Research Report

Stallion sperm concentration measurements: experience and equipment

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Abstract

Accuracy is paramount in evaluating sperm concentration and can be a challenge to those with minimal laboratory experience. Purpose of the study was to determine the effect of operator experience on sperm concentrations using 4 methods: Makler® counting chamber, equine densimeter, iSperm, and NucleoCounter®. There was no difference (p = 0.64) between experienced and novice processors for Makler® counting chamber; was difference (p = 0.005) for equine densimeter; was no difference (p = 0.35) for iSperm; and was tending toward difference (p = 0.068) for NucleoCounter®. Correlation between bias and magnitude for Makler® counting chamber was –0.74 (p = 0.003); for equine densimeter was –0.44 (p = 0.11); for iSperm was 0.06 (p = 0.83), and NucleoCounter® was –0.52 (p = 0.06). Makler® counting chamber produced a mean sperm concentration similar to an experienced processor but had significant variation within novice processors. Equine densimeter significantly overestimated sperm concentrations with novice processors but had the least variation. The iSperm performed poorly for both experienced and novices and produced significantly different concentrations than other methods. Thus, iSperm cannot be recommended to accurately measure sperm concentration. Lastly, NucleoCounter had no difference in mean sperm concentrations or variation and was the best system for a novice processor to gain accurate and repeatable sperm concentration measurements.

Introduction

Laboratory work can be challenging for veterinarians new to processing semen, since a few receive formal training. Accurately determining sperm concentration sperm in an ejaculate is an essential step in analyzing and processing semen[1] and depends on proper dilution technique, laboratory skills, and equipment knowledge. Several types of equipment exist to determine sperm concentration with varying working principles. The simplest method relies on a microscope and visual differentiation and counting of sperm on a slide with a known volume (hemocytometer or Makler counting chamber). A second, common, type of equipment is a spectrophotometer. This measures light impedance through the ejaculate to determine sperm concentration using a standard curve. A third type of equipment coined Computer-Assisted Sperm Analysis (CASA) uses a computer system coupled to a microscope to identify and count sperm and can perform sperm motion analysis. Finally, another common system to determine sperm concentration uses fluorescent dyes to stain nuclei to count sperm via either flow cytometry or a cell counter.[1] There were differences[2,3] among photometric, direct counting and fluorescent cell counting methods in evaluating stallion sperm concentration.

Each of these methods of determining sperm concentration has their advantages and disadvantages in terms of accuracy, time required to perform, and operational skills. Purpose of this study was to determine the effect of operator experience level on determining sperm concentrations using each of these 4 distinct methods.

Materials and methods

Animal use was approved by the Institutional Animal Care and Use Committee. Three ejaculates from 2 stallions were utilized. Briefly, stallions were collected using a Missouri artificial vagina, phantom, and tease mare. After semen was collected the volume of semen was determined and semen was gently mixed and evenly divided among an experienced and 6 novice semen processors. Experienced processor was a diplomate of the American College of theriogenologists with multiple years of experience in equine theriogenology. Novice

processors were 4th year veterinary students with little to no previous experience in semen processing.

Four methods were selected to determine sperm concentration based on different principles of measurement. They were: Makler® counting chamber (Sefi-Medical Instruments, Ltd., Santa Ana, CA, USA) to represent manual analysis; equine densimeter (Model 591B, Animal Reproduction Systems, Chino, CA, USA) to represent a spectrophotometer-based system; iSperm (mCASA; Aidmics Biotechnology Co, Ltd, Taipei City, Taiwan) to represent a CASA based system; and NucleoCounter® (SP-100; ChemoMetec A/S, Allerod, Denmark) to represent a fluorescent-dye based system.

Prior to study initiation, novice processors (n = 6) were given a training session specific to each equipment. Throughout the study, processors had access to written instruction on equipment operation. Instructions for Makler® counting chamber were as follows. Semen was diluted 1:1 with 10% buffered formal saline[4] and placed on a slide. After semen mixture was well mixed and a 5 µl drop was placed on the center of the disc area using a micropipette. A coverslip was then placed on the 4 pins and gently pressed down. Sperm heads were counted within each grid and those that touched the top or left lines, whereas those touching the bottom or right lines were not counted. A line of 10 squares were counted and multiplied by 2 (to account for dilution), this represented the sperm concentration (10^6/ml). A second strip of 10 squares was counted and the sperm concentration determined as before; average of 2 counts was used to determine sperm concentration.

