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TECHNICAL NOTE

Nadia Khaldi,[1] *M.D.; Alain Miras,*[1] *M.D.; Koffi Botti,*[1] *M.D.; Larbi Benali;*[1] *and Sophie Gromb,*[2] *M.D., Ph.D., J.D.*

Evaluation of Three Rapid Detection Methods for the Forensic Identification of Seminal Fluid in Rape Cases

ABSTRACT: We sought to discover whether spermatozoa concentration and the delay between ejaculation and test influence the results of seminal fluid fast detection tests. Two hundred and twenty-seven anonymous samples divided into four groups (normospermia, oligospermia, azoospermia, and controls) after a semen analysis were subjected to three fast detection semen tests: Diff-Quick® fast coloration, Phosphatesmo Km Paper® for acid phosphatases (AP) detection, and PSA-Check 1® for prostate specific antigen (PSA) detection. The study was performed at three time points (0, 48, and 72 h). Unlike cytology, results obtained with AP and PSA were not influenced by spermatozoa concentration. PSA detection results remained constant up to 72 h and were more reliable after 48 h than those obtained by AP detection.

KEYWORDS: forensic science, rape, forensic examination, seminal fluid, rapid membrane test, PSA, staining method

The number of forensic examinations concerning rape victims is constantly increasing. Since relatively few genital injuries are discovered at the medical examination (1–3), the detection of other marks such as the presence of semen is essential in order to confirm the presumption of sexual assault. For many years, cytological examination and the detection of acid phosphatases were considered to provide forensic evidence. More recently, easy-to-use, sensitive immunochromatographic assays for prostate specific antigen (PSA) detection have also been employed. Several studies have shown these new kits to be free of false negative results, but no study has compared them with other techniques or has evaluated their sensitivity according to delay after rape (4–10). Therefore, we sought to find whether spermatozoa concentration, on the one hand, and delay between ejaculation and the test, on the other, influence the results of seminal fluid fast tests.

Aim of Study

The aim of the study was to determine among the tests used at present which one allows the best detection of sperm. Another requirement was that the technique should be easy to use in emergencies. Evidence in the literature shows that PSA detection kits have better specificity than AP kits, so it was interesting to compare their performances with regard to the delay between ejaculation and test (4–11). Moreover, given the not inconsiderable number of oligospermatic and azoospermatic subjects in the general population, we sought to determine the characteristics of each of these techniques according to quality of sperm (12–14).

Materials and Method

This was a prospective analytical study performed blind in vitro on anonymous sperm samples collected at the in vitro fecundation laboratory at Pellegrin Hospital Bordeaux and having already undergone a spermocytogram. Mean age was 33 ± 6.75 years, median 32 years (24–59 years). At the end of the tests, we were informed only of sperm count. We studied 227 samples divided into four groups according to purely quantitative criteria. The first group comprised 108 normospermatic samples (48%). The second comprised 31 oligospermatic (14%) samples defined as the presence of less than 20 million spermatozoids per milliliter. The group included cases of severe oligospermia (less than 5 million spermatozoids per milliliter). The third group contained 37 azoospermatic samples (16%) including two from vasectomized patients, and the fourth comprised 51 controls composed of sterile water (22%). One hundred microliters of each sample were placed randomly in aliquots numbered from 1 to 227 and were frozen at -20°C until analysis.

Materials

We studied three techniques: the Diff-Quick® staining kit, the Phosphatesmo Km Paper® kit and the PSA-Check 1® kit. They were all compared with a reference spermogram. The Diff-Quick® staining kit (Dade Berhing Laboratories) is a rapid staining technique usually used on freshly taken capillary or venous blood samples. It uses a technique similar to the Pappenheim technique

[1] Forensic Science Laboratory, Pellegrin Hospital, University Teaching Hospital Bordeaux, 33076 Bordeaux Cedex, France.
[2] Professor of Forensic Medicine and Medical Law at the Medicine University, Bordeaux II, 146, Rue Léo-Saignat, 33076 Bordeaux Cedex, France, and Head of the Forensic Science Laboratory, Pellegrin Hospital, University Teaching Hospital Bordeaux, 33076 Bordeaux Cedex, France.

