A New Method for Rapid Determination of Sperm Concentration in Turkey Semen

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ABSTRACT The routine determination of sperm concentration not only provides a measure of semen quality but also supplies a means of delivering precise sperm numbers in modern artificial insemination (AI) programs. Methods for estimating sperm numbers in laboratory settings (e.g., hemacytometer) are not practical for use in breeding facilities. A microprocessor-controlled semen analyzer (Densimeter, Model 534-B-Modl, Animal Reproductive Systems) is commercially available for evaluating stallion semen. We calibrated and modified the semen analyzer to determine sperm concentration in turkey semen and to provide information on the required dilution for a constant AI dose. To calibrate the semen analyzer for turkeys, six samples of pooled semen (10 toms per sample) were collected by abdominal massage. A dilution curve of undiluted and 1:0.5, 1:1, 1:1.5, 1:2, 1:3, 1:5, and 1:10 diluted semen was established using Beltsville Poultry Semen Extender. Sperm concentration in diluted samples was determined by hemacytometer counts. The hemacytometer counts were used to develop an equation for estimating sperm concentration from the semen analyzer’s absorbance readings and the equation programmed into the instrument. The newly developed program on the semen analyzer was subsequently validated by comparing instrument values for sperm concentrations with concurrent hemacytometer counts, which compared well to concentrations determined by a previously calibrated photoelectric colorimeter (Klett). Correlation coefficients between the semen analyzer and hemacytometer counts and the Klett and hemacytometer counts were \( r = 0.996 \) and \( r = 0.992 \), respectively (\( P < 0.001 \)). The semen analyzer method was accurate and precise and could be beneficial to commercial turkey AI programs because it is easy to use, requires limited technological skills, and provides results for determining sperm concentration and AI dose in ~1 min.

(Key words: sperm concentration, turkey, hemacytometer, photometer)

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INTRODUCTION

The routine determination of sperm concentration not only provides a measure of semen quality but also supplies a means of delivering precise sperm numbers in modern artificial insemination (AI) programs. Assessment of sperm concentration is necessary to calculate AI dose and evaluate semen output of individual toms. Because several insemination protocols for turkeys rely on increasing the insemination dose over the course of an egg production period, a reliable and accurate method of counting sperm and calculating an AI dose is essential (Christensen, 1981; McIntyre, 1993). There are several well-established methods to determine sperm concentration in poultry semen. These include the use of a hemacytometer for counting sperm (Brillard and McDaniels, 1985; Bakst and Cecil, 1991), a spectrophotometer for determining optical density or absorbance (Carson et al., 1955), a probe colorimeter for measuring absorbance (Brillard and McDaniels, 1985), and a spermatocrit (Shaffner and Andrews, 1943; Brillard and McDaniels, 1985) for determining sperm numbers from packed cell volume after centrifugation. The hemacytometer method, although tedious, is the most exact method available because sperm are visually observed on the microscope and counted. For this reason it is used to establish regression equations so that values from other counting procedures can be expressed in sperm numbers (Brillard and McDaniels, 1985; Bakst and Cecil, 1991). Because of its simplicity, spermatocrit readings are used frequently to determining sperm count in turkey ejaculates; however, results using this method are more variable when compared to the other methods of determining sperm concentration (Brillard and McDaniels, 1985; Bakst and Cecil, 1989; McIntyre, 1993).

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A portable spectrophotometer and data processing unit (semen analyzer, Densimeter\(^3\)) has been developed to determine sperm concentration in stallion semen. The semen analyzer allows for quick determination of sperm concentration and AI dose. This is achieved by a built-in microcomputer that automates the calibration procedures and calculations and supplies the necessary instructions for each step of the measurement process. This instrument could be beneficial to commercial turkey AI programs because it is easy to use, accurate, requires limited technological skills or instrument maintenance, and provides fast results for determining sperm concentration and dilution for a constant AI dose. Our goal was to calibrate and validate the semen analyzer method for use in determining sperm concentration and AI dose for turkeys.

**MATERIALS AND METHODS**

**Calibration of Semen Analyzer for Turkey Sperm**

To calibrate the semen analyzer for turkey semen, six samples of pooled semen (10 toms per sample) were collected by abdominal massage (Burrows and Quinn, 1935). Pooled samples were thoroughly mixed and a dilution series of undiluted, 1:0.5, 1:1, 1:1.5, 1:2, 1:3, 1:5, and 1:10 were prepared using Beltsville Poultry Semen Extender (BPSE).\(^4\) The dilution series covered the transmission scale from 15% T, for a highly concentrated sample, to 93% T, for samples with low numbers of sperm.

