"This stud's a dud!"- Canine semen evaluation protocols and pitfalls

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Abstract

Properly performed canine semen evaluation is a major component of breeding soundness examination. Various types of equipment and protocols are available to practitioners and laboratory technicians to accurately assess sample concentration, motility and morphology. However, it is vital that precise laboratory standardization and quality control procedures be followed to optimize accuracy and usefulness of a semen evaluation.

Keywords: Semen evaluation, canine, dog, breeding soundness examination

Applications of a semen evaluation

Importance of an accurately performed semen evaluation as part of a breeding soundness examination cannot be over emphasized. Results are used to monitor dogs in a breeding program and those undergoing therapies for subfertility or infertility. Complete semen evaluation should always be done as part of pre-purchase examination or evaluation of chilled or frozen semen for artificial insemination.

No single parameter from semen evaluation that practitioner can use to accurately assess fertilizing capability of a dog or predict outcome of a successful litter.[1,2] However, breeding recommendations to maximize likelihood for successful pregnancies are possible using dependable and precise semen evaluation results.

Breeding soundness examination and paperwork

Obtaining a detailed history and performing a thorough physical examination prior to collecting a dog is vital, as there are many outside factors that may affect quality and accuracy of semen that might otherwise be overlooked.[3] Society for Theriogenology (SFT; www.therio.org) has a comprehensive breeding soundness examination form (refer next page) to record reproductive history, pedigree, physical examination and semen evaluation, to promote consistency and accuracy.

Laboratory setting

Prior to collecting dog, microscope stage, slides and coverslips should all be pre-warmed to 37 C^{o} to avoid any temperature shock when transferring samples for evaluation.[4] Correct labeling of all slides, test tubes and containers used in evaluation with a permanent marker is essential for record accuracy.

Semen analysis reliability

World Health Organization laboratory manual recommends standardized, evidence-based procedures for human semen analysis to improve reliability, accuracy and comparability of results from laboratories.[5] Unfortunately, these protocols are deficient in most canine commercial laboratories and private practices. This is a dilemma not only for researchers trying to analyze data, but also for practitioners depending on semen analysis accuracy for breeding recommendations or to monitor success of canine reproductive therapies.

Semen volume

Volume of ejaculate collected is not correlated to semen quality, but is required to calculate semen concentration,[2] so must be accurately recorded prior to starting any laboratory testing. Volume of first fraction varies from 1 - 5 ml and second fraction from 1 - 3 ml.

CANINE BREEDING SOUNDNESS EVALUATION

Guidelines Established by the Society for Theriogenology
761 Tiger Oak Drive, Pike Road, AL 36064-3063 • (334) 395-4666

AKC Reg #/Other ID # _____ Exam Date: _____
Call Name: _____ Registered Name: _____
Client Name: _____ Address: _____
Breed: _____ Color: _____ Date of Birth: _____

HISTORY
Reason for evaluation: _____
Date of last litter: _____Brucellosis test: positive/negative Date of test: _____Test used_____
Infertile relatives: _____

PHYSICAL EXAMINATION
Body condition: _____ Weight: _____ pounds/kilograms
Pertinent health problems: _____
Penis/Prepuce: _____ Scrotum: _____
Prostate:_____
Epididymides: (R) _____ (L) _____
Testes Width: (R) _____ (L) _____ Spermatic cords:_____
Testes Consistency: (R) _____ (L) _____ Masses/fluid/pain/other: _____

SEMEN COLLECTION & EVALUATION
Libido/Ease of collection: Poor / Fair / Good / Excellent Teaser bitch present: Yes/No Stage of cycle: _____

	Color	Volume (ml)	Concentration (sperm/ml)	Total Sperm/Ejaculate
Fraction 1	_____	_____		
Fraction 2	_____	_____	_____	_____
Fraction 3	_____			

Total Motile Spermatozoa: _____% Progressively Motile Spermatozoa: _____% Speed/velocity of motility: 0-5_____

Morphology: Stain(s) utilized: _____
% Normal: _____ Number Cells Counted: 100/200
Head defects (%): _____
Midpiece defects (%): _____
Tail defects (%): _____
Other defects (%)_____

Longevity: Extender(s) used: _____ Diluent rate: _____
Motility: 24 hours_____ 48 hours_____
Cytology: Fraction(s) evaluated: _____ Stain(s) utilized: _____
Presence of RBC, WBC, Epithelial cells, Bacteria, Germ cells (0 – 4+):
_____

CONCLUSIONS / COMMENTS / INTERPRETATION

Signed: _____ Clinic Name: _____
(Member – Society for Theriogenology) Address: _____

Semen color

Subjectively, second fraction is normally opalescent. Blood causes a pink or red discoloration possibly from either prostatic disease or penile trauma. A yellow color could indicate urine contamination or riboflavin (often present from dogs on raw diets) whereas no color often is an indicator of few to no sperm in the sample. A green hue could indicate an inflammatory exudate or excess smegma.[6]

Semen pH

Validity of this parameter is controversial and it is not on the SFT breeding soundness evaluation form. Unfractionated canine semen pH ranges from 6.4 - 6.8. Inaccuracy of pH paper strips along with newer antibiotics that ionize at multiple pH values decreases pH testing validity in dogs.[7]

Semen motility

Evaluation of sperm motion cannot be used to accurately predict fertilizing potential of sperm in a given sample when used for artificial insemination.[8] Motility evaluation should be done as soon as possible after semen collection.[9] There are 2 main methods for evaluating progressive motility, which normally should be $\geq$ 70% for dog sperm.

