How to Evaluate Semen in the Field

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Fresh and cooled, transported semen can be effectively evaluated in the field using a minimal amount of equipment. These tools will aid the practitioner in evaluating transported semen for insemination as well as fresh semen for packaging and shipment. Author’s address: Dept. of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610. © 2001 AAEP.

1. Introduction
Transported cooled semen has become widely accepted in the equine industry. Equine veterinarians are increasingly involved with both insemination of mares using transported semen and packaging semen for shipment. Successful breeding using transported semen relies on several factors including:

- Fertility of the mare and stallion
- Quality of collected semen
- Quality of semen after cooling
- Number of sperm used for insemination
- Mare management

Field semen evaluation enables the practitioner to provide many services to the client. Horses can be evaluated for breeding soundness prior to the onset of the breeding season. Transported cooled semen can be fully evaluated at the time of insemination so that the veterinarian and client are informed of the quality of ejaculate used for insemination. Finally, veterinarians can evaluate, package, and ship semen knowing that they have sent adequate numbers of good quality sperm for insemination. However, the ambulatory veterinarian faces several challenges such as lack of equipment, inadequate space, and temperature control when evaluating semen. The objective of this article is to describe simple techniques for determining sperm concentration, motility, and percent morphologically normal sperm of ejaculates collected in the field. Methods for proper dilution and packaging semen are also described.

2. Materials and Methods
Semen evaluation includes gross evaluation of raw semen for color and estimate of concentration, determination of ejaculate volume, determination of concentration (sperm/ml), estimate of sperm motility, and determination of the number of morphologically normal sperm present in the ejaculate.

Tools
Tools needed for semen evaluation in the field are:

- inexpensive bright-field microscope
- hemocytometer
- Platelet/WBC Unopette® Microcollection systems (#5855)
- glass microscope slides and cover slips
Gross Evaluation of Semen

Methods of handling semen will depend on whether the ejaculate is fresh or cooled. Ideally, a fresh ejaculate is maintained in a light-shielded environment at 37°C during evaluation. Temperature control is especially important in colder climates. Semen can be insulated from dramatic temperature fluctuations in a small cooler during evaluation. If the ambient temperature is low, a 1-l bag of fluid can be heated in the microwave and placed in the cooler to keep the environment warm. Care should be taken to avoid placing the container of semen in direct contact with something that is hot. The insulated collection bottle cover designed to fit the Colorado model artificial vagina is useful for holding and protecting semen in a cooler.

The ejaculate can be evaluated grossly in the collection bag or a sterile urine specimen cup. A graduated urine specimen cup also allows for crude measurement of volume. The color of the ejaculate should be noted. A normal ejaculate should be white and opaque. A creamy appearing ejaculate typically is more concentrated while a watery ejaculate often has fewer sperm cells. Abnormal ejaculates may be yellow (urine contamination, white blood cells) or pink (hemorrhage).

Concentration

Determining the concentration of an ejaculate is one of the more important steps in semen evaluation, yet is one of the most overlooked steps. The concentration of sperm in the ejaculate is multiplied by the ejaculate volume to determine the total number of sperm in the ejaculate. Knowing the concentration of sperm per ml of semen is also important when extending semen for shipment.

Using a hemocytometer and the Unopette® system, one can easily determine the concentration of an ejaculate. The Unopette® system provides a simple method for obtaining a 1:100 dilution of semen to determine concentration using a hemocytometer. Dilution of semen to 1:100 in any solution that will immobilize sperm (i.e., buffered formal saline) will work. The Unopette® system is self-contained with a 20-μl capillary tube and a reservoir filled with 1.98 ml of buffered ammonium oxalate solution (Fig. 1). The plastic cover over the capillary tube is used to pierce a hole into the reservoir. A well-mixed sample of semen is obtained through capillary action into the 20-μl tube. After wiping the sides of the capillary tube, the tube is placed into the reservoir and gently rinsed with the ammonium oxalate solution. Care must be taken not to expel any of the fluid contents during this rinsing procedure. Once the fluid in the capillary tube is expelled into the chamber, the capillary tube is inverted and seated into the top of the reservoir. A hemocytometer is fitted with a cover slip that will cover both chambers. The reservoir is inverted several times for mixing and the first drops of fluid are expelled from the tube by squeezing the reservoir. A drop is loaded into each chamber using care to avoid overfilling the chambers. The hemocytometer should be allowed to stand for approximately 5 min to allow the sperm cells to settle before counting.

