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Examination of the stallion for breeding soundness - what a five stage vetting doesn’t tell you

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Semen collection techniques
Semen samples can be obtained from intact stallions by manual stimulation, chemical ejaculation, condom or artificial vagina. For decades stallion semen has been collected using artificial vaginas (AVs) for the purpose of evaluating semen production and quality and for procurement of sperm for artificial insemination or preservation. A variety of models of AVs have been developed and many will work well to reliably obtain semen samples provided they meet certain basic requirements. These requirements include; the ability to provide sufficient stimulation to the stallion’s penis through pressure and elevated temperature (typically 45–50°C), the ability to adjust size and temperature for requirements of individual stallions, a nonspermicidal receptacle to recover the ejaculated semen and protect sperm from changes in temperature and the ability to be positioned or held in place alongside an oestrous jump mare or phantom. The inner liners used for the AV must be lubricated, nonspermicidal and soft enough to promote ejaculation. The AVs most commonly used in the United States are the Colorado model and the Missouri model. In both of these models, elevated internal pressure and temperature is achieved by filling the space between 2 rubber or latex liners with warm water. Many AVs have liners or collection receptacles that include an in-line filter to prevent the mixing of sperm rich fractions of the ejaculate with the gel-fraction (contributed by the seminal vesicles) and any debris that may be introduced during collection. Nylon mesh filters are preferred over cotton or polyester ‘milky-line’ filters as they do not absorb significant numbers of spermatozoa when the semen is filtered as is the case with the milky-line filters.

In some European countries, open-ended AV’s are commonly used to collect stallions. This technique allows the collection of ‘fractionated ejaculates’ or only the first few (sperm-rich) fractions of the ejaculate leaving behind or collecting separately the fractions of the ejaculate that contain few sperm and primarily secretions from the accessory sex glands.

Regardless of the AV used it is imperative that during the collection process the fragile spermatozoa are not damaged. Stallion sperm are extremely susceptible to damage from changes in temperature and osmolarity and from exposure to spermicidal substances such as soap residues on collection equipment. One criticism of the older Colorado model AV’s was that because they were so long (21 inches [53.34 cm]) that a stallion with a short penis was ejaculating in the interior of the AV possibly trapping the sperm in the AV where they could be exposed to elevated temperatures of >45°C if the collector was not careful to quickly release the internal pressure. The introduction of shorter Colorado model AV’s (15 and 19 inches [38.1 and 48.26 cm]) eliminates this concern as the stallion’s penis now can penetrate all the way through the body of the AV allowing for the sperm to be ejaculated into the inner AV liner in an area that protrudes out the back and is not exposed to elevated temperatures. Similarly, cold environmental temperatures can easily ‘cold shock’ sperm that are collected into a receptacle that is not protected by an insulated cone or cover of some type. An insulated cone that is warmed in an incubator and placed around the back of the AV to protect the collection bottle will protect the sperm.

The interior of any AV must be lubricated to allow the stallion to thrust without causing irritation or friction. Lubricants that are commonly used are water soluble to allow for easy cleaning of the liners. If excessive amounts of water soluble lubricants are used then it is possible that some of that lubricant will be pushed down into the collection bottle and dissolve in the ejaculated semen. Most of these lubricants have a very high osmolality and will result in osmotic damage to the sperm from the resulting increased osmolarity of the semen lubricant mixture. In the last few years there have been brands of water soluble lubricants introduced to the market that have an osmolarity similar to semen and therefore will not cause damage to the sperm if inadvertently mixed with semen during collection. Some ‘sterile’ lubricants also contain chlorhexidine to prevent growth of unwanted organisms. Chlorhexidine is highly spermicidal and exposure of sperm to chlorhexidine containing lubricants will also cause damage.

Another potential concern is that spermicidal residues from soap, disinfectants or tap water on collection equipment (liners, bottles, phantom, etc.) will result in sperm damage. New disposable liners can be used to eliminate the need for cleaning of latex or rubber AV liners to prevent this problem provided the stallion accepts the liners. Many stallions do not like the feel of a plastic AV liner because of the inevitable wrinkles that can cause discomfort and may require the use of a latex liner. If reusable latex liners are used they must be disinfected, washed and thoroughly rinsed. I have attached a copy of the ‘Select Breeders Service Standard Operating Procedure for Semen Collection and Disinfecting the Phantom and Collection Equipment’ to illustrate the protocol currently in place in SBS laboratories.