First method is a subjective evaluation of semen motility and involves assessing a drop of semen flattened between a microscope slide and a coverslip. Normal sperm should traverse microscopic field in ~ 2 - 3 seconds in a forward progressive movement.[2] Motility is best assessed using a properly aligned phase contrast microscope at 250X magnification.[10]

Layer of sperm should be only 1 layer thick so that motility can be accurately assessed in 1 plane and there should be enough room between sperm to assess individual sperm movement. If viewer detects several layers of sperm, sample should be diluted with an isotonic diluent and a less concentrated sample is viewed to improve subjective assessment. One drop of semen from a Pasteur pipette (pipette capacity, ~ 7 ml) is the correct volume to allow for even distribution and movement of sperm under a 22 x 22 mm coverslip placed on a slide.[11] Use of a smaller coverslip (18 x 18 mm) may put less pressure on sperm and allow more free movement.

Sperm motility is generally highest at center of coverslip and decreases towards edges. Therefore, viewer should assess those regions across coverslip equator and average motility observed in several viewing fields to increase subjective score accuracy. It is recommended that 2 trained viewers assess slide and average motility value of 2 independent results is recorded. Velocity of movement is also recorded, usually on a scale of 0 - 5 (with 0 being no movement, and 5 being very fast progression).

It is important to differentiate total motility from progressive motility; to do this, individual sperm movement is assessed. If sample is too concentrated, nonmotile sperm may be pushed around by motile ones, leading to an inflated motility score.

Second method for evaluating semen motility is computer-assisted sperm analysis system (CASA), an expensive, automated system that visualizes and digitizes successive sperm images, processes them, and then analyzes information giving precise data regarding motion of individual sperm. When properly calibrated and used with appropriate software parameter settings, a CASA system provides both accurate and precise information on detailed kinematic parameters such as curvilinear velocity, straight line velocity, and amplitude of lateral head displacement.[12,13] However, machine parameters such as imaging hardware and software, recording quality and camera frame rate can alter motility results.[14] Concentration of sperm, extenders, amount of debris, crystals and immotile dead sperm heads present in sample also alter motility results when using CASA systems.[13] These issues, compounded by intensive staff training and continuous standardization and machine maintenance, make CASA use challenging in private practice.

Sperm concentration and number

Sperm reservoir depletion decreases sperm concentration,[15] so frequency and interval of prior collections before performing an evaluation should be known. Sperm concentration can be determined manually or using automated methods. Once the concentration is known, total sperm number is calculated by multiplying concentration (# sperm/ml) by total volume (ml) of ejaculate collected. In dog, normal total number of sperm in ejaculate is 300 million - 2 billion.[2] Sperm production is dependent on grams of testicular tissue; total sperm number is ~ 10 million sperm per pound of body weight. For example, a typical value for a 10-pound dog would be 100 million sperm.

Manual hemocytometer has long been considered the gold standard for precisely counting canine sperm. However, the Nucleocounter® SP-100™ (Chemo-Metec A/S, Allerod, Denmark) discussed below has replaced it in recent years.[16,17] This manual method is very inexpensive and accurate when performed by trained staff. It is important to use specified cover slip corresponding to hemocytometer and to count both chamber sides. If there is more than 10% discrepancy between sides, chambers should be refilled, equilibrated and recounted. To maintain errors < 5%, it is recommended to count a minimum of 400 sperm per sample.[18] Care must be taken to properly fill chambers and count all sperm heads in designated counting grid only once to improve accuracy.

Differences in sperm concentration between CASA systems and hemocytometer are largely due to the Segre-Silberberg effect, which does not occur when using a hemocytometer due to a deeper sample chamber compared to most disposable slides used in automated systems.[17] Problems with sperm count are similar to assessing motility, sperm count can be inaccurate when low or high concentration semen samples are analyzed. CASA reliability is very dependent on users technical competence.[18-20]

Another automated method is the Nucleocounter® SP-100™. Sperm treated with a detergent are aspirated into a cassette lined with propidium iodide, which crosses cell membrane and binds specifically to DNA. Fluorescently labeled sperm are then quantified. Because sperm identification is so specific to DNA, there is no interference from debris, concentration, or extenders.[21]Pipetting must be done correctly for this machine to provide accurate counts.