The count is performed using 200× magnification and bright-field microscopy (Fig. 2). The central square that is subdivided into 25 smaller squares should be counted. All intact sperm and sperm heads within the 25 squares should be counted. Sperm falling on upper or left borders are counted while sperm on lower or right borders should not be counted to avoid double counting sperm. Both chambers on the hemocytometer are counted and the numbers averaged if the difference between the two is not greater than 10%. The averaged number is multiplied by 10⁶ to get the final concentration.
Example

325 sperm in chamber 1
329 sperm in chamber 2

\[(325 + 329) \div 2 = 327 \times 10^6 \text{ sperm/ml} \]

Automated methods for determining sperm concentration include the Equine Densimeter, the Spermacue and the HRI Sperm Counter. All of these systems are portable. While the automated sperm counting systems provide a concentration more quickly than hand-counting, the systems are also substantially more expensive. Furthermore, the automated systems can only be used with raw semen. Milk or egg yolk proteins in semen extend the automated systems can only be used with raw semen. Milk or egg yolk proteins in semen extend the systems are portable. While the automated sperm counting systems provide a concentration more quickly than hand-counting, the systems are also substantially more expensive. Furthermore, the automated systems can only be used with raw semen. Milk or egg yolk proteins in semen extend.

Sperm Motility

Temperature is a critical factor when evaluating sperm motion. Most field microscopes will not be fitted with a stage warmer, therefore measures must be taken to maintain samples at 37°C during motility evaluation. Simple tricks can be used to warm slides, cover slips, and pipettes. Any square glass bottle can be filled with warm water and laid on its side to serve as a slide warmer. Warmed, full fluid bags can also serve this purpose. A heating pad may be used as a slide warmer. Small, portable slide warmers are also available. Again, care must be taken to maintain components as close to body temperature (37°C) as possible for the most accurate assessment of motility.

Both raw and extended samples of semen should be examined for motility when evaluating a fresh ejaculate. Semen extender should be warmed to 37°C prior to mixing it with semen. Extender can be warmed by placing the diluent into a zippered plastic bag and immersing it in warm water in a stainless steel bucket. The bag prevents the diluent from becoming contaminated with tap water, which may contain elements that are detrimental to sperm. If rapid warming of the extender is required, it is important to warm the extender diluent before adding the milk-based components. Proteins are quickly denatured at high temperatures and the beneficial effects of the extender will be lost.

For motility analysis, semen can be mixed with warmed extender at a ratio of 1:3 or 1:4. A drop of semen is placed on a warmed slide, covered with a slip and quickly evaluated for total motility and progressive, linear motility at 200×. The sample will quickly cool without a warm microscope stage, so several fresh samples should be evaluated rather than prolonged examination of one sample. If excessive sperm circling is noted, it is likely that the sperm are cold-shocked by some component of the system. If the sperm are sluggish or die rapidly, the sperm may be stressed by temperatures that are too high.

