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The acid phosphatase test two minute cut-off: An insufficient time to detect some semen stains

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ABSTRACT

The ability to detect semen in sexual offence cases is a crucial first step to locating stains which may be suitable for DNA profiling. Since the development of the acid phosphatase test in the late1950s by Stuart S. Kind, the process undertaken to perform the test has gone largely unchanged. The method currently accepted by operational forensic science laboratories allows 2 min for a reaction to be obtained, and until relatively recently, this has not been challenged. In this research, samples of semen were obtained from three donors and a range of dilutions for each sample were prepared. Each dilution was subjected to acid phosphatase testing using both direct testing and the 'press test' method. The results showed that semen could be detected in excess of 15 min in dilutions up to 1 in 400 using the press test method and in dilutions up to 1 in 1000 using the direct method. Of further significance was the observation that using the press test method, the two minute cut-off was insufficient to detect the majority of stains and in some cases, semen stains as strong as 1 in 20 dilutions. This research provides compelling evidence for protocols currently utilised in forensic practice to be reviewed in order that forensic scientists do not overlook potential evidential material that may prove suitable for body fluid identification such as DNA STR profiling.

1. Introduction

When considering the prevalence of crime, particularly the prevalence of sexually motivated crime (a six percent increase from the time period 2008/09 to 2009/10 [1]), any improvements to current practices and techniques which would aid the conviction of offenders and lead to the successful exoneration of the innocent would be welcomed. In cases involving the investigation of sexual offences, forensic science laboratories frequently receive items such as intimate swabs from individuals and items of clothing to examine for the presence of semen. Utilising a robust and reliable method for detecting semen is therefore paramount in order to locate potentially evidentially significant material. Furthermore, given the improved sensitivity of DNA profiling tests that have been introduced in to routine forensic casework over recent years [2–4], the need for improved sensitivity at this first stage of detection has never been higher.

In 1957 Stuart S. Kind reported the Acid Phosphatase Brentamine Test for routinely screening items of evidence, such as clothing, submitted to a forensic laboratory for the presence of semen [5,6]. The method utilised the uniquely high levels of the enzyme acid phosphatase found in seminal fluid as its detection point. The acid phosphatase (AP) test has now become the most routinely utilised method of presumptively testing for the presence of semen within forensic laboratories. Since the development of the acid phosphatase test, the process and method of conducting the test have been accepted and adopted by forensic scientists and have remained largely unchanged. In his publication, Kind states that a reaction, by way of a colour change (orange to purple), will occur "within a few seconds to a few minutes", and current procedures at forensic laboratories have accepted that 2 min is an acceptable length of time for a reaction to take place in the presence of semen; beyond this time, a negative result is declared. Although this method has been universally accepted for many years within the forensic community, the authors felt the need to challenge this cut-off given there was no scientific justification to support it. Allard et al. [15] concurred with this view and indicted that research was being carried out with an increased reaction time of up to 10 min and since the research reported here was completed, Lewis et al. [7] have described their findings, again, utilising a time for the test of up to 10 min.

The acid phosphatase test is a presumptive test and relies on the fact that acid phosphatase is water soluble. The two methods commonly utilised for detecting semen are the direct test and the indirect test. In the direct test, an item such as an intimate swab will be moistened and rolled over a piece of filter paper or alternatively, an extract from a possible semen stain will be prepared and a drop placed onto a piece of filter paper. In the indirect test, often referred to as the press test and from herewith referred to as such, screening of a large item for the presence of semen stains is undertaken by placing a large sheet of filter paper over the item, dampening with water, and then pressing down firmly by hand to transfer some of the semen onto the paper. In both

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tests a chemical reagent comprising sodium acetate, acetic acid, α-naphthyl phosphate disodium salt and brentamine fast black K salt is then applied to the papers. In the presence of acid phosphatase, the α-naphthyl phosphate is hydrolysed to produce α-naphthol which then combines with the brentamine fast black K to produce a purple azo dye. As described above, this reaction is then allowed 2 min to take place under the original guidelines, and if no reaction occurs within this time, a negative result is recorded and the papers discarded.

