ABSTRACT

A semiautomated computerized system analyzed 35-mm negatives obtained from dark-field microscopy and determined velocity of each sperm and percentage of motile sperm in the sample. Precision and accuracy of the system for percentage of motile sperm were evaluated in comparisons with track motility and videomicroscopy. Motility was evaluated at four times during processing for cryopreservation or after thawing. Based on the 95% confidence intervals for percentage of motile sperm, computer and track motility evaluations were more precise than videomicroscopy. Computer and track determinations of the percentage of motile sperm were correlated with the ratio of live to killed sperm in prepared mixtures. The computer system was reasonably precise and accurate. Correlations of spermatozoal velocity or motility with fertility were studied. The competitive fertility index, based on semen from nine bulls, was correlated with the computer-determined percentage of motile sperm at 0 to 1.5 h after thawing, velocity, or the combination of motile sperm and velocity. Such a computerized system may aid in prediction of fertility.

INTRODUCTION

Visual estimation of the percentage of motile sperm, or their velocity, is the most common method of evaluating spermatozoal quality. Unfortunately, such estimates are neither repeatable (4) nor reliable in predicting fertility (9, 17). A simple technique to evaluate objectively spermatozoal motility was a goal when Rothschild (16) reported a new method, termed track motility, based on recording a 1-s time exposure, on photographic film using dark-field illumination. Motile sperm left a track whereas immotile sperm gave a sharp single image. Projection of 35-mm negatives onto white paper enabled measurement of the length of tracks to provide information on spermatozoal velocity as well as percentage of motile cells (6). Use of a chamber 20-μm deep and containing 7 to 10 × 10⁶ sperm/ml in filtered egg yolk-citrate extender was satisfactory for bull sperm that had been frozen, then thawed (3), and six fields (photomicrographs) per sample were prepared using a 2-s exposure and 180× magnification. Similar procedures to evaluate motility of bull sperm have been used (7, 10, 13, 15, 17).

Makler (11) introduced the concept of using, spaced over 1 s, a series of six short light pulses (.02 s) rather than a time exposure, to provide a chain-like track. These sperm tracks were measured manually, and velocity of each sperm was calculated. Makler (12) also developed a digitizer-computer system to measure the velocity of motile sperm. Each negative was projected onto white paper and the track traced with a primitive electric pencil. Only 5 to 8 min were required to obtain the percentage of motile sperm and average velocity of motile sperm from a population of 200 to 400 sperm, whereas manually measuring sperm tracks required 15 to 20 min.

Track motility estimated precisely the percentage of motile bovine sperm (4) and was
correlated with fertility or relative fertility of bull sperm (9, 13, 14, 17). Measurement of the percentage of motile sperm by track motility requires only a microscope and an inexpensive camera. However, it is tedious to evaluate the negatives, and the distance sperm move (velocity) typically is not measured to reduce labor cost. However, velocity data may be important for predicting fertility (19). Therefore, we developed a semiautomated, microcomputer-photographic method for evaluating track motility, which economically provides information on percentage of motile sperm and spermatozoal velocity. The objective was to determine whether the resulting data correlated with fertility of bull sperm.

**MATERIALS AND METHODS**

These studies utilized cryopreserved bull semen prepared by conventional procedures using egg yolk-based extenders (5). After thawing, sperm were further diluted with egg yolk-citrate or egg yolk-Tris extender, which had been filtered through a .8-μm pore membrane filter. Use of filtered extender for postthaw dilution eliminated large egg yolk particles that might interfere with interpretation of negatives. An aliquot of 14 μl of diluted semen (10 x 10^6 sperm/ml) was placed on top of a prewarmed slide (37°C), and a 20 x 26 mm coverslip was placed over the semen. The dark-field microscope was equipped with a thermostage set at 37°C and a 10x objective. Negatives were prepared using a 1-s time exposure and Kodak Plus X film (13). Six fields at predetermined sites on each of three slides (see below) were photographed for each sample.

Special slides were fabricated (Rocky Mountain Microscope Co., Ft. Collins, CO) to ensure that all sperm were in focus during photography. By vacuum deposition of tungsten, a series of parallel lines 10 mm long and approximately 200 μm apart, were placed on a 75 x 25 mm glass slide to run parallel with the long axis. Similar lines were deposited perpendicular to the long axis of a 20 x 26 mm coverslip. When the coverglass was placed (lines down) on top of 14 μl of extended semen on the slide (lines up), the slide and coverglass formed a grid. If the grid was in focus, all sperm were in focus. The special slides were effective in ensuring that all sperm remained in focus, but the parallel lines were too closely spaced, too wide (about 13 μm), and concealed some of the sperm. Therefore, all photomicrographs were taken just outside of the grid to circumvent this problem, but the preselected fields all were >6 mm from the edge of the coverglass.

