Clinical methods for counting canine sperm: automated and manual techniques
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Introduction
The breeding soundness examination (BSE) in the canine primarily involves obtaining pertinent breeding history, assessment of overall health, careful examination of penis, testes and prostate, and semen analysis.1,2 Semen analysis is arguably the most integral part of the BSE. Assessment of semen quality requires determination of ejaculate volume and pH, assessment of all cell types present in ejaculate, determination of sperm concentration, assessment of total and progressive motility, and assessment of sperm morphology.1,3

Clinical theriogenologists and small animal reproductive veterinarians routinely make important decisions based on semen analysis. Accurately determining quality and concentration of the sperm has become increasingly important for the small animal practitioner, as semen freezing and artificial insemination techniques have become more commonplace. Also, many companies are promoting and selling instruments to small animal veterinary practitioners that aid semen analysis. These instruments include: computer assisted sperm analysis (CASA) systems, cell counters such as the NucleoCounter, and photometric devices such as the Spermacue and Densimeter. It has become evident that with the significant increase in the prevalence of more advanced artificial insemination techniques and the increasing availability of new technology used for semen analysis, there is an increased need to standardize techniques for evaluating canine semen. In fact, it has been shown in other species that external quality control and adherence to recommended, standardized procedures are extremely important for consistent, accurate semen analysis.4,5 This is well accepted in human medicine with adherence to the World Health Organization (WHO) standards for semen analysis. The Society for Theriogenology and American College of Theriogenologists have published “Guidelines For Canine Breeding Soundness Examination” which is intended to “promote consistency” for the canine BSE. In discussing these guidelines with SFT/ACT members, many find these guidelines very useful and regard them as an important standard. This paper will focus on clinical methods used for determining sperm concentration in the canine semen sample with some discussion comparing automated to manual counting methods and hopefully further the discussion on standardizing techniques for determining sperm concentration as part of the canine semen analysis.

Hemocytometer
The hemocytometer is a special microscope slide with a grid system etched into its surface and raised rails where a specifically designed cover slip sits exactly 0.1mm above the surface. The grid consists of 9 large squares, with the corner squares made up of 16 smaller squares and the remaining 5 squares made up of 25 smaller squares, each containing 16 very small squares. This pattern is replicated, as there are two identical chambers on each side of the slide. The design, when loaded and equilibrated properly, allows for a known sample volume to be evaluated. The operator counts sperm cells present on the grid system using one of a few counting methods.6,7 The central large square is known as the “counting area” by some counting methods. The hemocytometer is considered the gold standard for sperm counting in many species, including the canine.8-10

Dilution of the semen sample is often necessary for proper use of the hemocytometer. One must be able to count individual sperm cells with a high degree of certainty and overcrowding of sperm cells on the hemocytometer makes proper evaluation difficult. Historically, dilution was somewhat easy and consistent for the practitioner when using the Becton Dickinson Unopette system. Semen dilution was described using acetic acid (and later ammonium oxalate) diluent (1.98ml) and the included capillary pipette (20ul) to make a 1:100 dilution. However, with the discontinuation of Unopette system, the ease and consistency of semen sample dilution changed. Practitioners were left to make their own dilutions using bulk chemicals and pipettes, or pursue alternative methods of sperm counting while searching for a commercially available alternative. A few companies now offer Unopette alternatives including...
Biomedical Polymers (BMP) Leukocheck® and Animal Reproduction Systems (ARS) Thrombo-tic®. Research is ongoing comparing these, and other dilution methods, for canine semen samples for use with the hemocytometer.

Once the semen sample is appropriately diluted, several methods for counting the sperm using the hemocytometer have been described. Once, perhaps the easiest method for counting canine semen using the hemocytometer, and the method described by the ACT/SFT guidelines, is counting all of the sperm cells present in the central square, the “counting area”. The number of sperm counted in this area represents the number of sperm cells present in the sample, in millions/ml, when using a 1:100 dilution. Counting both sides of the hemocytometer using this method is important, making sure they are within 10% and taking the mean as million/ml sperm present in the sample. It is advised that if each side is not within 10%, the hemocytometer should be reloaded, equilibrated and counted again.

Another method that has been described for counting sperm using the hemocytometer is to count sperm cells present in five squares of the 25 squares that make up the “counting area”. The 5 squares are typically counted in either a diagonal or “star” pattern. The number of sperm cells present are multiplied by five, giving the number of sperm present in 0.1ul. Simply multiplying by 10,000 (to get number of sperm present per ml of sample) and the dilution factor, gives the number sperm cells, in millions/ml, present in the sample.

The final counting method that will be considered for this paper is to count the sperm cells present in three of the large nine squares of the hemocytometer. This is typically done using three of the corner squares, not the central “counting area” square. The number of sperm cells present in each square are added together, multiplied by three and 10% of this value is added back in. The subsequent value is divided by 10 to obtain number of sperm cells present in the sample, in millions/ml, when a 1:100 dilution is used.

While the hemocytometer certainly has its limitations and drawbacks, it remains the “gold standard” for determination of sperm concentration to which all other methods are compared.