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(Giemsa-May-Grumwald). The smears are first air-dried and then dipped several times in staining solutions. They are then fixed and stained in only 15 s. The reagents used are eosin G in phosphate buffer at pH 6.6 (1.22 g/L) for solution I, a thiazine stain in a phosphate buffer at pH 6.6 (1.1 g/L) for solution II, and fast green in methanol (0.002 g/L) as fixative.

The Phosphatesmo KM Paper® kit is a rapid staining kit (Macherey–Nagel, GmbH&Co., Düren, Germany) for acid phosphatases using a rapid qualitative enzymatic staining procedure.

The PSA-Check 1® kit (VEDALAB, Alençon, France) detects prostate-specific antigen (PSA) and is used as a marker in the screening of prostatic adenocarcinoma. PSA is a glycoprotein synthesized by prostatic tissue and highly present in sperm (15,16). However, its spermatic concentration is very variable (0.2 mg/mL up to 1.55 mg/mL) so we diluted all the samples 200-fold with sterile water, as suggested by Hochmeister et al. and others (5,6,15–18).

Method

All samples were frozen at the same time. We then performed the three tests on every sample (T0). The samples were maintained in a heat chamber for 48 h and then underwent the three tests again (T48). Samples positive at T48 for PSA or AP were maintained for 24 h longer in the heat chamber, and then underwent another series of tests at T72.

The cytological study was performed with and without centrifugation of the samples with a Megafuge® 20.0 centrifuge (Heraeus Instruments), for five minutes at 1800 g. Suspension of the pellet required 100 μL and we used only 10 μL to spot each coverslip. Coverslips were examined under an Olympus BX 45® microscope at ×40 magnification.

To test the Phosphatesmo KM Paper® kit, we placed 100 μL of the dilution on each support. For the PSA-Check 1® kit, if the result was still negative at 1/200, we performed a dilution at 1/1000 in order to rule out the possibility of a hook effect due to a high initial concentration of PSA in the sperm examined, which might have given a false negative result. Each test required 200 μL of diluted sperm.

The kappa test was used to establish the concordance between the three tests. We used the McNemar khi^2 test for matched series to calculate significant differences between tests. A value of $p < 0.05$ was considered significant.

Results

There were 65% of positive cytologic tests and 33% of negative ones in a total of 176 sperm samples. Concordance with the spermogram was 74.4% ($\kappa = 0.47$), i.e., a fair concordance (Table 1). Sensitivity (Se) was 67%, specificity (Sp) was 100%, positive predictive value (PPV) was 100%, and negative predictive value (NPV) was 47% (Table 1). In the subgroup of normospermatic samples, there were 88.9% positive cytologic samples, whereas in the oligospermatic group there were only 51.6% of positives ($p < 0.05$) (Table 2). However, centrifugation did not modify the concordance between the cytological test and the spermogram (74.4% versus 74.4%) (Table 3). As expected, the sensitivity of the cytological test in the normospermatic group was better than in the oligospermatic group ($p < 0.05$). Finally, delay to test did not influence the cytological result since results at T0 were similar to those at T48 and T72.

The Phosphatesmo KM Paper® test was positive at T0 for 96% of sperm samples and for 1.9% of controls. Concordance with the spermogram was 96.5% ($\kappa = 0.9$), i.e., an excellent concordance. Se was 96%, Sp 100%, PPV 100%, and NPV 87.7%. As expected, there was no difference in Se between the subgroups, the sperm count not influencing AP detection (Table 4). At T48, the efficacy of the test decreased dramatically since PPV was 40.3%, Se 40.3%, and NPV 32.3%. Concordance was 53.3% ($\kappa = 0.22$), i.e., low concordance. At T72, the test was able to detect only 23.9% of the samples which had been positive at T48. Se was 26.8% for the normospermatic group, 33.33% for the oligospermatic subgroup, and 6.67% for azoospermatic samples. Concordance between the Phosphatesmo KM Paper® test and the spermogram was better at T0 (96.5%) than at T48 (53.3%) ($p < 0.05$), and was much better than at T72 ($p < 0.01$).