The ability to detect semen using this method is not 100% reliable, however, for a number of reasons. The most significant of these is the fact that although acid phosphatase is present in very high concentrations in semen, it is also known to be present in other biological material such as semen-free vaginal material, faeces and some foodstuffs and as such can produce false positive reactions [5,8,9]. The test is therefore only a presumptive test for the presence of semen and is not confirmatory. In order to confirm the presence of semen, one must establish the presence of spermatozoa microscopically, or in the case of azoospermic semen, by conducting another confirmatory test such as the choline test [10–12]. In addition to this specificity issue, seminal acid phosphatase levels are also known to vary between male individuals [13,14]. The ability to detect semen will therefore depend on the concentration present in different samples and in terms of forensic casework, this is an unknown factor which may yield stains undetectable utilising the current methods employed.

Although the limitations for using this method for detecting semen are widely known amongst forensic practitioners and it has been universally accepted for many years within the forensic community, the authors felt the need to challenge this cut-off given there was no scientific justification to support it. Allard et al. [15] concur with this view and indicted that they were undertaking research utilising an increased reaction time of up to 10 min. In addition, since the research reported here was completed, Lewis et al. [7] have described their findings, again utilising a time for the test of up to 10 min. This research was therefore conducted to challenge this method even further and to assess whether weak semen stains could be detected if the reaction was allowed to take place for up to 20 min and also to determine the degree of variation that exists between different donors.

2. Materials and methods

2.1. Semen dilutions

Semen dilutions of samples from three separate donors (A, B and C) were made using de-ionised water as specified in Table 1. Sufficient volumes of each dilution were prepared to ensure there were ample quantities to cover all replicates of both methods to ensure consistency in the results. One of the samples, (sample B), was known to be azoospermic, the remaining two were spermic.

Table 1
Volumes of semen and deionised water used to create dilutions.

Dilution	Volume of semen (μL)	Volume of water (μL)
1 in 20	300	5700
1 in 40	150	5850
1 in 60	100	5900
1 in 100	60	5940
1 in 200	30	5970
1 in 400	15	5985
1 in 500	12	5988
1 in 700	10	6990
1 in 800	10	7990
1 in 900	10	8990
1 in 1000	600 of 1 in 100	5400

2.2. Preparation of AP reagent

The AP reagent was prepared by dissolving 20 g of sodium acetate 3-hydrate (Fisher Scientific) in 1 L of deionised water. 5 mL of glacial acetic acid (Fisher Scientific) was then added followed by 1 g of α-naphthyl disodium phosphate salt (Sigma Aldrich) and 2 g of fast black K salt (Sigma Aldrich). The reagent was then vacuum filtered and stored in a refrigerator until required. The reagent was allowed to warm to room temperature before using.

2.3. Direct test method

100 μL of each dilution of sample A was pipetted onto separate filter papers (Fisher Brand 110 mm). 100 μL of the neat semen sample A was also pipetted on to a separate filter paper for use as a positive control and a blank filter paper was used as a negative control. The filter papers were allowed to dry naturally. Once dry, the filter papers were transferred to a fume cupboard and the AP reagent applied to the side containing the semen.

A record of any reaction that occurred was taken every minute for a total of 20 min. Reactions were recorded based on the strength of the purple reaction obtained and were categorised as strong, moderate or weak. This procedure was then precisely repeated using samples B and C and the dilutions made from these samples. The whole process was then repeated ten times for all samples and dilutions to ensure reliability of the results.

2.4. Press test method

Pieces of new white cotton fabric were cut into rectangles of approximately 30 cm × 40 cm and divided in to fourteen equal sections using a permanent marker pen. 100 μL of each dilution (11 in total) of sample A was then placed on a different segment. 100 μL of neat sample A was placed on a section of the material for use as a positive control and two sections were left blank as a negative. The material was then allowed to air dry.