When collecting semen using an AV, the stallion is encouraged to mount a properly restrained oestrous mare (jump mare) or can be trained to mount a stationary breeding mount (also known as a phantom or dummy mount). Ovariectomised mares can be reliably used as jump mares and usually require exogenous oestrogen (oestradiol cypionate - ECP 2–5 mg) to encourage oestrous behaviour. Stationary breeding mounts should be constructed of materials to provide a secure, solid foundation with limited movement while the stallion is mounted. The body should be of an appropriate diameter and be well
Padded to prevent injury to the stallion. Ideally, the height and angle of the horizontal ‘body’ of the mount should be adjustable if stallions of various sizes are to be collected. Breeding mounts should be positioned in a large open area to provide adequate space around the phantom. Ceiling height in the breeding shed must also be sufficient to allow the stallion to mount and dismount the phantom without risk of injury. The material used to cover the phantom must be made of a durable material that can be readily disinfected to minimise cross contamination of potentially pathogenic organisms that may be present on the external genitalia or in pre-ejaculatory secretions between stallions. Recently, phantom covers or drapes with built in ‘hips’ have become available and are useful to provide additional stability for the stallion.

Nearly all stallions can be readily trained to mount a phantom for semen collection with an AV. Care should be taken when training young inexperienced stallions to mount a phantom as improper techniques that lead to negative experiences for young stallions can lead to long-term negative behaviours in the breeding shed. An experienced team including a competent, quiet yet firm and patient stallion handler as well as an experienced collector and capable mare handler are needed to ensure that these early breeding experiences are positive for the young stallion.

Once the stallion has mounted the phantom or jump mare, the collector should carefully deflect (not firmly grasp) the stallion’s penis and direct it into the AV. When collecting semen for cooled or frozen preservation it is desirable to obtain an ejaculate that is low in volume and high in sperm concentration. Seminal plasma is not a good extender for stallion semen and preservation protocols, both cooled and frozen require adequate dilution or removal of much of the seminal plasma for optimal retention of sperm motility. Most stallions will emit some sperm-free pre-ejaculatory fluid when they mount. We will routinely attempt to void as much of this pre-ejaculatory fluid as possible by holding the stallion’s penis out of the AV for a few seconds while the fluid is expelled. Some stallions may void as much as 40 or 50 ml of this fluid. An ejaculate that contains semen with a sperm concentration of 90 million per ml in 100 ml of volume may have enough that volume reduced by voiding pre-ejaculate fluid resulting in the same 9 billion sperm in 70 ml at a concentration of 128 million per ml. As discussed in a later presentation on cooled semen processing, we would routinely concentrate sperm by centrifugation prior to cooling for an ejaculate with a sperm concentration <100 million per ml whereas the semen containing 128 million sperm per ml could be sufficiently diluted for cooling without centrifugation.

This technique does not work for all stallions as some will not emit any pre-ejaculatory fluid outside of the AV and others may protest being held out of the AV and dismount. Every effort should be made to obtain an ejaculate on a single mount without excessive prior teasing which leads to increased accessory sex gland production, greater amounts of seminal plasma and lower concentration of sperm in the ejaculated semen. Numerous factors can affect sperm production in the stallion and those are addressed by another speaker at this conference so will not be addressed here. It is important to reiterate, however, that poor collection technique and improper semen handling can definitely have a negative impact on the quantity and quality of the ejaculate obtained when collecting semen with an artificial vagina.

Many stallions can be trained to have semen collected with an AV while standing on all 4 legs (ground collecting) or without an AV by manual stimulation. These techniques are useful for stallions with hindlimb injuries that cannot physically mount a mare or phantom. Ground collecting has also been advocated for some situations where personnel or collection facilities are limited.