To represent the spectrophotometer-based systems, equine densimeter was used. Novice processors were trained to operate by following directions on the screen. Briefly, 3.42 ml of formalin 10 (Animal Reproduction Systems) was added to a cuvette and system was zeroed. Next, the cuvette was removed and 180 µl of raw semen was added to formalin 10 solution in the cuvette, mixed and placed back in the machine to determine sperm concentration.

Prior to use, iSperm was set up according to manufacturing instructions. Novice processors were instructed to use iSperm according to manufacturer directions. Initially, semen was diluted 1:1 with semen extender (INRA96, IMV Technologies, Osseo, MN, USA) and if needed, further dilutions were made with semen extender. A base chip was then mounted on the sample collector. One of 3 sampling loading methods were taught, and the method used was at operator discretion. Method 1 added 7.5 µl of extended semen sample on a vertical base chip, Method 2 used a dropper to add the extended semen sample into the cover chip, and Method 3 dipped the base chip into the extended semen sample. After the application of the extended semen sample, base chip was pressed vertically into the cover chip until a 'click' was heard and continued to be pressed for an additional 1–2 seconds. Once this was completed the sample collector/base chip/cover chip combination was attached to the iPad and sperm concentration was determined. Sample collector was rotated to perform a 4-view analysis. If any errors occurred, analysis was repeated.

For Nucleocounter® novice processors were instructed on operation and SemenView software including how to select the species for analysis and change the dilution factor to correspond to the ideal reading range for sperm concentration. Novice processors were free to set the dilution factor (DF), select the appropriate amount of reagent (S100; Chemometec) and sample volume based on the DF utilized. Briefly, depending on the DF used a volume of reagent was added to a clean tube. Next, the desired volume of sample was added to the reagent. These volumes (reagent and sample) were based on manufacturer tables. Next the sample was gently mixed, and a cassette tip was placed in the solution, sample aspirated, and the cassette placed in the machine and sample was analyzed. After the training sessions novice processors determined sperm concentration without supervision.

Data analyses

Mean sperm concentrations were analyzed using SAS (9.4) General Linear Model procedure by operator experience (novice vs. experienced), method of semen concentration analysis (Makler counting chamber, equine densimeter, iSperm, and NucleoCounter®) and interaction between experience and method. Means statement with least squares difference was used to determine mean sperm concentration by method and make pairwise comparisons. The SGPlot procedure was used to generate Bland–Altman plots (difference and mean concentration between experienced and novice processors). T-test was used to compare experience level for each method (test for zero bias). Independence of bias was tested using the Corr procedure using the bias (difference between experienced and novice) and magnitude (average of experienced and novice). Data were represented as mean ± standard deviation.

Results

Mean sperm concentration for Makler® counting chamber, equine densimeter, iSperm and NucleoCounter® for experienced operator was 107.1 ± 13.7, 97.6 ± 13.1, 148.6 ± 63.4, and 109.3 ± 16.4 (10^6/ml), respectively (Table 1). Mean sperm concentrations for the Makler® counting chamber, equine densimeter, iSperm, and NucleoCounter® for novice operator were

Table 1. Mean sperm concentration (10^6/ml) and standard deviation (STD) for each method by operator experience.

Analysis method	Experienced operator		Novice operator		p value
	mean	STD	mean	STD	
Makler counting chamber	107.1	13.7	109.9	29.2	0.64
Densimeter	97.6	13.1	109.2	19.1	0.005
Isperm	148.6	63.4	161.7	61.3	0.35
NucleoCounter	109.3	16.5	121.9	27.7	0.068

109.9 ± 29.2, 109.2 ± 19.1, 161.7 ± 61.3, and 121.9 ± 22.7 (10^6/ml), respectively (Table 1). There was no significant effect of experience level ($p = 0.72$), there was a significant effect of method ($p = 0.18$), and no significant interaction between experience and method ($p = 0.99$). There was no difference in mean sperm concentration values between Makler® counting chamber, equine densimeter, and NucleoCounter®; however, iSperm had significantly higher sperm concentration values ($p < 0.05$) than other methods. Bland–Altman plots for Makler counting chamber, equine densimeter, iSperm, and Nucleocounter are provided (Figure 1). Mean difference in sperm concentration assessments between experienced and novice operator for Makler® counting chamber was -2.8 ± 24.7; for equine densimeter was -11.6 ± 14.7; for the iSperm was -13.1 ± 56.5; and NucleoCounter® was -12.6 ± 26.6 (10^6/ml). T-test result between experienced and novice processors for Makler® counting chamber) was not different ($p = 0.64$); for equine densimeter was different ($p = 0.005$); for iSperm was not different ($p = 0.35$); and for NucleoCounter® there was a tendency toward difference ($p = 0.068$). Correlation between the bias and magnitude for Makler® counting chamber was -0.74 ($p = 0.003$); for equine densimeter was -0.44 ($p = 0.11$); for iSperm was 0.06 ($p = 0.83$); and NucleoCounter® was -0.52 ($p = 0.06$).

