Improved viability and acrosome staining to frozen-thawed semen samples - technical note

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Abstract
The viability and acrosome staining by trypan blue – Giemsa, or Chicago sky blue – Giemsa was improved and also the time of the procedure was shortened by elevated temperature. The quicker Giemsa staining at 50°C reduced the disturbing background caused by the freezing extenders. The mounting medium containing methyl-yellow improved the differentiation of the acrosome and tail viability status.

Keywords: acrosome, frozen/thawed semen, live-dead staining,

1. Introduction

There is an increasing demand to establish ex situ in vitro gene banks for preservation of farm animal genetic resources, the most commonly used method is freezing semen samples. Quality control of frozen thawed semen consists of motility assessment and live/dead evaluation of spermatozoa. The trypan blue – Giemsa, or Chicago sky blue – Giemsa staining gives important information about the status of spermatozoa of different mammalian species after different treatments. There are some problems with frozen - thawed samples making the evaluation difficult, like disturbing background caused by extender components, and the weak acrosome staining of boar spermatozoa. Authors present an improved staining method to determine live/dead and acrosomal status of frozen/thawed semen samples in different species.

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2. Materials and methods

Both combined staining are routinely applied in our laboratories using a yellow filter helping the better differentiation of the acrosome status. Preparations from frozen – thawed ram (Black Racka, Barbados Black Belly) and boar (Blond Mangalica) semen samples were mounted with immersion oil (Merck 4699) saturated with 0.2% dimethyl yellow (Sigma D6760) and centrifuged or filtered with a 0.2 micron acrodisc filter. The overnight staining was shortened to 3-4 hours by elevated temperature to 50°C and comparing different Giemsa solutions.

The staining procedure:

Dilute the sample with isotonic PBS (0.825% NaCl + 0.06% K_2HPO_4, anh., pH: ~6.8) 5-10x.

The viability testing stain consists of 0.16, 0.2, or 0.24% Chicago sky blue 6B (Sigma C-

8679; 2.4% stock solution diluted with PBS) kept in 15 mL dropper bottles at room-temperature.

The fixative composed of 86 mL 1N HCl plus 14 mL 37% formaldehyde solution and 0.2 g neutral red (Sigma N-2880) is in a staining jar at room-temperature and used repeatedly.
The acrosome stain is a 7.5% Giemsa stock solution prepared freshly before use in a staining jar.

Staining procedure:

1. Put one drop of Chicago sky blue and one drop of diluted semen onto the middle of a slide, mix with the edge of another slide, and make two smears by attaching and pulling them parallel.
2. Air drying in near vertical position on adsorbent paper at room temperature.
3. Fixing in a jar for 4 min.
4. Rinsing with tap, and distilled water.
5. Staining in Giemsa in uncovered jars.
6. Rinsing with tap, and distilled water.
7. Differentiation in distilled water for 2 min.
8. Air drying.
9. Mounting and cover slipping.
10. Evaluation with 40x dry, or 100x oil immersion objectives.

Classification:

For live/dead assessment the posterior part of the head and the tail, for the acrosome status the anterior part of the head provides information.

Heads:

live	white - light pink
dead	black - dark violet - grey

Tails:

live	pink
dead	black - dark violet

Acrosomes:

intact	purple
loose	dark lavender
damaged	pale lavender
no	white - light pink (live heads) white - pale grey (dead heads)