TABLE 1—*Results of different tests and agreement with the spermogram.*

	Positive Semen	Negative Semen	Agreement
Cytology +	118	0	74.5%
Cytology −	58	51	$\kappa = 0.47$
AP +	169	1	96.5%
AP −	7	50	$\kappa = 0.9$
PSA +	175	1	99.1%
PSA −	1	50	$\kappa = 0.97$
AP 48 +	71	1	53.3%
AP 48 −	105	50	$\kappa = 0.22$
PSA 48 +	169	5	94.7%
PSA 48 −	7	46	$\kappa = 0.85$
AP 72 +	17	0	25%
AP 72 −	54	0	
PSA 72 +	69	0	95.8%
PSA 72 −	2	0	

Cytology +/− positive/negative cytology.
AP +/− positive/negative phosphatase acid test.
AP 48 +/− positive/negative phosphatase acid test after 48 h.
AP 72 +/− positive/negative phosphatase acid test after 72 h.
PSA +/− positive/negative PSA test.
PSA 48 +/− positive/negative PSA test after 48 h.
PSA 72 +/− positive/negative PSA test after 72 h.
κ kappa, agreement.

TABLE 2—*Sensitivity of cytology with or without centrifugation and according to group.*

	Normospermic Group	Oligospermic Group
Centrifugation	90.7	38.7*
No centrifugation	88.9	51.6*

Table shows sensitivity of cytology (%) according to group.
Centrifugation does not increase cytology for spermatozoa detection.
The sensitivity of cytology is better for spermatozoa detection in normospermic group than in oligospermic group.
* $p < 0.05$ vs. normospermic group.

TABLE 3—*Parameters of tests.*

	Sensitivity	Specificity	PPV	NPV
Cytology	67.5	100	100	47
AP t 0	96	98	99.4	87.7
AP 48	40.3*	98	98.6	32.3*
PSA t 0	99.4	98	99.4	98
PSA t 48	96	90.2	97.1	86

Table shows parameters (%) of each test according delay.
AP t 0 phosphatase acid test at 0 h.
AP t 48 phosphatase acid test at 48 h.
PSA t 048 PSA test at 0 h.
PSA t 48 PSA test at 48 h.
* $p < 0.05$ vs. result at t 0.

TABLE 4—*Sensitivity of Phosphatesmo KM Paper test according to delay.*

	Normospermic Group	Oligospermic Group	Azoospermic Group
AP t 0	98.1	87.1	97.3
AP t 48	38*	48.4*	40.5*
AP t 72	26.8*	33.3*	6.7*

*$p < 0.01$ vs. results at t 0.
AP t 0 phosphatase acid test at 0 h.
AP t 48 phosphatase acid test at 48 h.
AP t 72 phosphatase acid test at 72 h.
At each time, results (%) of olgospermic and azoospermic group are not different from normospermic group.

TABLE 5—*Sensitivity of PSA-Check 1 test according to delay.*

	Normospermic Group	Oligospermic Group	Azoospermic Group
PSA t 0	99	100	100
PSA t 48	95.4	96.8	97.3
PSA t 72	97.6	93.3	100

PSA t 048 PSA test at 0 h.
PSA t 48 PSA test at 48 h.
PSA t 72 PSA test at 72 h.

Regarding the PSA-Check 1® test at T0, PPV was 99.4% in the sperm samples and 1.9% ($n = 1$) in the 51 controls. Concordance with the spermogram was 99.1% ($\kappa = 0.97$), i.e., an excellent concordance. Se was 99.4%, Sp 98%, PPV 99.4%, and NPV 98%. Sperm quantity did not influence the results. A T48, the test detected 96% of the sperm samples and 9.8% of the controls. Concordance was 94.7% ($\kappa = 0.85$), i.e., an excellent concordance. Se was 96%, Sp 90.2%, PPV 97.1%, and NPV 86.8% (Table 1). Se was not influenced by sperm quantity (Table 5). At T72, the test still detected 97.2% of samples positive at T48 with a concordance of 85.8% and Se was 97.6%. Concordance of the PSA-Check 1® test with the spermogram was better at T0 (99.1%) than at T48 (94.7%) ($p < 0.05$), but remained identical at T72 ($p =$ ns). Se and NPV were identical at T0 and T48 ($p =$ ns) while Sp and NPV decreased between T0 and T48 ($p < 0.05$).