**Microcomputer-Photographic System**

This system for evaluating spermatozoal motion relies on a human to discriminate sperm from debris but on the computer to distinguish immotile and motile sperm or calculate spermatozoal velocity. Thus, it could conveniently and objectively provide information about motility and velocity. The location or path of ~60 sperm (record) per track negative can be digitized and data for several negatives representing one sample can be merged. The system for these studies included a homemade rear projection screen (37 x 39 cm), 35-mm film projector, Grafbar GP-7 sonic digitizer (Science Accessories Corporation), a Zenith ZW-111-32 microcomputer with a Matrox ALT-512 graphics board, two Zenith Z-123 monitors, and a Hewlett-Packard 2225C ThinkJet printer (Figure 1). The sonic digitizer fed locational information to the computer.

The software is written in Fortran with some subroutines for data formatting, for interactions between the digitizer and computer, written in assembler language. A microcomputer with a S-100 buss, 64K of RAM, and two disk drives is required. The software consists of seven independent programs (Figures 2 and 3) individually called from a menu program. All options are displayed to the user for data input, updating, analyzing, output, and maintenance of files. At the beginning of a session, the user is presented with a menu of options (upper part of Figure 2). After initializing the data storage disk (program 1, Figure 2), program 3 (Figure 3) is selected. A screen display requesting 10 parameters is seen. The user then enters: sample identification number (1 to 999), slide number (1 to 9), field of view (1 to 9), date, duration of exposure used in

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2 Copies of the software "Sperm Motility" are available from R. H. Hammerstedt, but this software will run only on Zenith ZW-100 series computers equipped with a Matrox ALT-512 graphic board.

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Negatives were projected from below onto the lower surface of a horizontal, opaque glass screen. To locate each sperm in the projected image, the stylus of the sonic digitizer was touched to the image of an immotile sperm or used to trace the path left by a motile sperm. Because the digitizer was connected to the computer, the computer could detect immotile sperm (start and stop point essentially equal) or motile sperm (stop point not identical to starting coordinates; velocity > v micrometers per second). The locational coordinates of each spermatozoon were used to calculate the shortest distance from the beginning to the end of each sperm track, and this value was converted to straight line velocity by taking into consideration the exposure duration and magnification of the projected image. The operator controlled the threshold used to set the minimum velocity required to consider a spermatozoon as motile. Based on data from 10 samples studied in a preliminary experiment, a velocity of >20 μm/s was used as the threshold for motile sperm.

**Systems for Videomicrography and Track Motility**

Videomicrography (8) is a relatively inexpensive method to obtain objective data on motility and provides information without delay, but it is time-consuming, even though velocity data are not obtained. The system for videomicrography included a Leitz Dialux 20 microscope equipped with a thermostage (37°C), a 16× phase-contrast objective and 1.25× tube lens but without an ocular; an ITC-48 Ikegami video camera; VTG-33 FORA time generator; a JVC BR-6400U VHS recorder; and a Panasonic 1920 video monitor. For videomicrography, at each time of evaluation, 10 predetermined fields on each of three slides were recorded for 10 to 15 s. Spermatozoal motility later was determined (8) by replaying the videotape. For each scene (field), the videotape was “paused” and a wax pencil was used to mark the location of the neck of one sperm that was closest to each of 12 dots spaced uniformly (about 7 cm apart) on the video monitor. The videotape then was advanced .50 s. A sperm head that moved away from the wax pencil mark was considered motile, and one that stayed under the mark was considered immotile. There was little ambiguity, since a sperm head remained under...
Figure 2. The menu of seven programs constituting the Sperm Motility software package (upper) and the flow diagrams for programs #1 and #2 used to initialize the system and format the disk for data storage or to delete data for a particular field of view (record).

For track motility, six predetermined fields on each of three slides were photographed using Kodak Plus X film and a 1-s time exposure. The film was developed and the negative was projected onto a white poster board, 45 x 50 cm. Sperm that appeared as tracks were considered motile whereas a spermatozoal head with a sharp outline was considered immotile (velocity probably <15 μm/s). The numbers of motile and immotile sperm per field were recorded.

Experiment 1, Precision

To evaluate precision of the microcomputer-photographic system and whether it was better or worse than other methods, spermatozoal motility was determined using three ap-

the pencil mark or moved away a distance at least equal to the length of a sperm head; velocity >16 μm/s. Numbers of motile and immotile sperm in each field were recorded and later entered into a minicomputer.