Postacrosomal ring: red

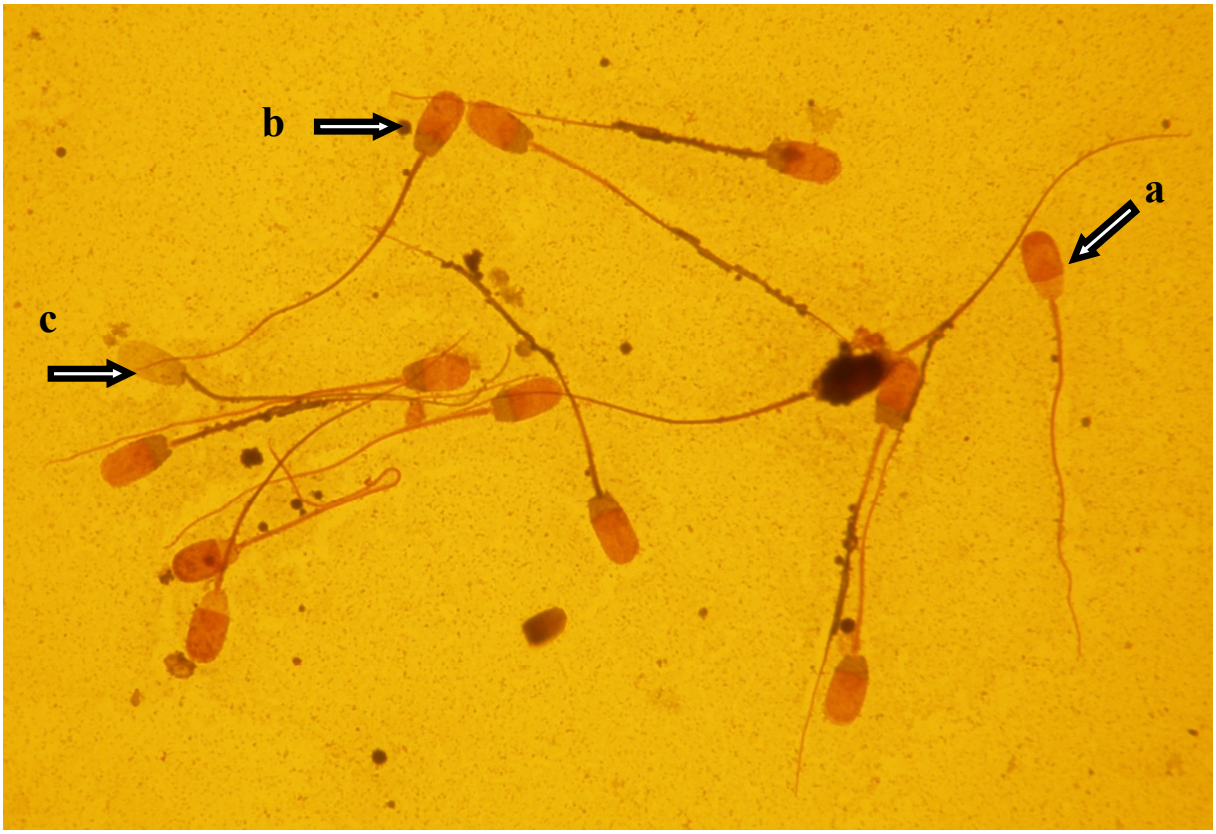
The staining was successful on all Mammalian animal spermatozoa, we tried (bull, yak, water buffalo, ram, goat, red deer, fallow deer, boar, stallion, dog, cat, rabbit, mouse, white rhinoceros, and two-toed sloth etc.).

3. Results and discussion

Dimethyl yellow solved in immersion oil resulted in not only a good differentiation of the acrosome status, but also a better distinguishing of membrane permeable „dead" and impermeable „live" tail domains of spermatozoa even in the case of background caused by the extender. The effect can be explained by the relief-like nature of the smears - the yellow mounting medium is thicker around the cells than above them. The yellow surroundings of the cells are lighter than the colored „dead", but darker than the unstained „live" sperm tails ensuring their easy and clear differentiation. The tail membrane of spermatozoa is more sensitive to the harmful effects of the freezing-thawing procedure than their head membrane, therefore its correct evaluation is extremely important for the quality control. Ram spermatozoa were well stained within 4 hours at 50^{o}C by the Giemsa (GS-500, Sigma) traditionally used by us, however the

acrosome staining of boar spermatozoa was too weak even after prolonged times. We got the best results at 50°C by freshly made 7.5% working solution in 0.1 borax (anh.) of the „Azur eosin methylene-blue solution according to Giemsa" (Riedel-de Haen, 32884 – also distributed by Sigma-Aldrich) after 3-hours staining for ram, and after 4 hours for boar spermatozoa (Figure 1.).

Figure 1. Improved live/dead staining of frozen/thawed Mangalica boar semen



a. live head and tail, intact acrosome; **b,** dead head and tail; **c,** dead tail, no acrosome

4. Conclusions

The staining quality was improved and its time was shortened by using the Riedel-de Haen Giemsa and using dimethyl yellow counterstain. Percentage of live spermatozoa with no morphological aberrations is a practical index of semen quality.

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