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Comparative study of the sensitivity and specificity of the zinc and acid phosphatase spot tests for the detection of seminal stains

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Summary. The sensitivity and specificity of a zinc spot test for the detection of semen were compared with those of an acid phosphatase detection method. As screening techniques both tests were found to be very sensitive, but the zinc test was more specific and was more reliable in older and especially in deteriorated specimens. It is concluded that the zinc spot test deserves at least the same place as the acid phosphatase test in the primary investigation of suspected semen stains and might well be the test of choice in older and poorly preserved stains.

Key words: Acid phosphatase spot test – Zinc spot test – Seminal stains

Zusammenfassung. Sensitivität und Spezifität eines Fleckentests zum Spermanachweis auf der Basis der Zinkkonzentration im Samen wurden mit den genannten Gütekriterien der sauren Phosphatasetechnik verglichen. Beide Tests waren als Suchtests geeignet, aber der Zinktest erwies sich als spezifischer und zuverlässiger für ältere und denaturierte Proben. Demnach kommt dem Zinktest als Sperma-Suchtest zumindest derselbe Stellenwert zu, wie der Phosphatase-Methode. Bei alten und schlecht erhaltenen Spuren sollte dem Zinktest der Vorzug gegeben werden.

Schlüsselwörter: Spermanachweis – Phosphatase-Methode – Zinktest, Spermasuchtest

Introduction

Absolute proof of the presence of semen in stains or on swabs can only be established by microscopical identification of spermatozoa. False-negative results, however, are common for a variety of reasons: the individuals involved may be

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naturally oligospermic or azoospermic or have previously undergone vasectomy, or natural degeneration of the spermatozoa may have taken place as a result of delay, poor sampling technique, or poor storage conditions. Forensic scientists have therefore looked for additional techniques to identify seminal stains. Visual identification is unreliable, and fluorescence under ultraviolet light can be biased, since many washing powders and artificial brighteners give rise a to a brilliant fluorescence that masks the pale fluorescence of semen (Kind 1964).

In 1935 Kutscher and Wohlbergs reported the high acid phosphatase activity of semen. This inspired leading scientists such as Lundquist (1946, 1950), Rasmussen (1945), Hansen (1946) and Riisfeldt (1946) to use this activity for the forensic identification of seminal stains. The value and high sensitivity of this method of primary searching for semen stains was clearly demonstrated by Walker (1950) and Kind (1958, 1964). Since then, numerous variants of the acid phosphatase test have been proposed (Gaensslen 1983), and these tests have become standard practice in most forensic laboratories, in addition to microscopical examination. Acid phosphatase however, is not present only in semen, but in all human and animal tissues and body fluids, in plants, bacteria and fungi, albeit mostly in much lower concentrations than in semen. The high sensitivity of modern acid phosphatase tests can therefore be a disadvantage, as it inevitably leads to a lower specificity and may cause false-positive results with, for example, vaginal acid phosphatase (Hauck and Leithoff 1959; Kind 1964; Walter and Höhn 1971; McCloskey et al. 1975; Gomez et al. 1974; Sensabaugh 1979; Tamaki et al. 1989). It is debatable whether this can be solved using quantitative tests. The cut-off points used in these tests have been the subject of long discussions and are both artificial and arbitrary (Kaye 1949; Fisher 1949; Hazen 1955; Shiff 1978; Gaensslen 1983). In addition, acid phosphatase has been shown to be both inhibited and stimulated by the chemical action of some deodorants and spermicidal products (Riisfeldt 1946; Lundquist 1950; Walther 1967; Brown and Brown 1974), and its activity declines rapidly if stains or swabs are not properly dried or are stored under poor conditions.

One of the more promising alternatives for the primary investigation of seminal stains is the high level of zinc in the prostatic fraction of seminal plasma (Mawson and Fischer 1953; Eliasson and Lindholmer 1971), which greatly exceeds the concentrations found in other tissues and body fluids. As zinc is a far more stable element than the organic substances of semen (e.g. acid phosphatase), a test based on this element might well be a more reliable seminal tracer, especially in older or contaminated stains. A simple and very straightforward zinc spot test was first proposed for forensic work by Suzuki in 1983, and his preliminary results suggested a high sensitivity and specificity. Most laboratories, however, continued to use the more familiar acid phosphatase tests, and the zinc spot test received little further attention. In this study the sensitivity and specificity of the acid phosphatase spot test are compared with those of the zinc spot test.

