taking oral contraceptives [16].

miRNAs are small pieces of RNA approximately 23 nucleotides in length and are intrinsically less vulnerable to degradation than mRNA. Tissue specific miRNA expression profiles have been observed [18]. To date the published results for miRNA in the identification of menstrual blood are conflicting: Hanson et al. [19] identified a marker for menstrual blood that the later study by Zubakov et al. [20] could not replicate. Confirmed miRNA markers for body fluid identification have been reported only for blood and semen though it may be a matter of time before a specific miRNA for menstrual blood is identified.

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doi:10.1016/j.forsciint.2011.06.013

The application of RNA technologies to reliably identify menstrual blood holds much promise. However, these assays are likely to remain specialised techniques until the methodology is compatible with an automated process and fully validated for casework. Currently the use of mRNA and miRNA requires the availability of clean facilities, a high level of expertise and represents a relatively high cost manual process.

The focus on mRNA assays may have led to simpler, more rapid tests such as latex agglutination assays for soluble fibrin degradation products being overlooked. One such fibrin degradation product is D-dimer, for which there are multiple assays in clinical use for the diagnosis of thrombosis. Menstrual blood has previously been demonstrated by Miyaishi et al. to have a mean concentration of D-dimer 2000 times greater than that of peripheral blood [30]. Post-mortem blood, which also shows a high D-dimer concentration, was distinguished from menstrual blood by analysis of myoglobin levels [30]. Post-mortem blood is unlikely to be present in a typical sexual offence case.

D-dimer assays may represent a quick and easy test for the presence of menstrual blood with a possibility for application in the field following minimal training. This is something that could not be achieved using the mRNA or miRNA methods. Consequently, there is a need for a re-evaluation of D-dimer assays as a method for menstrual blood identification.

2. Materials and methods

All donors of body fluid samples used in this study were employees of the Forensic Science Service (FSS), Birmingham, who gave written consent for the use of their samples.

Menstrual blood samples upon sterile cotton swabs were obtained for each day of menses from four anonymous donors and stored at −20 °C. Peripheral blood from each menstrual blood donor was collected into anticoagulant-free collection tubes by venipuncture and 10 μL aliquots immediately deposited upon glass microscope slides. Aliquots were allowed to dry and were then swabbed using a sterile cotton swab dampened with sterile distilled water (SDW). Swabs were stored at −20 °C.

2.1. Preparation of swabs and assay for D-dimer

The D-dimer assays used were the: D-Dimer Agglutination Kit (Teco, Niederbayern, Germany), Atlas D-Dimer Latex Kit (Atlas Medical, Cambridge, U.K.), and Pacific Hemostasis D-Dimer Assay (Fisher Diagnostics®, Virginia, U.S.A.) which utilise monoclonal antibodies conjugated to latex beads, and Clearview Simplify D-dimer (Alere, Cheshire, U.K.) which uses monoclonal antibodies for immunochromatography. Hereafter these kits will be individually referred to as the Teco, Atlas, Fisher and Clearview kits, respectively, whilst the Teco, Atlas, and Fisher kits will be referred to collectively as the latex kits.

The Teco and Fisher kits utilise the MA-8D3 monoclonal antibody, whilst the Clearview kit uses the DD3B6/22 monoclonal antibody. The monoclonal antibody employed in the Atlas kit is not stated. Claimed D-dimer sensitivities of the assays are as follows: Teco 250 ng mL^{-1}; Atlas 200 ng mL^{-1}; Fisher 250 ng mL^{-1}; Clearview 80 ng mL^{-1}.

In all experiments, the head of the swab was removed using a scalpel and placed into 200 μL of either sterile distilled water (SDW) or phosphate buffered saline (PBS). Samples were manipulated using a pipette tip to encourage the release of their contents into the surrounding solution, then vortexed prior to centrifugation for 5 s. The resulting solution was assayed for the presence of D-dimer as per the instructions for each kit, with the exception that for the latex kits 10 μL of sample was placed onto a supplied test card with 10 μL of latex reagent instead of the 20 μL sample aliquots and 20 μL latex reagent specified.