Track motility requires inexpensive equipment and has been used in many laboratories, but it is labor intensive and films must be processed before objective evaluation is possible. The system used to prepare negatives for track motility or computer analysis included an Olympus BH-2 microscope equipped with a Rocky Mountain Microscope Corp. (Fort Collins, CO) thermostage set at 37°C, dark-field illumination, a 10x objective, a 3.3x photoocular, and an Olympus C-35 35-mm camera.

For track motility, six predetermined fields on each of three slides were photographed using Kodak Plus X film and a 1-s time exposure. The film was developed and the negative was projected onto a white poster board, 45 x 50 cm. Sperm that appeared as tracks were considered motile whereas a spermatozoal head with a sharp outline was considered immotile (velocity probably <15 μm/s). The numbers of motile and immotile sperm per field were recorded.

Experiment 1, Precision

To evaluate precision of the microcomputer-photographic system and whether it was better or worse than other methods, spermatozoal motility was determined using three ap-
Figure 3. Flow diagrams for programs #3 and #4, of the Sperm Motility software package, which are used to enter data (or display/modify data) for one field of view (record) and to summarize and analyze data for a number of fields of view representing a given sample or series of samples. For brevity, programs #5, #6, and #7 are not described.

approaches: videomicrography (8), track motility (3), and the new system. Semen representing one ejaculate from each of three bulls was processed using two extenders [egg yolk-citrate (EYC) and egg yolk-Tris (EYT)]. To minimize particulate matter, both the A (no glycerol) and B (12% glycerol) fractions of the EYC or EYT extenders were allowed to sediment overnight at 5°C and the supernatant was decanted and filtered through an .8-μm pore membrane filter before use.

Semen in both extenders was evaluated by all three systems at four time intervals. Times of evaluation were: 1) immediately after extension in the A-fraction (no glycerol), 2) after cooling to 5°C and 60 to 75 min after addition of the last aliquot of B-fraction (with glycerol) extender, 3) after 2 to 17 min of postthaw incubation at 37°C, and 4) after 173 to 188 min of postthaw incubation at 37°C. Semen was diluted to 10 × 10^6 sperm/ml at the time of evaluation. The same samples were used to prepare concurrently video tape and 35-mm negatives, but three different slides were used for each method. An interval of 12 to 15 min was required to record each of six fields on each of three slides. The same negatives used for track motility were analyzed with the microcomputer-photographic system.

To compare the relative variability in percentage of motile sperm associated with fields and slides for sperm in each extender, at each time of evaluation, a nested analysis of variance was performed with fields nested within slides and slides nested within bulls. Because fields were independent of one another, slides were
used as the error term in further analyses. A repeated measure analysis of variance was performed for percentage of motile sperm at each time to evaluate effects of bull, extender, and evaluation method. The 95% confidence intervals were calculated for slides and fields to obtain estimates of precision using each of the three systems.

Experiment 2, Accuracy

To study accuracy of the microcomputer-photographic system, known mixtures of live and killed bull sperm were evaluated. For each of four bulls, samples of sperm that had been frozen, then thawed were mixed with killed sperm at ratios of 4:0, 3:1, 2:2, 1:3, and 0:4. A different straw of live sperm was used for each mixture for a given bull. Both killed and frozen then thawed semen had been extended at 8 x 10^6 sperm/ml in filtered EYC extender. Six fields on each of three slides were photographed for each mixture. All negatives were evaluated by the computer system and track motility served as a control. Percentage of motile sperm in each mixture from a given bull was expressed as a proportion of the value for the 4:0 mixture. The resultant data were used to calculate the correlation of spermatozoal motility and the proportion of killed sperm in the mixture.

Experiment 3, Correlation with Fertility

To evaluate relative merit of the microcomputer-photographic and track motility methods, the percentage of motile sperm and spermatozoal velocity, or track motility, were determined using representative straws of cryopreserved bull sperm previously used in a fertility trial (17). This experiment had a one-factor design (interval of incubation of sperm after thawing) and the data reported are based on 9 bulls (replicates).

Although the fertility experiment had been designed using 10 beef bulls, data for 1 bull were deleted because too few offspring were produced (17). To enable calculation of a "competitive fertility index," especially prepared cryopreserved semen was thawed at 35°C, and equal numbers of sperm from 2 bulls were mixed to provide 25 combinations of mixed semen. The resultant mixtures of 30 x 10^6 sperm/insemination dose were used to inseminate 785 beef cows. Based on the sire of each calf born (n=229 for 9 bulls), as established by phenotypic markers and bloodtyping, the competitive fertility index was calculated (17). This index ranked the 9 bulls on the basis of their competitive fertility with values ranging from -45.1 to 24.5. The competitive fertility index is a ranking based on relative fertility and not an estimate of fertility, although the two are correlated (1).