Discussion

Differences between and within human[5,6] and bovine[7] andrology laboratories were observed and thus it is not surprising in our study to find differences in agreement among different equipment and operator experience level.

Makler® counting chamber had similar mean sperm concentration values between experienced and novice processors. The correlation was significant, and the negative value indicated significantly more variation in the sperm concentration values with novice processors. The variation in sperm concentration assessments with novice processors can be due to several factors such as accuracy of dilutions, uniformly mixing semen prior to performing dilutions, mixing prior to pipetting samples, chamber filling, how the sperm were counted under the microscope, sample variation and mathematical errors. The accuracy of dilutions can be impacted by both the precision of pipetting and type of pipet used. Two types of pipets were available, air displacement and positive displacement. Positive displacement pipets were more accurate than air displacement pipets.[3] Additionally, with air
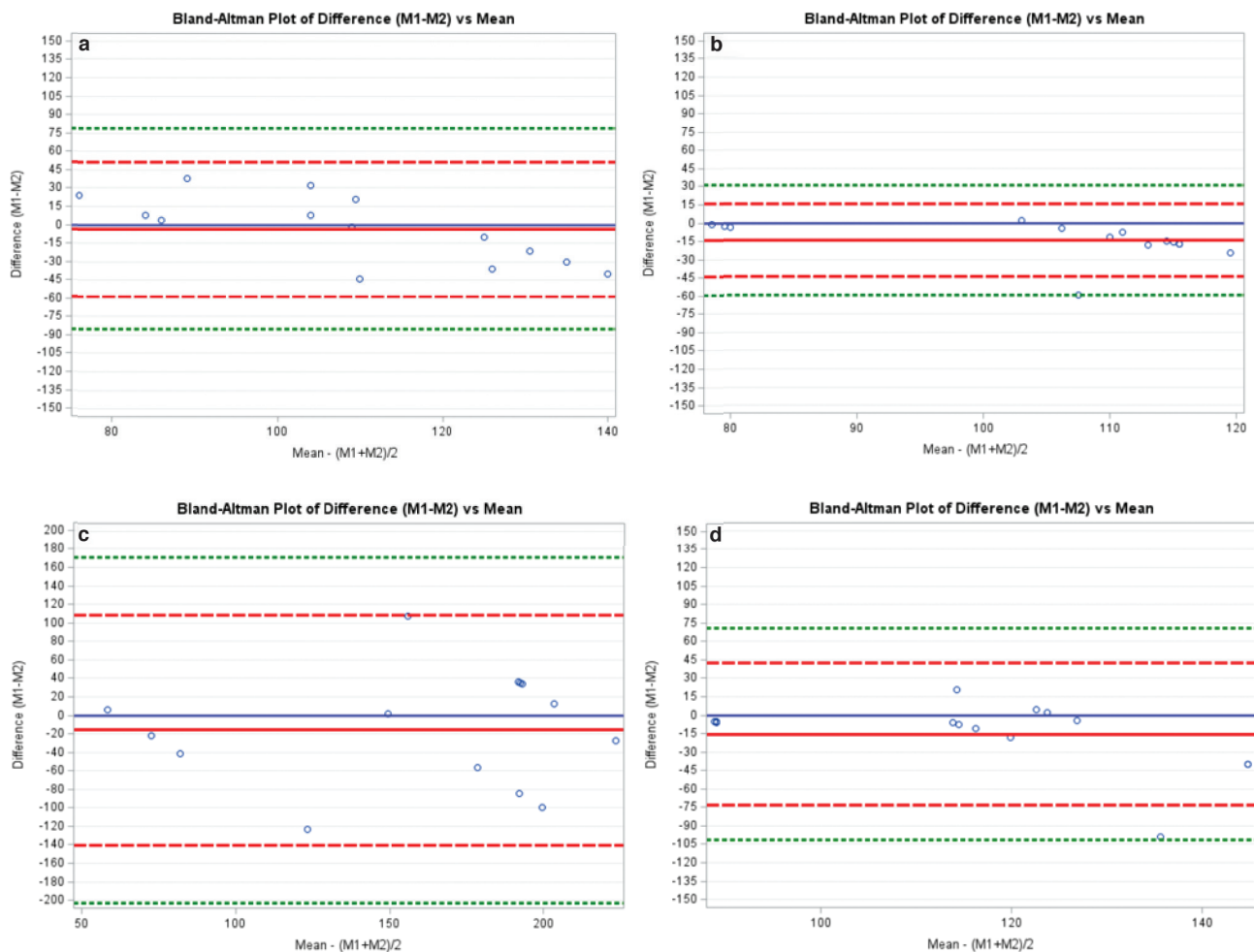