In certain circumstances, when a stallion cannot mount a mare for collection or will not respond to ground collecting techniques with AV or by manual stimulation, ejaculation may be induced by use of pharmacological agents such as xylazine and imipramine. One recommended protocol is to administer 2.0 mg/kg bw t.i. or i.v. If this does not induce erection and ejaculation within 10–15 min, xylazine is administered i.v. at a dose of 0.2–0.3 mg/kg bw t.i. in this scenario. Xylazine used alone can also induce spontaneous ejaculation without erection as the stallion enters a state of sedation. Some stallions will respond well to this treatment and in those cases, high density, low volume ejaculates of good quality semen can be obtained. However, the effective dose required to illicit a response seems to vary between individuals and many stallions either will not respond at all or will respond very inconsistently. In these cases chemically induced ejaculation is not a reliable means to consistently obtain semen for routine use.

**Semen evaluation**

Whether semen is collected for the purpose of evaluating fertility potential, diagnosing subfertility, use for artificial insemination of mares or for long-term preservation, a basic evaluation of semen quality should be performed. A proper evaluation of semen starts by first doing no harm. The results of your semen evaluation no matter how elementary or sophisticated are invalid if the sperm obtained are damaged during the collection process or mishandled during evaluation. Sperm are fragile and extreme care must be taken to avoid thermal shock, osmotic shock, exposure to direct sunlight or exposure to substances that are spermicidal. All equipment that comes into contact with the sperm must be warmed, clean and free of residues. Whenever possible, new disposable specimen cups, pipettes and AV liners should be used. The basic goals of semen evaluation are to measure the quantity and quality of sperm in the ejaculate and also to identify potential pathogens that may be present.

**Gross appearance**

Raw semen should be evaluated for colour. Red or brown semen may indicate blood in semen (haematospermia) or genital tract infection. Yellowish semen may indicate urine in the semen (urospermia). Urospermia can be confirmed by the presence of a urine smell to the semen and microscopically by the presence of urine crystals in the semen. Clumps, mucous or excessive debris in the semen may indicate a genital tract infection or a cleared occlusion of sperm sometimes occurring in the ampullae of sexually rested stallions.

**Volume**

In order to determine the total sperm produced in the ejaculate an accurate measurement of sperm volume should be made using a graduated cylinder or centrefuge tube. The gel-fraction of the ejaculate should be removed using an in-line filter during collection and should not be included in the recording of ‘gel-free volume’. Weighing raw stallion semen using an accurate balance is also an effective way of determining semen volume. We recently performed an experiment in our laboratory to comparing the weight of raw stallion semen with the volumes as measured by reading the volume in a graduated centrefuge tube and found that our visual measurements correlated very well (r² = 0.99) with volumes measured by weight.

**Concentration**

The concentration of sperm in semen can be measured by both manual and automated techniques. One very important criterion
for the accurate measurement of sperm concentration is accurate mixing, pipetting and dilution of raw semen. Proper pipetting technique and use of properly calibrated pipettes is critical as a very small error made in measuring raw semen will be magnified when the sample is diluted for counting and that error is multiplied as the concentration of the original sample is calculated. Regardless of the counting technique used, extreme care must be exercised when pipetting raw semen and making dilutions.

**Photometric technique (Densimeter, SpermaCue, etc.)**

There are numerous photometric-based counting devices commercially available for the automated estimation of sperm concentration. All of these devices are based on the following principle. A light source is passed through a sample chamber and the amount of light transmitted through the sample is detected by a photocell positioned on the other side. If the light is passed through a chamber that is empty or contains only optically clear diluent, 100% of the light will be detected by the photocell. When a known volume of semen is added to the chamber (raw semen for the SpermaCue and diluted semen for the other photometers) some of the transmitted light is blocked by the novel replicates of a given sample be measured to provide a reliable measurement. Nevertheless, if done properly using an adequate number of replicates and a standardised and consistent protocol for dilution, chamber loading and cell counting, haemacytometry can be an excellent way to determine sperm concentration. I have attached the SBS Standard Operating Procedure for counting sperm with a haemacytometer for reference. Our research indicates that one must count a minimum of 4 individual chambers from each sample to provide a measurement with an acceptable level of variation.