For each of the nine seminal samples, one straw was thawed and the contents were diluted 1:7.5 with filtered (.2 µm) EYC extender to provide 8 x 10^6 sperm/ml. At 0 and 1.5 h of incubation at 37°C after thawing, six fields/slide on each of three slides were photographed. Later, the negatives were analyzed by the semi-automated computer system and track motility methods. For each characteristic, a two-way analysis of variance was performed with interactions, time fixed (0 to 1.5 h incubation), and samples random (n=9). Sample means for percentage of motile sperm and velocity of motile sperm were correlated with the competitive fertility index.

RESULTS

Experiment 1

Variance in the percentage of motile sperm associated with slides, fields within slides, and bulls was significant (P<.01) at each time of evaluation for each method. At the initial extension and prefreeze evaluations, there was an effect (P<.01) of method of evaluation on percentage of motile sperm (Table 1); values were highest for the video system. Values determined by track motility or computer were inexplicably low for prefreeze observations. At 3 h, but not 0 h postthaw, the video system resulted in a higher percentage of motile sperm than the track or computer system. At 0 and 3 h postthaw, there were effects (P<.05) of extenders on the percentage of motile sperm.

The 95% confidence interval for the percentage of motile sperm was calculated for each method using data for one, two, or three slides with EYC and EYT extenders at 0 h postthaw (Figure 4). Variation decreased by 29 and 42% when two or three slides were evaluated rather than one slide (about 81, 370, and 270 sperm/slide were evaluated by video, track motility,
TABLE 1. Percentage of motile sperm in Experiment 1.1

<table>
<thead>
<tr>
<th>Evaluation at</th>
<th>Method VCR</th>
<th>TRACK</th>
<th>COMP</th>
<th>Extender EYC</th>
<th>EYT</th>
<th>Pooled SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial extension</td>
<td>80.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74.6</td>
<td>76.1</td>
<td>.82</td>
</tr>
<tr>
<td>Prefreeze</td>
<td>62.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.7</td>
<td>46.1</td>
<td>1.00</td>
</tr>
<tr>
<td>0 h Postthaw</td>
<td>37.4</td>
<td>36.3</td>
<td>39.0</td>
<td>41.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.66</td>
</tr>
<tr>
<td>3 h Postthaw</td>
<td>18.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.55</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Within data arranged by extender or method of evaluation, means in a row with a different superscript differ (<i>P</i>&lt;.05).

<sup>1</sup>Means based on three samples each in two extenders. Sperm were evaluated by videomicrography (VCR), track motility (TRACK), and computer (COMP). EYC = Egg yolk-citrate, EYT = egg yolk-Tris.

and computer). This decrease was a result of evaluating <i>n</i> independent means; as <i>n</i> increased, variation decreased (18). Because the video system was not as precise as the track or computer methods, at least for sperm in EYT, it was not used for further experiments.

**Experiment 2**

The percentage of motile sperm as determined by computer was correlated (<i>r</i>=.90; <i>P</i>&lt;.05) with the proportion of live sperm in the mixture (Figure 5A). There was no difference (<i>P</i>&gt;.05) in mean velocity of motile sperm in the four mixtures, and the overall mean was 50 μm/s. There was a difference (<i>P</i>&lt;.05) in the mean velocity of sperm from the four bulls (means ranged from 45 to 54 μm/s). Evaluations by track motility also were correlated (<i>r</i>=.95; <i>P</i>&lt;.05) with the proportion of live sperm (Figure 5B). Values for individual samples (n=20) determined by computer and track motility were correlated (<i>r</i>=.93).

**Experiment 3**

Means for the percentage of motile sperm in the nine samples used for the competitive fertility trial were similar for computer and track evaluations (Table 2). Two-way analyses of variance revealed effects (<i>P</i>&lt;.05) of time (Table 2) and time by bull for all parameters. Correlations of the percentage of motile sperm or spermatozoal velocity with the competitive fertility index were significant (Table 2). For evaluations by the microcomputer-photographic method, the multiple correlations based on the percentage of motile sperm and spermatozoal velocity for measurements at 0 to 1.5 h were .87 and .87 (<i>P</i>&lt;.05). Values for the percentage of motile sperm determined by the track and computer methods at 0 to 1.5 h were correlated (.98 and .93; <i>P</i>&lt;.01).