Photometric devices such as the Spermacue® or Densimeter ® that depend on quantitative light transmission measurement through a diluted semen sample are easy to use, and are relatively inexpensive. Samples that contain a lot of debris (e.g. leukocytes, bacteria, cytoplasmic droplets, etc.), are extremely concentrated, or very dilute should not be run using photometric devices, as results will be inaccurate.[20] Only raw semen can be used in photometric devices; extended semen can only be used if t extender has no optical density (i.e. is clear).

Flow cytometry using detection of light scatter and fluorescence of individual sperm is a very precise and accurate method to determine sperm concentration. However, equipment cost, need for skilled technicians, and involved sample preparation techniques limit this technique to research.

Quality control is critical, irrespective of whatever form of counter is used. When semen is evaluated grossly and microscopically, an estimate of sperm concentration should be performed. Motility samples should be assessed for extraneous cellular debris (epithelial cells, WBC, RBC) that may interfere with accurate counting. If semen is centrifuged, the size of the sperm pellet can be used to estimate total sperm in the ejaculate. If the count calculated by whatever method is used does not match with estimated count based on the above factors, count should be repeated or calculated using another (ideally more accurate) method.

Sperm morphology

Assessment of sperm morphology is a subjective evaluation susceptible to large discrepancies between evaluators. A 79.4% inter-laboratory variability is reported in assessing canine sperm morphology.[22] Phase contrast or differential interference contrast microscopy is one technique to view sperm fixed with formol buffered saline as a wet mount, thereby limiting damage done to sperm using staining techniques necessary for light microscopy.[2,23]

Specific structural defects are known to be associated with male infertility. Staining techniques help visualize sperm defects when viewed under oil immersion, but compared to phase microscopy, the staining preparation technique may contribute to morphologic artifacts.[24] Four common staining techniques (conventional, dipping and blotting, direct mixing and ignition) were compared and it was concluded that as long as slides were made carefully according to protocols, percentage of abnormal sperm was constant across all 4 techniques.[25]

Eosin-nigrosin (Hancock's) stain (available from SFT) is a common stain for sperm morphology. Typically, 1 drop of semen is mixed with 1 drop of stain, spread like a blood smear and allowed to dry. Dark slide background from nigrosin facilitates visualization of sperm head, midpiece and tail. This stain is a "vital" stain, meaning that eosin is able to permeate damaged plasma membranes of dead sperm,

staining them pink, compared to white or clear live sperm.[2,20] Sometimes there is only partial staining of sperm and staining may not be consistent if there are fat globules in seminal fluid[2] making it more challenging to clearly designate sperm as alive or dead. Round cells (germ cells, erythrocytes and leukocytes) and acrosomes cannot be easily differentiated.

Giemsa-Wright stain (Diff-Quick ™ Baxter Healthcare, Miami FL; Romanowski stain) is a quick, inexpensive, 3-step stain that most clinics use for blood smears. Slide is immersed 5 minutes sequentially in fixative, safranin, and crystal violet, lightly rinsed with water to remove stain and then dried. Artifacts may be minimized by drying the stained smear on a slide warmer set at 37 degrees C or by blowing.[2] This stain is good for round cells but cannot be used to assess acrosome and cytoplasmic droplets are difficult to observe.

Spermac stain is more labor intensive and costly, however, an excellent stain for evaluating acrosome, equatorial, midpiece and tail regions of sperm.[20]

Normal percentage of morphologically normal sperm (MNS) for canines should be $\geq$ 80%[6,9] and fertility appears to be affected if there is < 60% MNS.[2] A minimum of 100 sperm are evaluated and the abnormal cells classified as: 1) primary (occurring during spermatogenesis) or secondary (occurring during maturation or sample preparation); 2) major (negatively affecting fertility) or minor (not associated with fertility); or 3) compensable (improved fertility by increasing sperm numbers) or non-compensable (fertility not increased by increasing sperm numbers).

Other cell types in semen samples

In addition to sperm, semen samples may contain erythrocytes, leukocytes, epithelial cells and immature germ cells. These are usually noted on the breeding soundness evaluation sheet as numbers of cell type/100 sperm.

Advanced tests

Many specialized stains, assays, and tests are available to evaluate sperm morphology, function, chromatin, membrane integrity, etc. in dogs displaying suboptimal fertility with normal semen evaluation values. One example is the hypoosmotic swelling test, used to evaluate membrane integrity of sperm by assessing tail curling after incubation in hypoosmotic solution.

Conclusion

Routine semen evaluation is an integral part of a canine breeding soundness exam, easy to perform and a valuable tool to evaluate sperm. Results will only be reliable and accurate if equipment is routinely maintained and calibrated. To minimize error, laboratory personnel should undergo training protocols and run standardized procedures with quality control. Perhaps someday we will be able to perform a semen evaluation and, using a new parameter, reliably predict fertilizing potential.

Conflict of interest

There are no conflicts of interest to declare.

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