Once dried, the material was placed on to a sheet of polythene. A large sheet of filter paper (Fisher Scientific, Whatman grade 1) slightly larger than the material was then divided into fourteen sections, corresponding to the sections on the material and this was placed on top of the material ensuring the sections lined up with the sections of the material. The filter paper was then gently sprayed with deionised water ensuring that the whole paper was dampened as evenly as possible. A second sheet of polythene was then placed over the filter paper and pressed down by hand for 2 min to allow absorption of the acid phosphatase within the semen to the filter paper. Next, the filter paper was placed in a fume cupboard and AP reagent applied to the entire sheet.

A record was made of any reaction that occurred every minute for a total of 20 min. Reactions were recorded and the method was repeated as outlined in Section 2.3 above for the 'Direct test method'. The method was then repeated using samples B and C and the dilutions made from these samples. The whole process was then repeated ten times for all samples and dilutions to ensure reliability of the results.

3. Results

3.1. Direct method

Fig. 1 shows the mean time taken for a positive reaction to occur where various dilutions of semen were applied directly to filter papers then subjected to the acid phosphatase reagent. The mean time was calculated from the results of ten repeats of the test for each donor. These results show that semen dilutions up to 1 in 1000 can be detected after a mean of 11 min. Furthermore, Table 2 shows the minimum and maximum times for a dilution to produce a positive reaction, if a reaction was obtained. The maximum time for the direct testing methods was

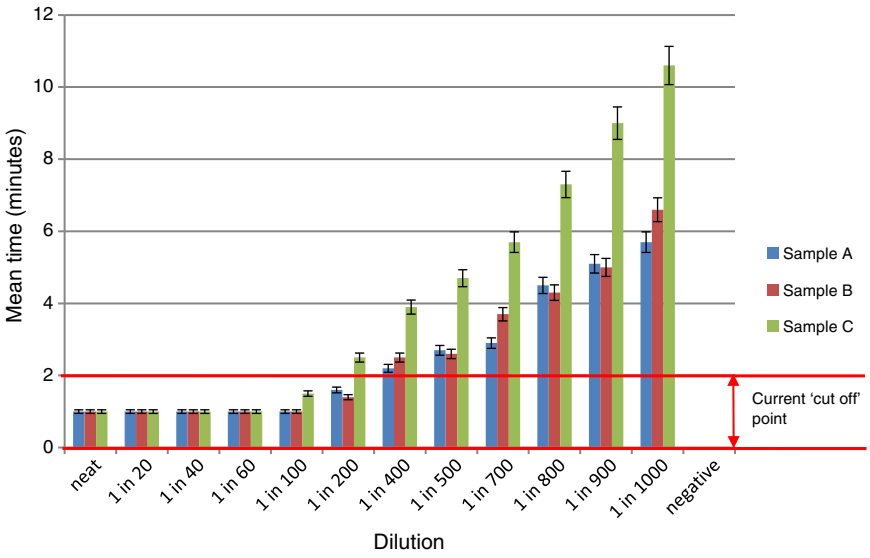

Fig. 1. The mean time taken for a positive acid phosphatase reaction using the direct method.

16 min for a 1 in 1000 dilution. The direct method also shows wide inter and intra-donor variation within the replicates. For a 1 in 800 semen dilution, for example, sample A produced reactions within a range of 2 to 7 min whereas sample C produced reactions within 5 to 10 min. It can clearly be seen, as would be expected, that as the semen becomes more dilute the length of time for a reaction to occur increases. All three samples were producing positive reactions in dilutions as high as 1 in 1000 when tested using the direct method, although samples A and B produced their reactions in a quicker mean time than sample C.

3.2. Press test method

Fig. 2 shows the mean time taken for a positive reaction to occur when the press test method was utilised to detect dilutions of semen. The mean time was calculated from the results of ten repeats of the test for each donor. These results show that semen dilutions up to 1 in 400 can be detected after a mean of 11 min. Furthermore, Table 2 shows the minimum and maximum times for a dilution to produce a positive reaction, if a reaction was obtained. The maximum time for a reaction to occur using the press test method was 16 min for a 1 in 400 dilution.