Materials and methods

Chemicals. First, 10 mg 1-(2-pyridylazo)-2-naphthol (Sigma, St. Louis, USA) was dissolved in 2 ml of Triton X-100 (Aldrich Chemicals, Brussels, Belgium) and mixed with 98 ml 0.5 *M* Tris solution [6 g tris(hydroxymethyl)-aminomethane (Merck, Darmstadt, FRG) in 100 ml distilled water] to obtain 100 ml working solution for the zinc test. Stock solutions used in the acid phos-

phatase test were (A) Michaelis Veronal-acetate buffer (9.75 g sodium acetate · $3H_2O$, 14.71 g sodium barbiturate, distilled water to give 500 ml), (B) 10 mg naphthol AS-BI in 1 ml *N*-*N*-dimethylformamide, and (C) 2 g *p*-rosaniline in 50 ml 2*N* HCl. All reagents were of the purest grade. From these stock solutions a working solution was made by mixing 15 ml A with 3 ml B and 39 ml distilled water, after which 2.4 ml C and 2.4 ml of a freshly made 4% aqueous sodium nitrite solution were added. This working solution was heated to 37°C in a waterbath and adjusted to pH 5.0 before use.

Methods. The in vitro sensitivity of both tests was evaluated using twofold dilution series of 1 ml human semen in physiological saline. Tests were performed in test tubes as well as by making stains from the dilutions on cotton cloth. Two drops of the test solutions were added and the colour reaction was read after 1 min. For stains, the test reagents were sprayed onto the cotton cloth. To assess the influence of contamination and deterioration of the semen on the sensitivity, the experiment was repeated on fresh semen dilutions and on dilutions stored for different time periods (1 day, 1 week, 2 weeks, 1 month, 2 months) under different conditions (−18°C, 4°C, room temperature and 37°C). The stains were kept in sealed petri dishes both in a dry and in a wet state. The latter storage method was used to facilitate deterioration, as it is known from daily forensic practice that wet stains deteriorate rapidly when packed in small non-ventilated containers.

The specificity of both tests was evaluated using air-dried stains and swabs of different body fluids (breastmilk, urine, blood, faeces, vaginal swabs from women after sexual abstinence for at least 3 days, nasal discharge, saliva, and sweat), fruit and plant extracts and beverages.

Results

Tables 1 and 2 give the results of the sensitivity analysis. For fresh semen samples and for frozen (−18°C) or refrigerated (4°C) samples, the sensitivity of the acid phosphatase test largely exceeded that of the zinc test. A clear and rapid breakdown in sensitivity of the acid phosphatase test was observed, however, if the semen dilutions had been kept at room temperature or at 37°C, or when the stains were not properly dried before storage at temperatures from 4°C to 37°C. The zinc spot test did not show this loss in sensitivity and the minimal detection titers remained stable throughout the study.

Table 3 gives the results of the specificity analysis. The zinc test was found to be more specific that the acid phosphatase test, which gave positive results, with

Table 1. Mean minimal detection titer for a clear result in twofold dilutions of semen stored under different conditions for different time periods. AP, Acid phosphatase test; Zn, zinc test

Storage conditions		Storage period					
		Fresh	1 day	1 week	2 weeks	1 month	2 months
−18°C	AP		512	256	512	256	256
	Zn		64	64	64	32	64
+4°C	AP		1024	1024	512	512	128
	Zn		64	64	32	64	32
Room temp.	AP	1024	64	2	–	–	–
	Zn	64	32	16	32	32	16
+37°C	AP		16	–	–	–	–
	Zn		32	32	32	16	16

Table 2. Mean minimal detection titer for a clear result in dried and wet stains made with two-fold dilutions of semen stored under different conditions for different time periods