Sperm Morphology

Specific morphologic features of sperm have been associated with pregnancy rates in bred mares. Morphologic evaluation of sperm can be also used as a predictor of fertility in stallions. To prepare a semen sample for morphologic evaluation, a drop of well-mixed semen is mixed with a drop of stain on a microscope slide. A second slide is used to make a smear. Eosin-nigrosin stain is a background stain, therefore the best slide is obtained when the smear is not overly thin (higher contrast between sperm and stain). The slide is allowed to dry and can be evaluated immediately or stored and evaluated later in the day. Sperm abnormalities are optimally detected using immersion oil and 1000× magnification. A minimum of 100 cells should be evaluated for the following abnormalities:

- normal sperm
- abnormal sperm head
- abnormal acrosome
- detached sperm head
- proximal cytoplasmic droplet
- distal cytoplasmic droplet
- abnormal midpiece shape
- bent midpiece
- hairpin tail
- coiled tail
- premature germ cell

In a recent study, the morphologic abnormalities indicated in bold were significant predictors of stallion fertility.

Evaluation of Cooled, Transported Semen

Transported semen can be evaluated as rigorously as fresh semen using the tools outlined above. A small aliquot of well-mixed extended semen should be reserved in an all-plastic tube or in the bottom of the shipment bag. Procedures for concentration and morphology are the same as those outlined for fresh semen. A hemocytometer count is the only reliable method for determining concentration in extended samples that are not optically clear. The insemination dose can be determined by multiplying the volume of the extended semen by the concentration.

Example

40 ml extended semen/dose × 25

\[40 \times 10^6 \text{ sperm/ml} = 1 \times 10^9 \text{ sperm/dose} \]

For optimal motility analysis, cooled semen should be allowed to warm to body temperature (37°C) as cooled semen frequently will be sluggish or circling. However, an immediate overall motility
estimate is useful for comparative purposes in the event the sample becomes markedly worse over time. The reserved semen sample is then warmed by placing it in a breast or pants pocket for 15 to 30 min and evaluated again.

Packaging Semen for Transport

The overwhelming popularity of breeding with cooled transported semen is increasing the demand on equine practitioners to collect and package semen for shipment. Traditionally, practitioners have diluted semen at a standard ratio of 1:4 (semen:extender) for shipment. This dilution ratio maintains sperm viability in many cases. However, in vitro work\(^2\) has shown that equine sperm diluted to a concentration of 25–50 \(\times 10^6\) sperm/ml maintained motility parameters longer than samples diluted to higher sperm concentrations. Furthermore, ejaculates with a low initial sperm concentration can be over diluted which may result in an inadequate insemination dose.

In the best-case scenario, the sperm concentration should be determined from an ejaculate and the ejaculate diluted to a final concentration of 25–50 \(\times 10^6\) sperm/ml. A small aliquot of raw semen can be reserved to determine concentration. The remainder of the ejaculate can be extended 1:1 initially and diluted further once the concentration is determined.

Certain constants can be remembered when packaging semen for shipment:

*Constants to Remember*

Usage: 25–50 \(\times 10^6\) sperm/ml

Minimum dilution of 1:4 (semen:extender)

Common shipment dose = 1–2 \(\times 10^9\) sperm

- \(1 \times 10^9\) sperm \(\div 25 \times 10^6\) sperm/ml = 40 ml dose
- \(1 \times 10^9\) sperm \(\div 50 \times 10^6\) sperm/ml = 20 ml dose

- \(2 \times 10^9\) sperm \(\div 25 \times 10^6\) sperm/ml = 80 ml dose
- \(2 \times 10^9\) sperm \(\div 50 \times 10^6\) sperm/ml = 40 ml dose

*Examples*

**Ejaculate 1:**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Concentration</th>
<th>Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 ml semen</td>
<td>(250 \times 10^6) sperm/ml</td>
<td>90/80</td>
</tr>
</tbody>
</table>

- 35 ml semen \(\times 250 \times 10^6\) sperm/ml = 8.75 \(\times 10^9\) sperm available in this ejaculate
- (Approximately 8 full doses at 1 \(\times 10^9\) sperm/dose and 4 doses at 2 \(\times 10^9\) sperm/dose)

You want to send 2 billion sperm/dose, and you want to send 2 doses (total 4 billion sperm).