The press test method also shows wide inter and intra-donor variation within the replicates as was observed with the direct method. For a 1 in 60 semen dilution, for example, sample A produced reactions within a range of 1 to 7 min whereas sample C produced reactions within 4 to 12 min.

It can clearly be seen that as the semen becomes more dilute the length of time for a reaction to occur increases. All 3 samples were producing positive reactions in dilutions as high as 1 in 400 when tested using the press test method, although samples A and B produced their reactions in a quicker mean time than sample C (as per the direct tests). With the press test method reactions were observed up to a maximum dilution of 1 in 400 in one of the samples and 1 in 200 for the other two.

4. Discussion

The results of this research clearly show that the current two minute cut-off point utilised routinely for the acid phosphatase test is inadequate for detecting weak stains. Of the 36 total reactions observed in the direct test method (all dilutions of all samples from three donors), a total of 19 (57%) were observed after the 2 minute mark. In the case of the press test method, a total of 19 reactions were observed, 15 of

Table 2
Minimum and maximum times for a reaction to take place.

Minimum and maximum times for reaction to occur (min)												
	Press test						Direct					
	Sample A		Sample B		Sample C		Sample A		Sample B		Sample C	
Dilution	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Neat	1	1	1	1	1	1	1	1	1	1	1	1
1 in 20	1	2	1	4	2	6	1	1	1	1	1	1
1 in 40	1	5	2	7	2	10	1	1	1	1	1	1
1 in 60	1	7	4	9	4	12	1	1	1	1	1	1
1 in 100	2	9	6	12	7	13	1	1	1	1	1	2
1 in 200	5	12	N/R	12	N/R	14	1	2	1	2	2	3
1 in 400	11	16	N/R	N/R	N/R	N/R	2	3	1	4	2	6
1 in 500	N/R	N/R	N/R	N/R	N/R	N/R	2	4	1	4	3	6
1 in 700	N/R	N/R	N/R	N/R	N/R	N/R	2	4	2	5	5	8
1 in 800	N/R	N/R	N/R	N/R	N/R	N/R	2	7	3	7	5	10
1 in 900	N/R	N/R	N/R	N/R	N/R	N/R	3	8	3	7	6	15
1 in 1000	N/R	N/R	N/R	N/R	N/R	N/R	3	9	4	10	N/R	16
Negative	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R

N/R = no reaction obtained.

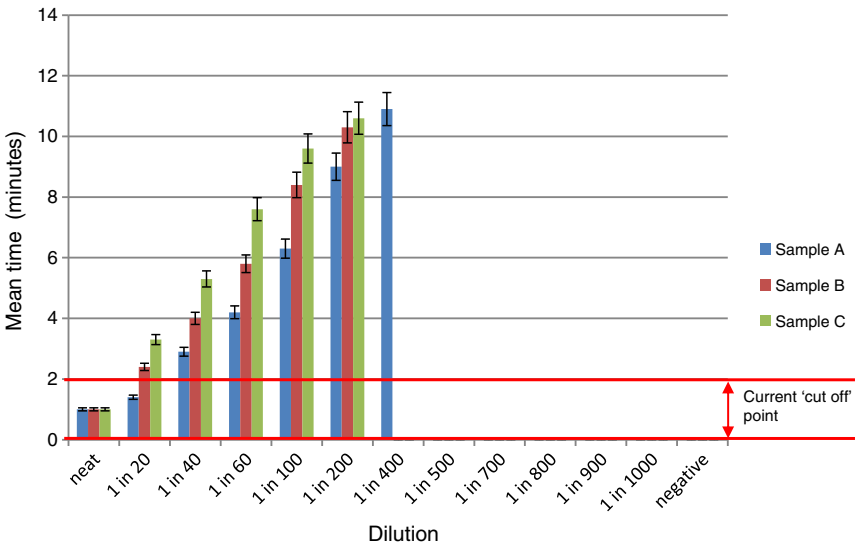

Fig. 2. The mean time taken for a positive acid phosphatase reaction using the press test method.