Discussion

This study sought to investigate two parameters: delay before performing tests and the effect of sperm concentration. Apart from ease of use and rapidity of sample collection, this in vitro study controlled these two parameters as well as possible by avoiding sampling errors, variable delays before analysis, and environmental factors impossible to evaluate such as cell destruction and problems due to handling. For this reason, our sampling and analysis conditions do not exactly reflect real live sampling conditions. Our dilutions were based on previously published data showing that a 200-fold dilution probably corresponds to an in vivo dilution. Since previous studies had already evaluated various dilutions and had demonstrated that the detection threshold was very low with PSA tests, we did not consider it necessary to perform extremely weak dilutions but preferred a factor of 1/200 (5–10). Moreover, according to Hochmeister, the PSA concentration at this dilution does not induce a hook effect, which is responsible for false negatives (5). We used sterile water as a control population since vaginal secretions contain more than 80% water (19). Moreover, the same procedure was used in the studies by Hochmeister et al., Maher et al., and Sato et al. (5,9,20).

The cytologic test was not performed at the three time points (T0, T48, and T72) since sperm could not be lost in the heat chamber irrespective of the time point, contrary to what occurs in real life. This technique requires the intervention of a biologist and therefore cannot be easily performed in consultations, where the requirement is a rapid response. Nevertheless, since it is the method most widely used and because we sought to demonstrate its limitations in the case of oligospermia and azoospermia, it was essential to use it in our study. In order to approach real-life conditions, and if this technique is ever applied, we decided to use a rapid staining method. Hochmeister et al. mentions the possibility of working on a supernatant after centrifugation in order to detect PSA and AP, thus conserving the pellet for DNA investigation (5). Despite the apparent contradiction between simplifying the staining procedure, on the one hand, and increasing the duration of the technique, on the other, we preferred to centrifuge the samples in order to detect any eventual increase in sensitivity. We performed sperm detection after 72 h at 37°C only on samples found positive at T48 for both tests, since we considered that those which were negative at T48 would remain so at T72. Moreover, we only have a limited number of kits. In statistical terms, the specificity of PPV and NPV at T72 could therefore not be calculated because at that time point, there were only positive cases. On the other hand, there was a significant difference in sensitivity.

The age of the population studied was slightly higher in our study (24 to 59 years, median 32 years) than in the population of sexual aggressors of adult women investigated by Meurisse et al. (58% of sexual aggressors aged 18 to 30 years, mean 24 years) and Daligand et al. (75% between 16 and 40 years, mean 24 years) (21,22). However, the mean age in our study was between 30 and 40 years, corresponding to the age of all perpetrators of rape taken together. Since our population was obtained from in vitro fecundation, it was not exactly representative of the general population, with oligospermatic and azoospermatic subjects more highly represented. For example, there was 14% oligospermia and 16% azoospermia in our population, as opposed to a mean of 8% azoospermia (7.2 to 18%) in the study by Willot (14). However, it was interesting to study these subgroups that the population of perpetrators of rape probably also contains. Moreover, if we had studied the general population, the subgroups would have been insufficient in number to obtain significant results. In this respect, these subgroups may in part account for the low-powered results of certain clinical studies (23–25). In addition, in certain circumstances such as fever or high alcohol levels, men are known to produce a lower quantity of sperm (11,12), so they may temporarily form part of the oligospermatic subgroup.

