Precision of the Neubauer Hemocytometer in Quantifying Concentration of Canine Spermatozoa Within and Between Operators of Differing Experience Levels

Kaitlyn Caraway, Robyn Wilborn, Aime Johnson, Jay Barrett, Todd Steury

Abstract

The importance of measuring accurately and precisely the concentration of spermatozoa in semen is increasing as artificial insemination becomes widely practiced in veterinary medicine. This study tested the precision of the Neubauer hemocytometer with canine spermatozoa of varying concentrations within and between operators of different experience levels (Figure 1). We calculated the standard deviation in estimates of spermatozoa concentration between two chambers of each hemocytometer and between replicate samples from the same ejaculate for both an experienced operator and an inexperienced one. The Neubauer hemocytometer precision was also evaluated across varying concentrations of spermatozoa. We determined that the operator's experience level did not have a significant effect on precision when using the Neubauer hemocytometer. For both the experienced and inexperienced operator, the standard deviations between the two chambers of the hemocytometer and between replicate samples were 10.22 (±1.22) million spermatozoa and 10.33 (±2.16) million spermatozoa, respectively. Both these deviations exceeded 10 million spermatozoa, which many veterinarians consider to be significant when calculating a semen dosage for artificial insemination. However, this degree of deviation was present within the estimates of both operators, implying that it was not an effect of operator experience or expertise, but rather an inherent risk in using the hemocytometer.

Introduction

The analysis of concentration of spermatozoa in canine semen samples is becoming popular with the increased use of artificial insemination (AI) in veterinary medicine (Hansen et al., 2006). Accurately measuring the concentration of spermatozoa of a semen sample is important because it allows veterinarians to determine how many breeding dosages can be obtained from a single ejaculate. Common dogma is that at least 150 to 200 million progressively motile spermatozoa are recommended per insemination dose, but this number can vary greatly depending on the type of semen used (fresh, chilled or frozen) (Linde-Forsberg, 1991) and even the size of the dog breed. For example, an adequate breeding dose for a toy breed (e.g. Chihuahua, expected litter size of 2 to 4 puppies) requires much fewer progressively

Figure 1. Microscopic (400x) image of a canine spermatozoa on a hemocytometer grid.

motile spermatozoa than a breeding dose for a giant breed (e.g. Great Dane, expected litter size of 9 to 12 puppies), which correlates to size of the male testes of the corresponding breeds (Amann, 1986). This calculation of breeding doses is especially important with limited quantities of frozen semen from a valuable animal.

For this study, we chose to evaluate the Neubauer hemocytometer because it is the oldest, most common, and simplest method of measuring the concentration of spermatozoa cells in a sample (Kuster, 2005). For quantifying the spermatozoa concentration in semen samples, the Neubauer hemocytometer has long been considered the standard by the World Health Organization (WHO) (Cooper, 2010). It is the standard method for quantifying boar semen (Hansen et al., 2006), bull semen (Atiq et al., 2011), stallion semen (Samper, 2000), goat semen (Leboeuf et al., 2000), and human semen (Cooper, 2010). The hemocytometer has two chambers with a total area of 1.0 mm^2 on each end where the spermatozoa cells are counted. A coverslip sits 0.1 mm off the grid, allowing a diluted semen sample (1:100 dilution) volume of 0.1µl (Cooper, 2010). All spermatozoa cells within this area are counted manually under a microscope to quantify the concentration as millions of spermatozoa cells per milliliter, and the two chambers are averaged to obtain a spermatozoa concentration reading for that hemocytometer. The hemocytometer is cost-effective and relatively simple to use. However, it is much more time-consuming compared to other methods of quantification.

While computer-automated semen analysis (CASA) methods have become very popular in recent years to increase efficiency, these methods are influenced by the Segre-Silberberg (SS) effect. The SS effect causes the spermatozoa to be pulled through the chamber faster and drives them toward the walls of the chamber, causing an underestimation of the concentration of spermatozoa, particularly in thin, capillary-loaded slides. This phenomenon was seen when analyzing human and boar semen samples on the CASA machine, but was not seen with the hemocytometer. It is believed that the SS effect does not have time to develop in the hemocytometer due to the increased chamber depth, as compared to the CASA slides (Cooper, 2010).