2.2. Sensitivity of the D-dimer assays

SDW-extracts for days 1, 2, and 3 for each donor were serially diluted 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 in SDW. The dilutions for Donors B and C were assayed for D-dimer presence using all three latex kits whilst the dilutions for Donors A and D were assayed using the Teco kit only.

2.3. Specificity of the D-dimer assay

The potential for false positive reactions for D-dimer with other commonly encountered body fluids was assessed using the following samples.

Peripheral blood from the four donors of menstrual blood was tested at volumes of 10, 20, 30, 40, 50 and 60 μL; saliva from three donors; urine from three donors; semen from three donors; vaginal swabs from three donors were also assayed. Liquid samples were pipetted in 10 μL aliquots onto microscope slides and allowed to dry prior to swabbing. Swabbed samples were prepared for assay as described above.

2.4. The effect of mixtures of body fluids upon D-dimer assay

Semen samples were obtained from three different donors. Mixtures were made up of peripheral blood with semen in the ratios: 3:1, 2:1, 1:1, 1:2, 1:3, and neat body fluid (where 3:1 represents peripheral blood:body fluid). 10 μL aliquots of each mixture were dried and swabbed as described above. The assay for D-dimer was then performed using the Teco kit. Similar mixtures were made for peripheral blood with saliva and peripheral blood with urine. Additional mixtures of peripheral blood with vaginal secretions and peripheral blood with menstrual blood were made by pipetting 10 μL, 25 μL and 50 μL of liquid peripheral blood onto cotton swabs on which vaginal secretions or menstrual blood had previously been collected. The swabs were allowed to dry before testing for D-dimer using the Teco kit.

2.5. The effect of exercise

A relationship between vigorous exercise and the level of D-dimer in peripheral blood has been previously reported [32–34]. Four volunteers were asked to jog for approximately half an hour. Blood samples taken from donors prior to and post-exercise were collected into non-EDTA collection tubes. 10 μL aliquots were dried and swabbed as described above prior to being tested using the Teco kit.

2.6. The effect of sample storage conditions

A set of 22 aged samples comprising stains on swabs and fabric from a number of different donors was tested. The stains had been stored for at least 3 years at −20 °C (6 swabs and 3 fabric samples), room temperature (10 swabs), or 37 °C (3 fabric samples). Each of these samples was tested for D-dimer with the Teco kit as before.

In order to test the effect of storage over a short timescale, five swabs containing menstrual blood were exposed to the air at room temperature for four different time periods: 24 h, 48 h, 1 week, and 2 weeks. The samples were then pooled by time point and tested using the Teco kit.

3. Results and discussion

3.1. Comparison of D-dimer assays, extraction medium, donors, and sample days

Initial observations found extraction buffers containing SDS prevented the assay from working, presumably due to denaturation of the protein target (data not shown). Fig. 1 shows the visual appearance of positive and negative results using the latex and Clearview assays, respectively. The Clearview assay proved the easier assay type to use due to less preparation and ease of interpretation.

Comparison of the kits was made by using all four of the kits to test the same extracts (Table 1). This showed the Teco and Clearview kits to have similar levels of sensitivity whilst the Atlas kit showed the lowest number of positive results. Whilst no false positives were obtained from peripheral blood with any kit, the Teco and Clearview kits showed fewer false negatives than the Atlas and Fisher kits.

3.2. The effect of sample dilution

The level of dilution at which D-dimer is detectable in a sample allows an estimate of D-dimer concentration within that sample. Variation for the dilution at which D-dimer was detectable, and therefore variation in D-dimer concentration of a sample, was seen both between donors and days. Donors B and C were the only donors for whom samples were tested using all three assays. Samples from Donor C were seen to have higher levels of D-dimer than samples from Donor B for all days and using all kits (Fig. 2). The variability in concentration of D-dimer between different sample days and donors may be attributable at least in part to the variability in amount of material collected on the swab during original sampling. It was clear that certain swabs contained more menstrual blood than others through visual examination.