Computer-assisted sperm analysis

Computer-assisted sperm analysis (CASA) has been in use for over 35 years. It was borne out of the need for a more objective method for assessment of sperm quality. Computer-assisted sperm analysis assesses parameters such as: motility (overall, progressive, hyperactive), morphology, morphometry, viability and concentration. Most CASA systems work by using a camera attached to a microscope that scans several fields of a specially designed slide (typically shallow well). The images are digitized and analyzed by a computer program.

There are many CASA systems available for sale that are marketed for use to small animal veterinary practitioners, especially over the last 10 years. The modern systems have become increasingly user friendly and more economically feasible for use in small animal reproductive practices. These systems provide the practitioner with a quick and easy assessment of several sperm parameters. However, CASA systems have several drawbacks, and proper use is imperative for accurate results.

Perhaps one of the most interesting and consistent inaccuracies with CASA is determination of sperm concentration, especially at lower concentrations. It has been written that sperm concentrations <20 x 10⁶ to 50 x 10⁶/mL are most likely unreliable and should be checked using manual counting methods. This illustrates the need to remain proficient in manual methods of canine sperm counting. Some of the causes that have been suggested for the inability of CASA to accurately determine sperm concentration are inappropriate slide/chamber use, Segre-Silberberg effect, mis-identification of debris as sperm and collision artifact.

Cell counter

NucleoCounter® is a cell counter that has gained popularity over the last several years in the small animal clinical reproductive setting for counting canine sperm. It can be used with both raw and extended semen samples. The NucleoCounter® essentially works like a fluorescent microscope which captures images with a CCD camera and displays them on a computer screen. It uses an LED light instead of a
laser, like a traditional flow cytometer cell counter uses. Also, it is fully enclosed and somewhat portable. The cassettes used with the NucleoCounter®, for sperm analysis, contain propidium iodide (PI) stain and an integrated pipette that mixes the appropriate amount of stain and semen sample for analysis. The result is fluorescence and counting of nuclear material only, not debris. The NucleoCounter® been shown to be an accurate method for counting animal cells, including the counting and estimated viability of sperm cells.17-20 The number of viable cells in the sample is determined by counting the non-viable cells and total number of cells. This is done by staining (PI) the non-lysed sample and performing a count, then comparing it to the total count (lysed sample). The (PI) will only stain nuclear material in a sperm cell whose plasma membrane has been compromised, which indicates a non-viable status, not a true live/dead status.19

The NucleoCounter® is an easy to use and an accurate automated device to count sperm. In fact, it has been suggested that it should become the new gold standard for determining sperm concentration.6 Research is ongoing comparing its precision and accuracy for counting canine sperm. (personal observation)

**Photometric devices**

The Spermacue® and Densimeter® are photometric measuring devices that have been marketed to small animal reproductive veterinarians for several years. They work by measuring the optical density of semen samples. The light transmission data is ultimately converted to an estimate of sperm concentration and reported in million/ml. The devices are relatively inexpensive and easy to use. Due to the nature of how they estimate the sperm count (light transmission), any nonsperm cells in the sample contribute to inaccuracies in the measurement. In addition, the manufacturers caution that the estimates of sperm concentration become significantly less accurate with very dilute or very concentrated semen samples. Manufacturers of other automated sperm counting devices (CASA) also make this caution.

When using photometric devices to estimate sperm concentration, each sample should be screened using a microscope prior to use. Most practitioners place a small drop of the sample onto a warm slide and quickly scan under different magnifications. It is extremely important to perform hand counts on samples that contain excessive numbers of other cells, such as white blood cells, bacterial cells or epithelial cells. Debris, such as fat globules or cytoplasmic droplets should also be considered. Dilution, centrifugation or hand counts are also necessary for sample concentrations that do not fall within the manufacturers concentration recommendation for accuracy.

**Comparative sperm analysis**

Scientific articles comparing sperm counting methods have been written for many years. However, most of the comparative research has focused on species other than the canine, with few exceptions. Recently, there is increased interest in determining canine sperm concentrations, with greater accuracy, as more advanced reproductive techniques utilizing minimal numbers of sperm become commonplace in small animal reproductive practices. Besides being an integral part of the BSE, it is important to accurately determine sperm concentration when working with small or compromised semen samples and accuracy is imperative when packaging frozen canine semen samples to ensure correct insemination dose.

Historically, a dose of 150 x 10⁶, progressively motile sperm cells has been described for insemination in the canine.3 However, different insemination doses for the canine have been reported, for alternative methods of insemination, with varying success.21-24 Ensuring that the assessed sperm concentration is accurate may lead to a better estimate of the most appropriate insemination dose needed for particular methods of insemination.

The aim of our current research is to compare several different, but commonly used dilution methods available for the small animal practitioner to use with the hemocytometer. We will also compare the previously described methods for using the hemocytometer to count canine sperm cells. Additionally, we will compare the accuracy and precision of the most common measuring devices used by small animal reproductive practitioners for determination of canine sperm concentration.
References

19. Morrell JM, Johannisson A, Juntila L, et al: Stallion sperm viability, as measured by the NucleoCounter SP-100, is affected by extender and enhanced by single layer centrifugation. Vet Med Int 2010;2010(0):659862.