One large source of error with using the Neubauer hemocytometer is that the operator must manually count cells. Consequently, an operator that is experienced with the hemocytometer, microscopes, and the identification and counting of spermatozoa cells should be able to obtain accurate and precise results. However, pipetting errors, clumping of spermatozoa cells, and variations in the size of the drop dispensed into each chamber could all create variations in spermatozoa count estimates between chambers in the hemocytometer and between replicate samples from the same ejaculate. For example, if the operator dispenses a small drop into one chamber of the hemocytometer and a large drop into the other, a large standard deviation will result in the estimated concentration of spermatozoa averaged across chambers of the hemocytometer. Such errors could be exacerbated by untrained operators and lead to clinical mistakes. The main goal of this study was to determine whether these errors coincide with the operator's level of experience.

Accuracy and precision are two terms used to describe a group of estimates. Accuracy is a measure of an estimate's closeness to the true value, and precision is a measure of how close a estimates in a group are to each other, regardless of how close they are to the true value. While it would be valuable to quantify the accuracy of the hemocytometer, doing so requires knowing the true values. For the purposes of this study, accuracy was not questioned. However, precision of the hemocytometer can readily be estimated by comparing multiple values from the same sample (ejaculate), including replicates by the same operator as well as another operator. For this study, we determine the standard deviation (i.e., precision) of estimates of spermatozoa concentration between chambers of the hemocytometer and between replicate samples from a given ejaculate, as influenced by the degree of operator experience.

Experimental Design

One hundred different samples (ejaculates) were obtained from a total of eighteen different dogs. The dog breeds used were Labrador Retrievers (8), Beagle (1), English Pointers (2), and Beagle-Corgi crosses (7). Dogs ranged from one to seven years of age with a mean of 4 years of age. All procedures were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC #2009-1580).

"...the Neubauer hemocytometer has long been considered the standard by the World Health Organization."

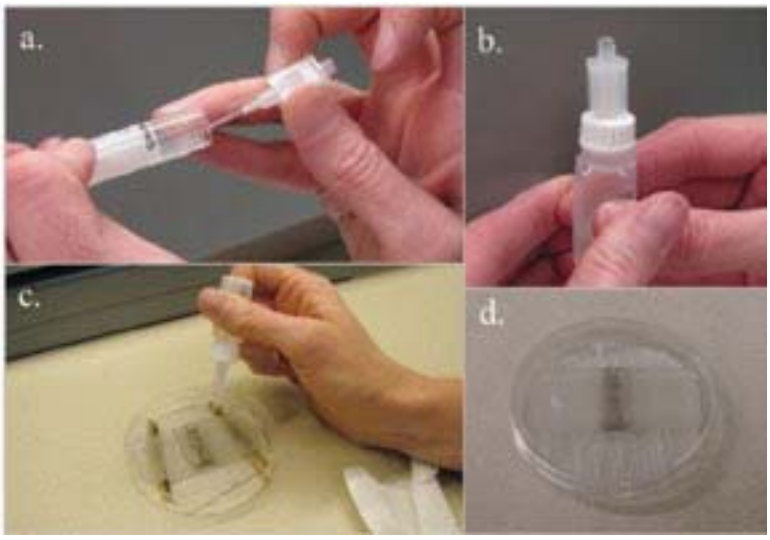

Figure 2. Method for preparing the Neubauer hemocytometer for this study: (a.) pipette is filled with 20 µl raw semen sample; (b.) sample is diluted 1:100 with reservoir mixture; (c.) each chamber of the hemocytometer is filled with 20 µl of raw sample (d.) loaded hemocytometer is incubated for four minutes in humidity chamber.

The inexperienced operator was trained in the proper use of the hemocytometer for a total of two hours. During this time, hands-on instruction was provided, and two practice samples, not included in the data collection, were counted. Proper procedure was posted

in the laboratory so that the inexperienced operator could quickly refer to protocol at any time, but no further hands-on instruction was provided.