This difference in sensitivity between assays has previously been observed by the clinical community and is the subject of several reviews [35,36] which point to the variation in D-dimer

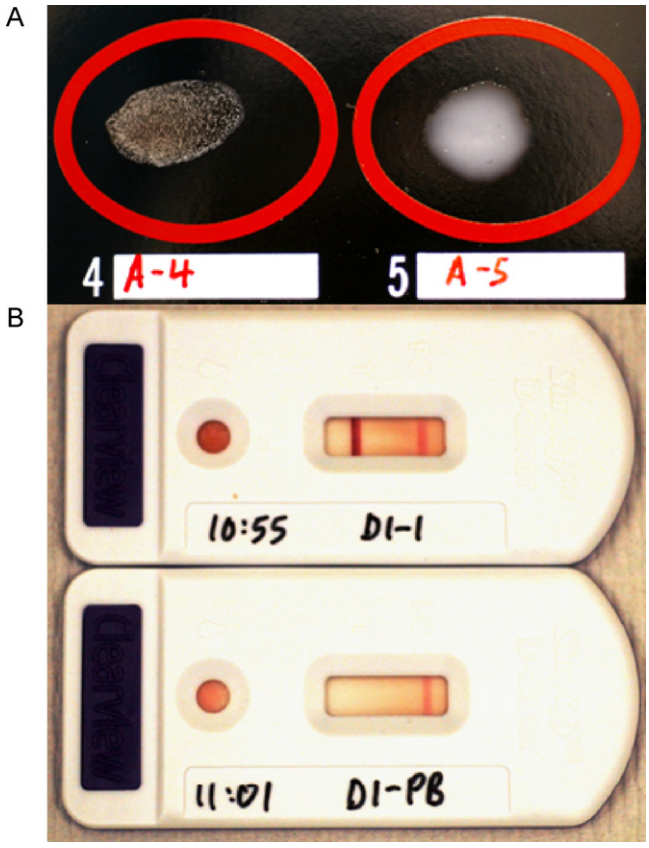

Fig. 1. (A) Results obtained using the Teco assay for the PBS-extract of Donor A for day 4 (left) and day 5 (right). The picture clearly shows the agglutination that occurs in D-dimer positive samples (left) and the absence of agglutination in D-dimer negative samples (right). (B) Results obtained using the Clearview assay for the PBS-extract of Donor A for day 1 (top) and peripheral blood (bottom). The presence of two lines indicates a positive result for the presence of D-dimer whilst one line indicates a negative result.

antigen, the size of degradation product that it is present on, and the difference in efficacy of the monoclonal antibodies used.

3.3. D-dimer kit specificity

Whitehead and Divall previously suggested that, "When blood and semen are mixed blood plasminogen is activated to plasmin and fibrin is degraded to soluble fibrin as the stain forms" [22]. This would render the D-dimer assay little value in the investigation of sexual offences were the levels of soluble fibrin found in such circumstances sufficient to produce a positive result. To investigate this possibility, a set of peripheral blood/semen mixtures were tested with the Teco kit. Using this assay, D-dimer was not detected in mixtures of peripheral blood and semen indicating that if D-dimer is formed it is at levels below the sensitivity of the test. Of the other body fluid/peripheral blood mixtures tested only the menstrual blood/peripheral blood mixtures gave positive results for D-dimer. The negative results for D-dimer from peripheral blood/vaginal secretions indicates that cases of vaginal bleeding through trauma would not be wrongly identified as menstruation.