Figure 1. Bland-Altman plot difference (experienced minus novice operator sperm concentration) to mean ([experience plus novice operator sperm concentration]/2) for A. Makler counting chamber; B. Densimeter; C. ISperm; and D. NucleoCounter. For each figure (A–D) blue line represents a difference = 0. Dashed red line represents 2 standard deviations (STDs) and green dashed line represents 3 STDs.

displacement pipets novice users may inadvertently aspirate too much fluid by depressing the plunger too far or not aspirate enough fluid all of which can impact accuracy. Another source of variation can be how the sperm were counted under the microscope. In general, sperm should be counted when the head is either in the grid or lying on 2 adjacent lines to prevent double counting with adjacent squares; whether this was carried out correctly each time is unknown but is a potential source of error. Lastly, since a 1:1 dilution was made the count needed to be doubled to determine the concentration. In this case it is unlikely mathematical errors contributed to the variation but this is more likely with hemocytometer as not only the dilution factor has to be accounted for but also the volume of sample analyzed.[8] Although the mean sperm concentrations were similar between experience level, the amount of variation within the novice group makes it difficult to recommend this as an accurate method.

With equine densimeter novice processors obtained significantly higher mean sperm concentration values than the experienced operator; however, the variation between experience levels was not different. The higher mean sperm concentration obtained by novices could be due to pipetting error either with the densimeter media or semen sample or mixing of sample. Interestingly, there was no difference in variation among groups. This could be due to the fact equine densimeter has direction for operation on the LED as the sample run that may result is less variation in the procedure. This suggested that novices using an equine densimeter will generally overestimate sperm concentrations but with less variation.

The iSperm assessed significantly higher concentrations than other methods but there was no difference related to experience on the concentration and variation. This system is subjected to the same problems as the Makler® counting chamber, such as mixing, dilution, and chamber filling but is also subjected to software misidentification.[9] For boar sperm, CASA system had higher agreement with the hemocytometer with dilute samples but this was lost in more concentrated semen samples.[10] CASA-based systems (e.g. iSperm) is considered to be inaccurate and only give a rough estimate of sperm numbers[10] and are not recommended by WHO[11] or the National Association of Animal Breeders.[12]

With NucleoCounter® there was no significant difference in sperm concentration values or variation between experience levels. Studies have used NucleoCounter as a gold standard for determining sperm concentration[13,14] but this system is subjected to errors with dilution and dilution factor settings. Using a dilution rate outside optimal limits will decrease accuracy.[1] Additionally, excessive time to read could impact accuracy since a fluorescent dye is used.[1] This appears to be the best method for novices to evaluate sperm concentration since the mean and variation in sperm concentration were not significantly different.

Each system that measured sperm concentration had their advantages and disadvantages in how they performed with novice processors. Makler® counting chamber produced a mean sperm concentration similar to an experienced operator but had significant variation. Equine densimeter significantly overestimated sperm concentration with novice processors but had the least variation. The iSperm performed poorly for both experienced and novices and produced significantly different concentration than other methods. Thus, iSperm cannot be recommended to accurately measure sperm concentration. Lastly, NucleoCounter had no difference in mean sperm concentrations or variation and was the best system for a novice processor to gain accurate and repeatable sperm concentration measurements. Regardless of the system employed to evaluate sperm concentration, it is important to take time to become familiar and comfortable with the laboratory techniques and operation of the equipment to ensure accurate results.

Conflict of interest

None to report.

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