Even with quick staining, sensitivity was good with cytology but decreased significantly in oligospermatic samples. This finding is not surprising and tends to demonstrate an as yet undescribed limiting factor in this technique. Moreover, as expected, cytology was unable to detect azoospermia, thus penalizing NPV, so it was not possible to establish the absence of sperm on the basis of a negative cytologic result. For this reason, this technique should not be used as a first intention technique, but may be used as a second intention if other tests are negative due to a loss of sensitivity due to too long a storage period. Unlike the other techniques, cytology remained sensitive for 72 h (26). Moreover, the results were no better after centrifugation. Indeed, the coverslips were more difficult to read and the spermatozoids were more difficult to identify. Even with the oligospermatic group, there was no significant difference, so centrifugation is of little use in the rapid search for sperm.

The Phosphatesmo KM Paper® test gave very good results at T0, but these were probably improved by the ideal conditions of the study. After 48 h at 37°C, the sensitivity and NPV of the test had decreased to the point that our negative result could no longer be considered reliable. Sperm count did not have any influence on the detection power of the test, so in this respect the test is better than cytology. On the other hand, delay before testing seems to be a really limiting factor with this technique and it can be used reliably only within the 24 h following rape. In 1985, Graves et al. (27) tested the presence of AP in vaginal samples according to delay after a sexual relationship; AP was detected only after a mean time of 14 h (27). Our results are in agreement with the literature since we detected AP in vivo only after approximately 18 h (see Dahlke et al. (4)) and there was a clear decrease in sensitivity as the time period increased (see Steinman et al. (28)). We therefore prefer to be cautious about using this test in isolation. Moreover, its cost, which is the same as that of the PSA-Check 1® kit, does not constitute a criterion for choosing it.

With regard to the PSA-Check 1® test, no hook effect was observed after 1000-fold dilution of tests which were negative at 200-fold dilution. The concentrations obtained were therefore not too high. The test gave excellent results both at T0 and T48 and were probably improved by our ideal in vitro conditions. However, all conditions being identical, there were differences compared with the other tests. Contrary to the cytological test, there was no significant difference according to the subgroups, nor was there any difference according to delay, contrary to the Phosphatesmo KM Paper® test. Agreement with the spermogram decreased significantly between T0 and T48, while it was identical to that obtained at T72. This is likely due to statistical errors caused by the low dispersion of the results. More samples would have been needed to increase the number of negative results. A lower NPV was observed at T48 but it was still greater than that obtained with the Phosphatesmo KM Paper® test. After 48 h at 37°C, the Phosphatesmo KM Paper® test became less reliable than the PSA-Check 1® test ($p < 0.001$). Unlike cytology, its great simplicity makes it usable in routine consultations, and the result may be read in the first two minutes. The presence of an internal control offers an added advantage compared with the colorimetric test, and it does not require any specialized skill. Moreover, its low cost makes it more accessible than the ELISA technique and as attractive as the Phosphatesmo KM Paper® test. Therefore, among the tests studied, the PSA-Check 1® test seems to be the best marker of the presence of semen, with constant satisfactory sensitivity over time. Moreover, in agreement with the literature, we found that it has very good specificity, thus making it an ideal marker (4–10).

Conclusion

An examination after rape requires the use of a reliable semen detection test that is sensitive and has very good negative predictive power. Previous studies have shown that cytology allows the detection of some but not all types of sperm up to 72 h whereas a search for acid phosphatases does detect all types of sperm but only in the first 24 h. Regarding the PSA detection kit, it allowed all types of semen to be detected up to 48 h (16). The commercialization of rapid detection kits would make the routine search for PSA more viable. There is an excellent concordance between the PSA-Check 1® kit and the spermogram, with very good negative predictive values even at 48 h. In our opinion, the PSA test is the best marker of the presence of semen and is well suited for use in emergency consultations. If necessary, a cytological test could be performed during the consultation if the PSA result were to be negative after 48 h.

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Additional information and reprint requests:
Sophie Gromb, M.D., Ph.D., J.D.
Head Departement of Forensic Medicine and Medical Law
Hôpital Pellegrin
Placa Amélie Raba Léon
33076 Bordeaux Cedex
France
E-mail: sophie.gromb@chu-bordeaux.fr