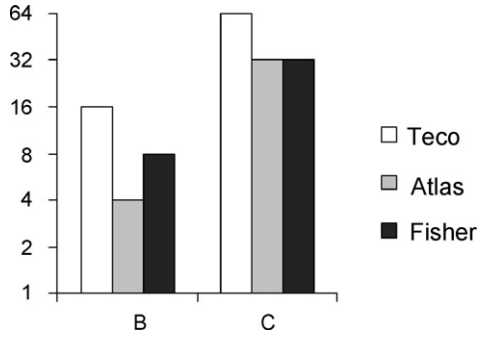

Fig. 2. The maximum possible dilution at which a positive result for D-dimer was observed for Donors B and C at sample day 2.

None of the pre- and post-exercise blood samples tested with the Teco kit tested positive for D-dimer. It may be that exercise simply does not cause a high enough concentration of D-dimer in the peripheral blood to give a positive result; the study by Weiss et al. [34] did not look at D-dimer concentration specifically but found "Fibrin Degradation Products" to be present at a concentration of approximately 150 ng mL^{-1}, after 1 h of heavy exercise. This level is below the detectable level for D-dimer of any of the latex kits used in this study, and was achieved after heavy exercise for 1 h unlike the moderate exercise for half an hour examined here.

3.4. The effect of sample age and storage conditions upon D-dimer assay result

In sexual offence investigations the amount of time may vary before a stain is available for testing, therefore it was important to ascertain whether age of stain has an effect upon D-dimer result. As shown in Table 2 storage of menstrual blood stains at room

Table 1
A table of results for D-dimer presence (+) or absence (−) using (A) SDW and (B) PBS as extraction medium. Donors are denoted as A, B, C, and D whilst D-dimer assay kits are represented as follows: Teco (T); Atlas (A); Fisher (F); Clearview (C). Note that the Clearview kit was not used for testing of extracts in SDW. Blanks indicate instances where no test was performed.

	Day 1			Day 2			Day 3			Day 4			Day 5			Peripheral blood		
	T	A	F	T	A	F	T	A	F	T	A	F	T	A	F	T	A	F
(A)																		
A	+	+	+	+		+	+	+	+	+		+	–	–	–	–		–
B	+	–	+	+	+	+	+	–	–							–	–	–
C	+	+	+	+	+	+	+	+	+							–		–
D	+	–	+	+	–	+	+	–	+	+	+	+	+	–	+	–	–	–

	Day 1				Day 2				Day 3				Day 4				Day 5				Peripheral blood			
	T	A	F	C	T	A	F	C	T	A	F	C	T	A	F	C	T	A	F	C	T	A	F	C
(B)																								
A	+	+	+	+	+		+	+	+	+	+	+	+		+	+	–	–	–	–	–		–	–
B	+	+	+	+	+	+	+	+	+	+	+	+									–	–	–	–
C	+	–	–	+	+	+	+	+	+	+	+	+									–		–	–
D	+	–	+	+	+	–	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–

Table 2
Results obtained for D-dimer presence (+) or absence (−) for menstrual blood and peripheral blood samples exposed to air at ambient temperature for different time-periods.

Replicate	Time since exposure			
	24 h	48 h	1 week	2 weeks
1	+	+	+	+
2	+	+	+	+
3	+	+	+	−
4	+	+	+	+
5	+	+	+	+
PB	−	−	−	−

temperature for up to 1 week does not appear to affect the detection of D-dimer, although by 2 weeks there is some indication that degradation of the samples has begun to take place. Peripheral blood samples tested negative at all time periods. The swabs used in this experiment were pooled from different donors and different days of menses. All swabs originated from sample days that had proven to be positive for D-dimer in previous testing.

Of the samples that had been in storage at the FSS for more than 3 years no positive results were obtained for any of the samples that had been stored at room temperature or 37 °C. In contrast, positive results were obtained for 4 (67%) of the swab samples that had been stored at −20 °C, and for 2 (67%) of the fabric samples that had been stored at −20 °C.