Storage conditions			Storage period				
		Fresh	1 day	1 week	2 weeks	1 month	2 months
Dried							
−18°C	AP		512	512	256	256	256
	Zn		64	64	64	64	32
+4°C	AP		1024	512	512	256	256
	Zn		64	64	32	64	32
Room temp.	AP	1024	512	512	256	512	256
	Zn	64	64	64	64	32	64
+37°C	AP		256	256	128	128	128
	Zn		32	32	32	32	32
Wet							
−18°C	AP		128	256	128	128	128
	Zn		32	32	32	32	32
+4°C	AP		256	256	128	128	64
	Zn		32	64	32	32	32
Room temp.	AP	1024	128	1	–	–	–
	Zn	64	32	32	32	32	32
+37°C	AP		4	–	–	–	–
	Zn		16	32	32	32	32

some other stains (e.g. cauliflower, vaginal swabs, blood, serum, and blood-contaminated body fluids). Especially in the latter case, the acid phosphatase test was found to be unreliable, since a total of 9 of the 20 stains of body fluids contaminated with blood reacted positively although no semen was present.

Discussion

Although acid phosphatase tests have become standard practice in most forensic laboratories for the primary identification of seminal stains, no definite solution has yet been found for the problems of non-specificity and of the fragility of this enzyme. The discussion and the refinements of the technique nowadays seem to drift away from the original idea of a ready-to-use test for on-site application, because of elaborate and complex laboratory technology. In daily forensic work, however, there is still a need for a reliable and straightforward on-site test for the detection of seminal traces.

The results of the experiments described above confirm and enhance the initial results of Suzuki, who suggested that the high level of zinc in seminal plasma might mean zinc testing could be performed as an alternative for the primary detection of seminal stains. Although the sensitivity of the acid phosphatase test in fresh specimens far exceeded that of the zinc test, it declined rapidly in cases

Table 3. Number of stains reacting positively with AP or Zn

	N	AP	Zn
Urine	20	0	0
Blood	20	6	0
Serum	20	7	0
Sweat	20	0	0
Faeces	20	0	0
Breastmilk	20	0	0
Vaginal swabs	20	4	0
Nasal discharge	20	0	0
Blood-contaminated stains[a]	20	9	0
Cauliflower	10	2	0
Potato	10	0	0
Grapefruit	10	0	0
Apple	10	0	0
Pear	10	0	0
Peach	10	0	0
Banana	10	0	0
Cow's milk	10	0	0
Beer	10	0	0
White wine	10	0	0
Red wine	10	0	0
Coffee	10	0	0
Tea	10	0	0

[a] 10 vaginal swabs from menstruating women after sexual abstinence of at least 3 days' duration; 5 urine stains and 5 faecal stains contaminated with blood in the lab

when the semen had deteriorated. This result might have been expected on theoretical grounds, since anorganic elements are generally more stable than organic compounds. As there is no reason why the total zinc concentration in any specific sample should decrease with time, the differences found in the mean minimal detection titers for the zinc spot test should logically be the result of inter-sample variation. This was in fact the case in the experiments described above, as all the dilutions and stains with which the reaction was weaker showed this weaker reaction throughout, from the very beginning. It can therefore be concluded that the zinc test might well be the test of choice in poorly preserved and older stains.

In addition, it was shown that the zinc test was more specific than the acid phosphatase test. Using the zinc test, no positive results were found with other body fluids or other materials containing no semen. The acid phosphatase test, however, reacted positively to cauliflower extracts, vaginal swabs from women following sexual abstinence for at least 3 days, blood and serum, and was very unreliable on specimens contaminated with blood. These results suggest that the zinc test might not be the test of choice only for deteriorated stains, but that it might also be more reliable in general.

Finally, it was observed during the experiments that the zinc test solution could be stored at room temperature in dark bottles for more than 2 months. This, together with the fact that the test is very straightforward, makes it suitable for on-site application. Taking all this into account, it can be concluded that the zinc spot test deserves at least equal ranking with the acid phosphatase spot test in primary searching for seminal stains.

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