Each ejaculate was quantified on a total of four hemocytometers, two by an experienced operator and two by an inexperienced one to obtain an inter-chamber and inter-replicate standard deviation for each operator. Both chambers of the hemocytometer were counted separately by each operator and averaged to provide an inter-chamber mean. This mean represents the concentration of spermatozoa for that hemocytometer. An estimate of the inter-chamber standard deviation was calculated from the individual counts of the two chambers. Each operator repeated this process a second time using the same raw ejaculate. The spermatozoa counts representing each hemocytometer of the same ejaculate were then statistically averaged to obtain an inter-replicate mean. An estimate of the inter-replicate standard deviation was calculated from the averaged counts generated from the two replicates.

The mean concentration of each individual ejaculate as measured by each operator was grouped into three concentration categories that represent realistic spermatozoa concentrations in canines: <100 million cells/ml, 100 – 200 million cells/ml, and >200 million cells/ml. The standard deviation within each concentration category was analyzed to determine at which concentration the hemocytometer was the most precise.

Materials and Methods

Neubauer Hemocytometer

Each operator followed a standard procedure for preparing each Neubauer hemocytometer (Figure 2). A 1.98 ml unopette reservoir and 20 µl capillary pipette were obtained. The spear end of the capillary pipette was used to create a hole in the top of the reservoir and then discarded. The raw semen sample was inverted three times to ensure homogenization, and the pipette was filled with 20 µl of the semen sample via capillary action. The pipette was then placed pipette end down into the reservoir, and a meniscus was made three times to ensure even mixing between the semen sample and reservoir mixture, resulting in a 1:100 dilution ratio. After allowing the reservoir and semen mixture to equilibrate undisturbed for ten minutes, it was then used to fill the chambers of the hemocytometer. This was accomplished by inverting the pipette and turning the reservoir upside down to dispense one full drop of the mixture (20 µl) into each chamber of the hemocytometer. Once both chambers were filled, the hemocytometer incubated for four minutes in a humidity chamber. This incubation period allows the sample to settle, while also preventing evaporation of the fluid. The hemocytometers were then counted using the method stated in the previous section.

Table 1. Standard deviation between experienced and inexperienced operators and among varying concentrations.

	Inter-chamber		Inter-replicates
Average Standard Deviation	10.22 (±1.22)		10.33 (±2.16)
Operator Difference	Inexperienced 1.97 (±2.03) higher		Inexperienced 2.80 (±1.96) higher
	<100 million cells/ml	100-200 million cells/ml	>200 million cells/ml
Average Standard Deviation	3.96 (± 0.58)	13.68 (±2.95)	29.16 (±11.52)

Statistical Analysis

A linear mixed-effects model was used to statistically compare the standard deviation in inter-chamber spermatozoa counts, as well as inter-replicate spermatozoa counts, between the experienced and inexperienced operators. Thus, in these models, the response variables were either the estimated standard deviation in inter-chamber spermatozoa or the estimated standard deviation in inter-replicate spermatozoa counts; the dependent variable was the operator experience level, and a random effect of ejaculate was included in these models as a blocking factor. A separate, similar mixed-effects model was used to compare the standard deviation between sperm count categories for the inter-replicate counts.

Results

The results of the precision tests are summarized in Table 1. The standard deviation in estimated spermatozoa concentration between chambers of the hemocytometer run by both the experienced and inexperienced operator averaged 10.22 (± 1.22) million spermatozoa with a 95% confidence interval (C.I.). Standard deviation in estimated spermatozoa concentration between the two chambers of the hemocytometer averaged 1.97 (± 2.03; 95% C.I.) million spermatozoa higher for the inexperienced operator relative to the experienced operator; however, differences were not statistically significant at the $\alpha = 0.05$ level ($t_{281} = 1.90$, $p = 0.058$).

Standard deviation in estimated concentration of spermatozoa between replicate samples for all ejaculates run by both the experienced and inexperienced operator averaged 10.33 (± 2.16; 95% C.I.) million spermatozoa. Standard deviation in estimated spermatozoa concentration between replicate samples of the hemocytometer was estimated to be 2.80 (± 1.96; 95% C.I.) million spermatozoa higher for the inexperienced operator relative to the experienced operator; however, differences were not significant ($t_{99} = 1.43$, $p = 0.1562$).