For each sample dilution, 37.5 \(\mu\)L of sperm suspension was added with a positive displacement pipet to 10 mL of 3% NaCl so that hemacytometer counts and percentage T from the semen analyzer could be determined without further manipulation of the sample. Four milliliters of this sample was added to a plastic cuvette, inverted 10 times and placed in the semen analyzer after zeroing the machine with 3% NaCl. For determination of sperm concentration by the hemacytometer method, chambers were filled with sperm suspension allowed to settle for 15 min and 5 of the middle squares of the center grid of 25 squares were counted on both sides of the chamber and averaged (Bakst and Cecil, 1989). Duplicate counts of the diluted samples were determined by two independent estimates.

**Utilizing the Semen Analyzer for Determining Sperm Concentration and AI Dose**

Data from the six trials were entered into a statistical graphics software package (Sigma Plot\(^5\)) and a polynomial equation was generated to the data (percentage T vs hemacytometer counts). This equation was incorporated into the microprocessor of the semen analyzer to provide a custom program for turkey semen. The AI dose was calculated by the following equation:

\[
\text{DOSE} = \frac{\text{NSPD}}{\text{VIA} \times \text{CONC}}
\]

where DOSE = volume of extended semen required for a single hen (milliliters); NSPD = number of viable sperm per dose (x 10\(^6\) viable sperm); VIA = viability factor in decimal form; and CONC = concentration of extended semen (x 10\(^6\) sperm per milliliter). Because recommended sperm concentration per AI dose varies across the egg production period (Christensen, 1981; McIntyre, 1993), the program was written so that the AI dose desired could be inserted. In addition, if sperm viability was to be determined by some other method (i.e., fluorescent staining or ethidium bromide stress test) these values could be inserted into the calculation to determine the concentration of viable sperm. The number of AI doses was calculated by the following equation:

\[
\text{NDPC} = \frac{\text{TVSP}}{\text{DOSE}}
\]

where NDPC = total number of doses in collection; and TVSP = total volume of extended sperm in collection.

**Validation of the Semen Analyzer**

To validate the calibrated semen analyzer, sperm concentrations were determined by hemacytometer, a previously calibrated photoelectric colorimeter (Klett)\(^6\) method (Bakst and Cecil, 1989), and the semen analyzer method. Pooled semen samples from 10 toms were diluted 0 (undiluted), 1:1, 1:5, and 1:10 in BPSE (four replicates). Hemacytometer readings were determined by diluting 37.5 \(\mu\)L into 10 mL of 3% NaCl and samples counted as described above. Klett readings were determined by taking 10 \(\mu\)L of sperm suspension added to 10 mL of 3% NaCl, absorbance recorded and semen concentration determined. For determination of semen concentration with the semen analyzer, 3.42 mL of 3% NaCl was placed into a cuvette and inserted into the semen analyzer to zero the instrument, then 12.8 \(\mu\)L of diluted semen was added using a positive displacement pipet. The sample was covered with parafilm, inverted 10 times and replaced into the semen analyzer. To demonstrate the repeatability of the semen analyzer, eight ejaculates from individual males were diluted 1:1 in BPSE and replicate measures of semen concentrations (n = 10 counts per ejaculate) were determined.

**Statistical Analysis**

The interrelationships between the semen analyzer versus hemacytometer and Klett vs hemacytometer were evaluated using Pearson Correlation Coefficients esti-
RAPID DETERMINATION OF SPERM CONCENTRATION

FIGURE 1. Percentage transmission generated by the semen analyzer plotted against turkey sperm concentration estimated by hemacytometer.

RESULTS

To calibrate the semen analyzer, the percentage T output and the concentration of sperm determined by hemacytometer readings of the dilution curve were plotted (Figure 1). The estimated parameters for the determination of sperm concentration from percentage T was:

\[
\text{CONC} = 27.3343 - 7.5399 \times 10^{-1} (T) + 9.364 \times 10^{-3} (T^2) - 6.3932 \times 10^{-5} (T^3) + 1.8326 \times 10^{-7} (T^4)
\]

where CONC = concentration of sperm per milliliter (x 10⁹ sperm per milliliter); and T = percentage transmission.