![Figure 4](https://example.com/figure4.png)

**Figure 4.** The 95% confidence intervals for percentage of motile sperm at 0 h incubation using egg yolk-citrate (EYC) (A) or egg yolk-Tris (EYT) (B) extenders, based on evaluation of one, two, or three slides (1, 2, 3) by videomicrography, track motility, or computer. Mean percentages of motile sperm in EYC were 41.5, 39.1, and 43.5 for videomicrography, track motility, and computer. Similarly, mean percentages of motile sperm in EYT were 33.2, 33.5, and 34.8.

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As anticipated, 95% confidence intervals for the percentage of motile sperm decreased as the number of slides evaluated increased (Figure 4). Precision when evaluating one slide was similar for track (±8 percentage units) and computer (±9 percentage units) evaluations at 0 h post-thaw. These values are similar to those reported by Elliott et al. (3) and Revell and Wood (15) but much better than visual estimates (2). The confidence interval was about ±5% when three slides were evaluated by either system. The 95% confidence intervals for percentage of motile sperm for track and computerized motility were lower using EYC extenders than EYT extender (Figure 4). This may result from a difference in how sperm swim that affects accuracy of their detection on the 35-mm negative.

Based on analyses of mixtures of live and killed bull sperm, the computer system was accurate. However, for one bull, a higher percentage of motile sperm in the 2:2 mixture than in the 3:1 mixture of live to killed sperm was detected by both track motility and computer (Figure 5); improper mixing or use of an atypical straw of semen probably occurred. Observed values for the relative percentage of motile sperm for track motility were closer to the theoretical plot than for the microcomputer-photographic system (Figure 5). This may have resulted from inability of the computer system to store data for >60 sperm/field whereas for track motility, evaluations of the same negatives included up to 108 sperm. With the computer system, the first 60 sperm encountered while systematically scanning the field were digitized.

As the studies reported herein were being completed, a fully automated computer system, CellSoft (CRYO Resources, Ltd., New York), became available and was used to analyze aliquots of the semen used for the competitive fertility trial of Experiment 3. Data obtained by CellSoft (P. R. Budworth et al., 1987, in press) and the microcomputer-photographic system for the percentage of motile sperm or mean spermatozoal velocity were significantly correlated (.95 and .75; P<.05) for observations at 0 h of incubation but not after 1.5 h of incubation at 37°C (r=.47 and .58; P>.05). Evaluations by CellSoft may be more accurate for determining the mean velocity of sperm, but the system is very expensive and requires 8

Discussion

The microcomputer-photographic system was reasonably precise (Figure 4). However, evaluations using videomicrography resulted in a higher (P<.05) percentage of motile sperm for three of the four evaluation times (Table 1). Possibly all dead sperm were not in sharp focus because of the higher magnification of the objective and nature of the optics used (16x phase-contrast vs. 10x dark-field) in the video system. Also, fewer sperm were analyzed with the video system (<120 cells/sample) than with the track or computer systems (about 370 and 270, respectively).
TABLE 2. Means for characteristics of spermatozoal motility and correlations between mean values and the competitive fertility index.¹

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Mean and range ²</th>
<th>Correlation ³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>1.5 h</td>
</tr>
<tr>
<td>Computer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motile sperm, %</td>
<td>27 (10–40)</td>
<td>11 (1–23)</td>
</tr>
<tr>
<td>Velocity, μm/s</td>
<td>43 (34–51)</td>
<td>32 (26–39)</td>
</tr>
<tr>
<td>Track</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motile sperm, %</td>
<td>28 (8–45)</td>
<td>12 (2–25)</td>
</tr>
</tbody>
</table>

¹ Based on data for semen from nine bulls evaluated immediately after thawing (0 h) or after 1.5 h of incubation at 37°C. The competitive fertility index was calculated by Saacke et al. (17) from data for 229 calves.
² Means differed between 0 and 1.5 h (P<.01).
³ All correlations were significant (P<.05).

to 10 min to analyze a sample (P. R. Budworth et al., 1987, unpublished).

The microcomputer-photographic system gave reasonably precise and accurate evaluations of frozen-thawed bovine sperm. It is much less expensive than a fully automated system, and the data generated probably are slightly better for prediction of fertility than those obtained by track motility. Multiple correlations for data (motility and velocity) obtained by computer at 0 or 1.5 h of incubation with the competitive fertility index were .87 whereas the simple correlations based on track motility were .84 and .81. However, evaluations by the microcomputer-photographic method are less tedious than analysis of track motility. The time to analyze one sample using the microcomputer-photographic system was 5 to 7 min, excluding the time to photograph the samples (2 min) or develop the negatives.

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