When the sample concentration averaged less than 100 million cells/ml, the standard deviation was 3.96 (± 0.58; 95% C.I.) million spermatozoa cells. The standard deviation for a sample concentration between 100 and 200 million cells/ml was 13.68 (± 2.95; 95% C.I.) million spermatozoa cells, and the standard deviation for a concentration over 200 million cells/ml was 29.16 (± 11.52; 95% C.I.) million spermatozoa cells. The categorical grouping of spermatozoa concentration was a significant predictor of standard deviation in spermatozoa counts ($F_{2, 98} = 43.29$, $p < 0.0001$), with all three categories significantly different from each other (all $t_{98} > 4.67$, $p < 0.0001$).

Discussion

Results indicate that even between the two chambers of a hemocytometer slide, estimated concentration of spermatozoa can easily vary by as much as 20 million spermatozoa from the mean. Similarly, even after minimizing such variation by using averages of both chambers, estimated spermatozoa concentration can vary between replicate samples by as much as 20 million spermatozoa from the mean. Although there is not a well-documented benchmark for a measure of clinical significance, an error of ≥ 15 million cells/ml is considered by some veterinarians to be a costly clinical mistake in canine reproduction (Wilborn,

personal communication). If sample concentration is overestimated, too few spermatozoa may be used for an insemination dose, resulting in lower pregnancy rates and reduced litter sizes. On the other hand, when sample concentration is underestimated, too many spermatozoa may be used for an insemination dose, and valuable semen that could have otherwise been used on an additional female may be wasted.

There was no significant difference between the precision of either inter-chamber counts or inter-replicate counts when run by an experienced operator versus an inexperienced operator. From a clinical standpoint, this is very important. Veterinarians are more efficient when time-consuming tests such as this one can be reliably performed by support staff such as veterinary assistants or technicians. Many veterinary practices have significant turnover in support staff and experience level varies greatly among these individuals. Therefore, it is critical to have a method of quantification that is highly repeatable with little training required.

In summary, the Neubauer hemocytometer is a simple method for determining the concentration of canine spermatozoa. Although this method is considered the standard for accuracy, precision did vary and could contribute to clinical errors when calculating breeding doses for canine artificial insemination. The level of operator experience did not have a significant effect on results, indicating that this method would lend itself to use by properly trained veterinary support staff with results comparable to those achieved by the veterinarian.

Acknowledgments

The authors would like to thank Drs. Bruce Smith and Rob Gillette for the use of the dogs, as well as Sara Beth Griffin and Steve Waters for their assistance with the animals.

References

Amann, R. P. (1986). Reproductive physiology and endocrinology of the dog. *in* D.A. Morrow (Ed.). *In Current Therapy in Theriogenology 2: Diagnosis, Treatment and Prevention of Reproductive Diseases in Small and Large Animals*. Philadelphia, PA.: W.B. Saunders, 532-538.

Atiq, N., N. Ullah, S. M. H. Andrabi, and S. Akhter (2011). Comparison of photometer with improved Neubauer hemocytometer and Makler counting chamber for sperm concentration measurement in cattle. *Pakistan Veterinary Journal*: 31 (1), 83-84.

Cooper, T. G. (2010). *WHO Laboratory Manual for the Examination and Processing of Human Semen*, Fifth Edition. WHO Press: Geneva, Switzerland.

Hansen, C., T. Vermeiden, J. P. W. Vermeiden, C. Simmet, B. C. Day, and H. Feitsma (2006). Comparison of FACSCount AF system, improved Neubauer hemocytometer, Corning 254 photometer, SpermVision, UltiMate and NucleoCounter SP-100 for determination of sperm concentration of boar semen. *Theriogenology*: 66 (1), 2188–2194.

Kuster, C. (2005). Sperm concentration determination between hemocytometric and CASA systems: why they can be different. *Theriogenology*: 64 (3), 614-7.

Leboeuf, B., B. Restall, and S. Salamon (2000). Production and storage of goat semen for artificial insemination. *Animal Reproduction Science*: 62 (1-3), 113-141.

Linde-Forsberg, C. (1991). Achieving canine pregnancy by using frozen or chilled extended semen. *Veterinary Clinics of North America: Small Animal Practice*: 21 (1), 467-485.

Samper, E. (2000). *Equine Breeding Management and Artificial Insemination*, Second Edition. Philadelphia, PA.: W.B. Saunders.

Wilborn, R. Personal interview. 11 November 2012.