**Automated Cell Counting Techniques (NucleoCounter SP100, CASA)**

The NucleoCounter SP100 is a fairly new piece of equipment that uses direct counting of fluorescently labelled sperm heads to accurately and rapidly determine sperm concentration. A known volume of semen is diluted with a solution containing a detergent that causes sperm membranes to be permeable to a specific DNA intercalating stain. The stain binds to the DNA and when illuminated with UV light, emits fluorescence in a specific wavelength of light. That fluorescent image (sperm heads only visible) is then digitised and software is used to count the sperm in a fixed given area. From that count the sperm concentration is calculated. Using this technique, sperm suspended in extenders, even those with a high percentage of nonclarified egg yolk can be accurately counted. The system uses a unique cassette preloaded with the fluorescent stain propidium iodide (PI) into which the diluted and membrane permeabilised sperm are loaded by depressing a plunger. The sperm then travel through a series of channels, are exposed to the PI and then dispersed into a viewing field for illumination and identification. We have validated this system with haemacytometry in our lab and find it to be accurate and highly repeatable.

Computer Assisted Semen Analysis (CASA) was developed in the 1980s as a way to use image analysis to objectively and accurately quantify the measurement of sperm motility (see below). It has also been used as a system for automatic cell counting. In this system, microscopic images of live sperm are captured by video camera, digitised, identified and tracked through successive video frames. Computer software is then used to trace the position of the sperm heads from one frame to the next and recreate the path of the sperm. By using a chamber with a known depth, the area of the field being viewed is calculated and the concentration of sperm in the sample can be determined based on the number of cells identified within that area. The principle is the same as haemacytometry but in this case the counting is being done by computer. There are advantages and disadvantages to this. CASA allows for many more cells to be counted in a shorter period of time and any bias or subjectivity introduced by a human is eliminated as the software uses defined algorithms to determine what will be counted. However, there are significant disadvantages. First is the accurate determination of sperm from nonmotile sperm. CASA uses the size and intensity of any object in the field to determine if it should be counted as a sperm or not. The algorithm first identifies all objects that have a size above a certain user defined minimum. If that object is moving it is considered a sperm. The average size and intensity of all the moving objects is then calculated and then compared to the size and intensity of all the nonmoving objects (nonmotile sperm and other objects). If the size and intensity of a nonmoving object falls within user defined ‘gates’ for size and intensity of the mean of the moving objects, then that nonmoving object is labelled and counted as a sperm. The number of moving objects and labelled nonmoving objects is combined and that cell count is used to calculate the sperm concentration. Issues associated with mislabelled objects and with inaccurate tracking of motile sperm due to ‘virtual collisions’ can lead to inaccurate measurements of sperm concentration using this system.

Another source of error is associated with the dynamics of particle flow within the fixed coverslip counting slides (Microcell, Leja slide) typically used for CASA. For any direct counting method the area that the counted sperm occupy must be known in order to calculate concentration. A typical slide with a floating coverslip does not provide a consistent depth under the coverslip and so these slides have been developed. There is a phenomenon of particle flow (known as the SS effect) that causes an uneven...
distribution of cells in a semen sample across the chamber of these fixed coverslip slides and this can lead to errors in sperm concentration measurements unless a correction factor is applied. We have developed a protocol in our laboratory that eliminates some of these sources of error and allows for accurate and rapid counting of sperm using a CASA system and fixed coverslip slides. To eliminate the effect of ‘collisions’ we dilute the samples with formalin or sodium citrate to immobilise the sperm and then count only static cells in the sample. We have also calculated a correction factor that we apply to the concentration to account for the SS effect for stallion sperm in this system. We validated this method using the NucleoCounter SP100 described below and use it to increase the number of cells counted and decrease the time associated with direct manual counting associated with the haemocytometer. One can also use DNA specific fluorescent stains and special software on some CASA models to eliminate errors due to misidentification of objects within a sample and allow the tracking and counting of sperm diluted in nonclear extenders.