Sperm concentrations derived from the semen analyzer and Klett were compared to those determined by hemacytometer counts to evaluate the accuracy of the semen analyzer method (Figure 2a,b). Correlation coefficients between the semen analyzer and hemacytometer counts and the Klett and hemacytometer counts were \( r = 0.996 \) and \( r = 0.992 \), respectively \( (P < 0.001) \). The regression line for the hemacytometer vs the semen analyzer method was \( Y = 1.034 (X) + (-0.0105) \) and that for the Klett vs the hemacytometer method was \( Y = 1.140458 (X) + (-0.12872) \). At high sperm concentrations the Klett values did not fit the line as well as the semen analyzer (Figure 2a,b). The overall mean coefficient of variation for replicate measurements \( (n = 8 \text{ ejaculates, 10 counts per ejaculate}) \) on the semen analyzer was 6.2%. The semen analyzer method was time efficient, reliable, and easy to perform; sample preparation and determination of sperm count, AI dose, and number of AI doses in a given sample required approximately 1 min.

DISCUSSION

An equation was developed for the semen analyzer to determine sperm concentration in turkey semen. The semen analyzer utilizes a computer-controlled narrow band light source with a dominant wavelength of 660 nm. The energy from this source is transmitted through a sample placed in the measurement chamber and evaluated by a silicon photo detector. A precision analog to digital converter translates the output of the photo detector amplifier into a digital representation of the energy transmitted and can distinguish differences of less than 0.01% transmission. The percentage transmission that corresponded with hemacytometer readings was used to develop a program determining sperm concentration in turkeys.

[^7]: Microsoft Corp., Redmond, WA 88052.
Validation of the programmed semen analyzer resulted in high correlations between hemacytometer counts and concentrations determined by the semen analyzer. Lower accuracy was observed at the highest sperm concentrations with the Klett than with the hemacytometer method. Determining sperm concentration using the semen analyzer method was reliable, as the CV of repeated counts averaged 6.2%.

Because of the programming and validation performed in this study, other laboratories could use this method for determining sperm concentration in turkey ejaculates without having to repeat tedious calibration procedures. Although setting up and calibrating spectrophotometers or spermatocrits can be accomplished in individual laboratories, these validations are only as good as the original hemacytometer reading, which can vary greatly (Bakst and Cecil, 1989), and technicians not accustomed to evaluating sperm may be even less accurate. This lack of experience is important because single hemacytometer counts are not highly accurate and differences of up to 20% have resulted from the same technician counting duplicate samples from the same source (Freund and Carol, 1964). These differences stem from many procedural steps, including filling the chamber correctly, focusing technique through the chamber to perform the count and determining what sperm to count (what sperm are actually in squares). Also, the hemacytometer counts are time consuming (15 to 20 min to set up and count the sample).

Use of the semen analyzer requires little technical skill and the results are rapid, and, as shown in the present study, data are reliable and reproducible. Many turkey breeders have neither the expertise, resources, nor time to reliably calibrate instruments. Using the semen analyzer method for determining sperm count allows for standardizing the procedure across labs and evaluation of different flocks with the same equipment. One advantage for using the semen analyzer in a field or farm set up is that for dilutions, 3% NaCl can be used instead of toxic or mutagenic substances such as formalin or ethidium bromide, which have to be carefully handled and disposed of according to proper hazardous waste procedures.

The amount of time it takes to determine sperm count is important. The semen analyzer can be standardized, and the determination of sperm concentration, total sperm numbers, AI dose, and number of AI doses for a sample can be completed in approximately 1 min.

FIGURE 2. Validation of sperm concentration estimates between the semen analyzer and hemacytometer methods (left) and between the Klett and hemacytometer methods (right), correlation coefficients were \( r = 0.996 \) and \( r = 0.992 \), and regression equations were \( Y = 1.034 (X) + (-0.0105) \) and \( Y = 1.140458 (X) + (-0.12872) \) for the semen analyzer and Klett, respectively.
Brillard and McDaniel (1985) reported an average of 9.44, and 2.3 min to determine sperm counts on a hemacytometer, spermatocrit, and spectrophotometer, respectively (n = 6 replicates per technique). However, these averages do not appear to take into account the 15 min standing time for the hemacytometer before reading or the 5 to 10 min centrifugation time required for the spermatocrit.

As with any assay, there are a number of constraints. First, use of the semen analyzer method is best applied to viscous white semen. Debris and particles in high concentration will yield incorrectly high results for sperm concentration. As with all laboratory procedures, proper technique is crucial for accurate results and because of the viscosity of turkey semen a positive displacement pipet is essential for accurate pipetting of samples.

The newly developed program on the semen analyzer was validated using hemacytometer counts and sperm concentration determined by a previously calibrated photoelectric colorimeter. The semen analyzer method could be beneficial to a commercial turkey AI program because it is simple, accurate, requires limited technological skills, and provides fast results for determining sperm concentration and AI dose.

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