which were over the current two minute cut-off point. This equates to a total of 79%. The AP reactions obtained were recorded as being either strong, moderate or weak, and whilst all those occurring after the longer time intervals in excess of 10 min were weak, the colour of purple obtained was that usually associated with the presence of semen. This is a very important observation when considering the likelihood of obtaining false positive reactions with this method [5,9]. A number of substances including semen-free vaginal material, faeces and some foodstuffs are also known to produce reactions with this test on occasion, and this can sometimes, but not exclusively, result in a reaction which has a different colouration and/or reaction time to that frequently observed with semen. For this reason, it is important to remember that the AP test is only a presumptive test and the presence of semen must be established by confirming the presence of spermatozoa, or in the case of azoospermic semen, by conducting another confirmatory test such as the choline test [10–12]. Allard et al. [15] have also reported that it is possible to obtain DNA STR profiles using the SGMplus® (Second Generation Multiplex system) from swabs bearing dilutions of semen up to 1 in 10,000; partial (incomplete) profiles were obtained at this weaker dilution and full or almost complete profiles were generally observed to 1 in 1000 and 1 in 2000. Given that one of the primary purposes for detecting semen is with a view to obtaining DNA STR profiles to determine the source of this body fluid material, this clearly shows the need to be utilising a method which can detect stains previously unsuitable for analysis. It may also be beneficial to conduct a study into the comparison of DNA detected from semen recovered from both swabs and fabrics. This may help determine whether swabs or fabrics retain more recovered DNA and also if there is a difference in AP reaction times, therefore saving valuable time in the laboratory.

The results of this study also show that there is a considerable variation in the time for a reaction to occur between samples from different donors, and also within the replicated samples from the same donor. The concentration of acid phosphatase levels in the dilutions utilised in this research was assumed to be constant given that the same sample dilutions were used throughout the entire process and the replicated tests were conducted at the same time. It is unclear, therefore, what factor or factors are responsible for the observed variation in this study. Furthermore, it is known that whilst acid phosphatase levels remain relatively constant in an individual regardless of the frequency of ejaculations [16], that acid phosphatase levels do vary between male individuals. This, in addition to the variation observed during acid phosphatase testing conducted in this research, highlights the need to ensure that a testing regime is employed that caters for such differences.

Another observation from this study is the large difference in the detection ability between the two methods utilised (direct test and press test) suggesting that the currently used press test method has a lower than desired sensitivity. Dilutions of up to 1 in 1000 were detected with direct testing, but only a maximum of 1 in 400 when using the press test. However, the direct tests were conducted by placing dilutions of semen onto filter papers to simulate the methods utilised for direct testing or spot testing in forensic laboratories, whereas the press tests were conducted using stains on cotton fabric. To some extent this large difference would be expected given the nature of the test, but nevertheless, is worthy of further investigation. Some work to determine whether the make and type of filter paper used can affect the ability to detect semen has recently been reported by Lewis et al. [7] but the authors here believe that there is also value in trying to assess how the length of time the paper is in contact with the item is attributable to the reaction obtained.

5. Conclusion

This research was designed to challenge the two-minute cut-off time for detection of semen stains in forensic casework when using the acid phosphatase test. The results obtained provide clear evidence to demonstrate that diluted semen stains potentially capable of yielding DNA STR profiles can be detected well in excess of the standard two-minute cut-off time and up to as long as 16 min in this study. In addition, whilst it is common knowledge that concentrations of seminal acid phosphatase in men can vary, this study shows to what extent the variation exists in the three donors tested. These findings support those recently published by Lewis et al. [7] where AP reactions were assessed within a ten-minute cut-off, but suggest that recording results beyond this time may be also advantageous.

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