TECHNICAL NOTE

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Cytological Detection of Spermatozoa: Comparison of Three Staining Methods*

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ABSTRACT: Sperm detection can be an important factor in confirming sexual assault in cases of rape. This paper compares three of the most commonly used staining methods cited in the scientific literature: Christmas tree, hematoxylin-eosin, and alkaline fuchsin. The population studied was composed of 174 consenting women seen at the Male Infertility Center in Toulouse, France. The date of their last sexual intercourse was accurately known. Alkaline fuchsin did not seem effective in detecting spermatozoa in vaginal samples. Compared with hematoxylin-eosin, Christmas tree stain appeared to be the most useful test in the first 72 h. Two external factors were associated with decreased detection of spermatozoa: time since intercourse and sperm volume.

KEYWORDS: forensic science, cytology, sperm, spermatozoa detection

Rape is usually an unwitnessed crime and evidence of semen can play a crucial role in corroborating the victim's allegations (1). The medical examination provides the opportunity to collect physical evidence and biological samples to confirm the presence of sperm and convict an assailant (2–6). Swabs should be routinely taken as semen may be recovered in the genital tracts even if the victim is so emotionally traumatized that penetration or ejaculation by the assailant was not disclosed during questioning (7).

The presence of spermatozoa is the current criterion confirming sexual intercourse. This biological evidence is accepted both by the medical forensic teams, for whom cytology is a gold standard even if other methods are also used (1,5,8–14), and by the judicial authorities (15).

Hematoxylin-eosin is the cytological stain most commonly described in the scientific literature (2,3,5,6,16,17). Some teams have used the "Christmas tree" stain (nuclear fast red and picroindigocarmine) in forensic routine (7,18–20). Hooft has used alkaline fuchsin (10–14). The Papanicolaou stain has also been studied (21,22) but the results obtained by Randall (22) in volunteers showed that only 25% of smears from women having had sexual intercourse in the previous 24 h presented spermatozoa, which excludes use of this technique in forensic routine.

The aim of our study was to determine the best cytological stain for routine forensic detection of spermatozoa by comparing the three most commonly used stains: hematoxylin-eosin, Christmas tree, and alkaline fuchsin.

Materials and Methods

Cytology

We studied 174 cervicovaginal samples from consenting women seen at the Male Infertility Center of the University Hospital of Toulouse, France, for in vitro or postcoital tests from November 1997 to July 1998. Mean age of the men was 32 years (range 23–48) and of the women 30 years (range 21–40). The date of the last sexual intercourse for each patient and time of sampling were noted to evaluate the interval between ejaculation and swab taking.

Three glass slides were prepared from each swab. They were air dried, fixed in alcohol and ether, and stained with either hematoxylin-eosin, nuclear fast red and picroindigocarmine (Christmas tree stain), or alkaline fuchsin. All slides were screened microscopically with a magnification X40 on 100 microscopic fields and the mean number of spermatozoa per field was counted.

In addition to the swabs, characteristics of the sperm (volume, spermatozoa count) of the partner were noted during a previous outpatient visit and the gynecologist observed the characteristics of the cervicovaginal secretions (cervical dilatation, quantity and quality of mucus) after pelvic speculum examination, using a three-grade classification system.

Statistical Analysis

The slides prepared with the three cytological stains were read blind by the authors. Statistical analysis was done using SAS software and the threshold of 5% was considered significant. Counts were compared using the chi-square test. Means were compared using a t-test for matched samples. A logit model was produced to analyze the results of the three stains according to time since intercourse and male and female factors.

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Results

Hematoxylin-eosin detected spermatozoa in 34.7% of samples, Christmas tree in 35.1%, and alkaline fuchsin in 28.4% (Fig. 1). The difference between alkaline fuchsin and the other stains was statistically significant, however, hematoxylin-eosin and Christmas tree did not differ significantly. Statistical analysis ruled out alkaline fuchsin as a reliable method and the results shown in Fig. 2 confirmed that alkaline fuchsin could not be considered a gold standard: when the two other stains were negative, alkaline fuchsin did not detect any more spermatozoa.

Particular attention was paid to the number of spermatozoa detected in each microscopic field according to time since intercourse. Christmas tree stain gave significantly higher results (8.3) than hematoxylin-eosin (4.6 with $t = 2.33$; $p = 0.023$) and alkaline fuchsin (4.2 with $t = 2.47$; $p = 0.017$) (Fig. 3). Christmas tree stain seemed markedly easier to read and this could be reflected in the results.

The number of spermatozoa detected was analyzed at three different time intervals since intercourse (Fig. 4). We observed that: (i) no spermatozoa were detected after 72 h with any stain; (ii) during the first 72 h, there was no significant difference between the two most efficient tests: 15.1 spermatozoa per microscopic field for hematoxylin-eosin and 14.1 for Christmas tree ($t = 0.23$; $p = 0.636$) in the first 12 h; 9.1 for Christmas tree compared with 2.6 for hematoxylin-eosin ($t = 1.49$; $p = 0.234$) between 12 and 72 h.

Factors which could theoretically affect spermatozoa detection on swabs taken at a maximum of three days after intercourse were studied: two male factors (sperm volume and spermatozoa count), three female factors (cervical dilatation, quantity, and quality of mucus), and time since intercourse. None of the women had douched since intercourse. The period of three days was chosen because in our study no spermatozoa were detected after this time. Christmas tree stain was chosen because of its significantly better results compared with the other two stains.