You have: \(2 \times 10^9\) sperm \(\div 25 \times 10^6\) sperm/ml = 80 ml extended semen/dose

Alternatively, to send 2 billion sperm/dose at 50 \(\times 10^6\) sperm/ml:

You want: \(2 \times 10^9\) sperm \(\div 50 \times 10^6\) sperm/ml = 40 ml extended semen/dose

You have: \(2 \times 10^9\) sperm \(\div 250 \times 10^6\) sperm/ml = 8 ml semen needed

40 ml extended semen/dose = 8 ml semen = 32 ml extender (≈1:4 ratio)

**Ejaculate 2:**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Concentration</th>
<th>Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 ml semen</td>
<td>(65 \times 10^6) sperm/ml</td>
<td>50/40 (2)</td>
</tr>
</tbody>
</table>

75 ml semen \(\times 65 \times 10^6\) sperm/ml = 4.875 \(\times 10^9\) sperm available in this ejaculate

(Approximately 4 full doses at 1 \(\times 10^9\) sperm/dose and 2 doses at 2 \(\times 10^9\) sperm/dose)

You want to send 2 billion sperm/dose, and you want to send 2 doses (total 4 billion sperm).

You have: \(2 \times 10^9\) sperm \(\div 25 \times 10^6\) sperm/ml = 80 ml extended semen/dose

You have: \(2 \times 10^9\) sperm \(\div 65 \times 10^6\) sperm/ml = 31 ml semen needed

80 ml extended semen/dose = 31 ml semen = 49 ml extender (≈1:1.6 ratio)

If this sample were empirically diluted to 1:4 (semen:extender), a suboptimal dose would be shipped (if 10 ml semen were diluted with 40 ml extender, each dose would only contain 650 \(\times 10^6\) sperm). If this ejaculate is diluted as outlined above, the ratio of semen to extender is far too low to maintain sperm viability. This ejaculate would be best managed by extending 1:1, centrifuging for 10 minutes at \(\approx 1200\) RPM and resuspending the pellet with enough semen extender to get a final concentration of 25–50 \(\times 10^6\) sperm/ml.

Once the ideal dilution ratio has been determined, the semen is mixed with extender that has been pre-warmed to 37°C. Several commercially available, nonfat dry skim milk–glucose extenders may be used. A stallion’s semen should be tested in several extender and antibiotic types prior to shipping semen for commercial purposes to determine which extender best maintains sperm viability.

The extended semen can be packaged for transport in an 8-oz baby-bottle liner. Excess air is removed from the bag, the neck of the bag twisted, folded over, and the bag secured with a rubber band. Double bagging is advisable in the event the original bag leaks. Once the semen is packaged, it is placed in a storage container for shipment. Several com-
Commercial containers for storage and transport are available such as the Equitainer™, Equine Express™, ExpectaFoal™, Bio-flite™ and Lane STSTM. The Equitainer™ is a durable semen transport system intended for repeated use. The other listed containers are disposable systems marketed for one-time use. There is significant variation in cooling rate and storage temperature between systems.3,4 Extended semen from a stallion should be tested in the transport container intended for commercial shipment to determine the predicted viability of the sperm after cooling and storage.

3. Summary

Using these methods, the practitioner can perform quality semen analysis in the field. Most of the items listed for semen evaluation are compact and can be easily carried in a truck. As the demand for breeding with transported cooled semen increases, veterinarians will find that semen analysis is an important component of their daily practice.

References and Footnotes


*Bright-Line® Counting Chamber with Neubauer Ruling, American Optical, South Bridge, MA.
*Becton Dickinson #5855, Rutherford, NJ.
*Society for Theriogenology, Nashville, TN.
*Animal Reproduction Systems, Pomona, CA.
*Animal Reproduction Systems, Pomona, CA.
*Minitube of America, Verona, WI.
*Hamilton-Thorne, Beverly, MA.
*MP and J Associates, Des Moines, IA.
*Expecta, Parker, CO.
*Bio-Flite™, Anaheim, CA.
*Lane Manufacturing, Denver, CO.