The negative result obtained at the 2-week time period (Table 2) and the complete lack of a positive result for D-dimer in samples stored for over 3 years at room temperature or 37 °C is most likely to be as a result of protein degradation. However, unlike the results obtained for shorter time periods, it is difficult to state with absolution that the samples stored for over 3 years were positive for D-dimer when they were initially taken, although we believe it is unlikely that all the 13 samples in this category would be negative prior to storage. It should be noted that samples do not test positive for D-dimer 100% of the time as shown by Donor A for sample day 5 (Table 1). This means that the negative results obtained for some of the fabric and swab samples stored at −20 °C may not be as a result of degradation but simply because D-dimer was not present on the samples in sufficient amounts to begin with.

4. Conclusions

In general the D-dimer kits proved easy to use and minimal sample preparation was required. Also, freezing and thawing of samples did not appear to affect the test result. The drawback of the latex assays however is the requirement for a subjective judgement to be made. For instance, in the testing of highly diluted samples it was sometimes difficult to decide whether agglutination had or had not occurred. Such "borderline" cases were treated as negative results, but the "borderline" itself may differ between examiners or with experience. Further to this, and mentioned in the instructions of the kits, is that in certain samples white flakes can appear. These samples require careful interpretation so as not to be confused with a positive result. Of the latex assays the Teco assay performed best due to a high sensitivity to D-dimer whilst the Atlas assay was shown to have the lowest sensitivity.

The Clearview assay proved much easier to interpret, although without testing of this kit upon diluted samples it is difficult to assert that it is any better than the latex assays. Sample extraction into SDW or PBS was shown to have no effect upon D-dimer result. It is clear that variation in D-dimer levels between different donors on different days did occur, as shown by the lower proportion of positive results seen for Donor A—day 5 samples (Table 1). As previously stated the variation observed between donors, sample days, and concentration of D-dimer, is potentially as a result of differing amounts of material recovered on each swab.

One of the main caveats in the application of the D-dimer test to the identification of menstrual blood is that its main function is as a diagnostic test for deep vein thrombosis. Accordingly, a peripheral blood sample from a person with deep vein thrombosis might be misinterpreted as a menstrual blood sample or vice versa. Other health conditions in which elevated D-dimer levels have been observed include cancer [37], coronary heart disease [38], and psychological states such as depression [39], whilst D-dimer is also found to increase as a result of ageing [40]. These health conditions may not cause the level of D-dimer in peripheral blood to reach that of menstrual blood, but may give a positive result for D-dimer especially when using more sensitive kits such as the Clearview test. A control blood sample from the victim would allow for the elimination of such conditions being the cause of a positive D-dimer test.

The last research upon D-dimer and its use in the identification of menstrual blood was performed by Miyaishi et al. [31] in the 1990s. Since then work has focused upon the use of mRNA [12–15] and miRNA [20,21] markers to fulfil this role. RNA-based tests promise a multiplexed approach with the capability to detect multiple body fluid types simultaneously and with high levels of sensitivity. However, issues remain to be resolved with markers for menstrual blood. Despite the sensitivity of the assays, known menstrual samples have provided false negative results and recently, increased sensitivity of the assays has put into question the menstrual blood-specificity of the MMP markers [15]. These observations suggest that the interpretation of the results can therefore be difficult. In a forensic sample demonstrating a low level positive for MMP could be the result of vaginal secretions mixed with peripheral blood, or a sample with low levels of menstrual blood. Robust interpretation guidelines are essential. Nevertheless, mRNA has been isolated from bloodstains of up to 15 years old [15] and this demonstrates the potential robustness of this technique.

For application to the forensic laboratory, both mRNA and miRNA methods are in need of further development, are costly, technically demanding and time consuming. In contrast, the D-dimer tests described here are cheap and extremely easy to implement, and the current study suggests they could be used as a presumptive test for menstrual blood at the bench or perhaps at a crime scene. The D-dimer tests did not produce any false positive results for peripheral blood, mixtures of peripheral blood or other body fluids including semen and vaginal secretions, or post-exercise blood, and were effective in detecting D-dimer in samples that had been frozen in storage for over 3 years. These results are encouraging, and the application of the results of the D-dimer test would doubtless improve the decision-making process of the forensic scientist.

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