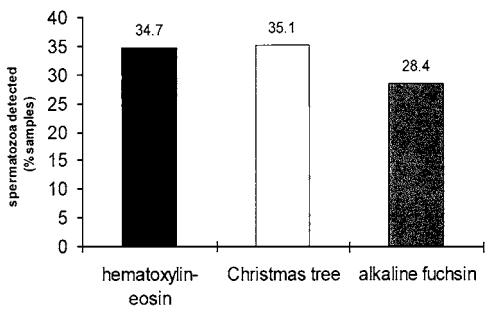

FIG. 1—*Detection of spermatozoa according to stain.*

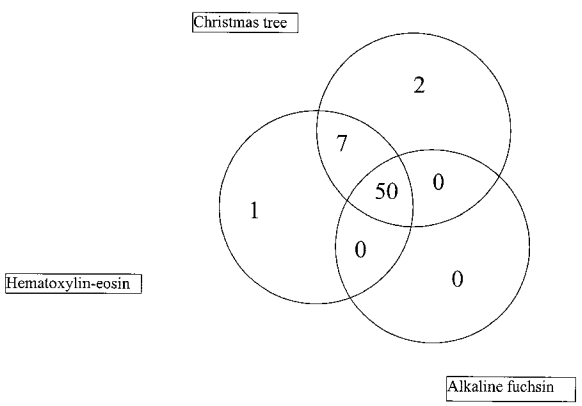

FIG. 2—*Number of spermatozoa detected by one, two, or three staining methods.*

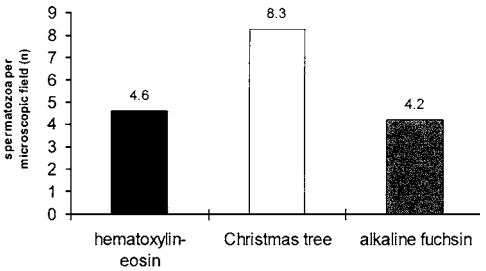

FIG. 3—*Number of spermatozoa per microscopic field according to staining method.*

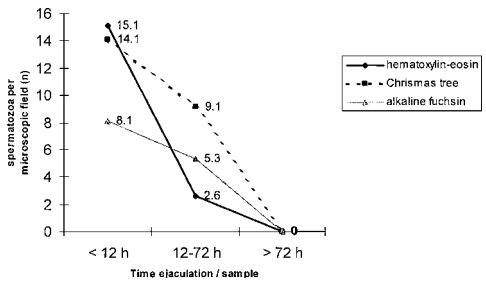

FIG. 4—*Number of spermatozoa detected with the three stains according to time between ejaculation and sample collection.*

Using stepwise logistic regression taking these factors into account, only two factors appeared to be associated with decreased detection of spermatozoa. The first was increased length of time since intercourse and the second was the partner's sperm volume (the greater the volume, the more spermatozoa detected: mean 42.7 million/mL, range 0.9–214) making it possible to classify 99.1% of the swabs studied ($p < 5\%$).

Discussion

The aim of this study was to guide the forensic physician in the choice of the best cytological method to detect spermatozoa. As far as we are aware, there has been no previous comparison of the sensitivity of cytological stains in spermatozoa detection nor any evaluation of their effectiveness according to time after intercourse.

Davies (3) found that vaginal samples from volunteers showed spermatozoa up to the 30th hour after sexual intercourse. The frequency of negative results (absence of spermatozoa) was 1% between 24 and 36 h, 16% between 36 and 48 h, 33% between 48 and 72 h, and 50% between 72 and 96 h (3). Spermatozoa may be present up to 144 h after sexual intercourse (5). Willott (5) detected no spermatozoa in a large proportion of swabs within 24 h after intercourse in rape victims, the longest time after intercourse being three days.

Christmas tree stain seemed to be equivalent to hematoxylin-eosin whereas alkaline fuchsin appeared to be ineffective in detecting spermatozoa. Closer comparison of Christmas tree and hematoxylin-eosin led us to conclude that these stains were of similar value in detecting spermatozoa, whatever the interval between intercourse and medical examination. Christmas tree had the advan-

tage of detecting more spermatozoa on each slide studied and was probably the easiest to read, making working conditions better for the technician.

Study of various factors affecting spermatozoa detection showed that time since intercourse is of major importance. During the time between intercourse and swab collection, an association of female factors and biological degradation of male cells in the vagina come into play. Sperm volume in an individual can vary depending on frequency of intercourse and other factors. Spermatozoa count was not a contributory factor, but it should be noted that none of our volunteers had azoospermia.

Conclusion

Compared with alkaline fuchsin, Christmas tree and hematoxylin-eosin stains appeared to be gold standard cytological methods for detecting spermatozoa. Their level of detection seemed similar, although Christmas tree stain had the advantage of being easier to read. One major factor influencing spermatozoa detection was the interval between ejaculation and swab collection. In our group of in-vivo volunteers, no spermatozoa were found after three days. The second major factor was sperm volume. We had expected the spermatozoa count to be important, but in our population without azoospermia this factor did not appear to be significant.

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