Motility

Once we have a good measurement of the quantity of sperm in the semen it is necessary to determine what percentage of those sperm may potentially be able to fertilise an oocyte when inseminated into a mare. Whilst fertilisation is a very complex process that requires the sperm to possess a number of functional attributes many of these attributes are unknown or cannot be readily measured using standard laboratory techniques. Sperm motility is the most widely used and readily assayed measure of sperm function and for decades has been used as an indicator of the potential of a semen sample to achieve pregnancy in the mare. Early work on determining the quantity of sperm required for optimal fertility in an artificial insemination programme relied on a subjective estimate of ‘progressive motility’. Sperm motility can be measured either subjectively by a technician visually observing a sample of semen under a microscope or by computer assisted semen analysis (CASA). Sperm are very sensitive to fluctuations in temperature and this is readily apparent when observing sperm motility. The velocity and ‘progressivity’ of sperm are significantly decreased when sperm are observed at temperatures that vary significantly from 37°C. After collection, the semen should be kept in a 37°C environment and all items coming into contact with the semen will significantly decrease when sperm are observed at temperatures that vary significantly from 37°C. After collection, the semen should be kept in a 37°C environment and all items coming into contact with the semen should be prewarmed to 37°C. Use of a heated microscope stage or slide warmer is highly recommended. In order to properly visualise the motility of individual sperm and, therefore, determine progressivity, raw semen should be diluted with an appropriate pre-warmed extender to a concentration of 25–40 million sperm per ml. After dilution the semen should be kept at 37°C for 5 min to equilibrate prior to reading motility. If the motility of the extended semen is very poor or the sperm look ‘shocked’ or appear to be dying quickly on the slide, examine a drop of the undiluted raw semen to see if the poor motility may be due to something wrong with the extender. If the extender is not at the right temperature (either too hot or too cold) or if it is not in the proper osmotic range it could kill the sperm very quickly.

An experienced technician can generally obtain consistent and reliable estimates of the percentage of total and progressively motile sperm in a semen sample and this can be sufficient for most breeding programmes. However, because of the subjective nature of motility estimation and lack of a standardised and quantifiable way to estimate motility, there can be great variations between technicians and laboratories in the motility of a given sample of semen. There is also a real possibility of inadvertent technician bias introduced during the process that can influence results. In addition, variation in the procedures used for dilution, incubation and motility evaluation of cooled transported or frozen-thawed semen can lead to disputes between stallion and mare owners over the perceived quality of semen received in fulfillment of a breeding contract.

Computer assisted motility analysis using CASA instruments has many advantages over subjective motility estimations. Use of this objective system eliminates much of the inter-laboratory and individual technician variability and bias inherent in subjective estimates. CASA also provides detailed quantitative information on other aspects of sperm motion such as velocity, linearity, lateral head displacement, etc. that are impossible to quantify using subjective estimates. It is also valuable in detecting slight changes in motility when evaluating processing protocols, extenders or tracking a stallion’s overall semen quality during the season or from year to year during his breeding career. I described above briefly how CASA systems identify sperm. For the purpose of evaluating motion characteristics, the labelled sperm heads are tracked during successive video frames and then the trajectory of that path is recreated. From this recreated path, a number of calculations are made. There are calculations of velocity (VCL, VAP) that reflect the distance travelled along the path over unit time, measurements of trajectory such as linearity (LIN) and straightness (STR) which reflect the ratio of distances along the path to the distance from a straight line (VSL) from first frame to last and also measurements of how far and how rapid the sperm head moves from side to side along the path (amplitude of lateral head displacement (ALH) and beat cross frequency (BCF)). Figure 1 illustrates how some of these calculations are made.

While use of CASA does provide accurate, objective and repeatable measures of sperm motility it is important to recognise that there are numerous technical factors that can influence the measured values. Standardisation of sample preparation and analysis algorithms is required for reliable comparison of values obtained between operating technicians and laboratories. Furthermore, motility, regardless of how sophisticated its measurement, is only one indicator of relative cell health and the true fertility of any semen sample can only be accurately determined by properly timed insemination of an adequate number of reproductively healthy mares.

Sperm morphology

Sperm morphology is a critical but often poorly measured or completely ignored aspect of semen evaluation. All semen samples have a mixture of viable and nonviable spermatozoa and an assessment of the percentage of sperm with ‘normal’ gross morphology as well as categorisation of the quantity of various
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of these microorganisms are not pathogens in the mare and will not cause disease. There are a number of potential pathogens, however, that can be found in semen and can have a negative impact on fertility as well as spread disease through natural mating or artificial insemination. With the advent of transported semen it is increasingly important to ensure that shipped semen does not act as a vector for the spread of disease around the country. Many bacteria and viruses can also survive cryopreservation and live in frozen-thawed semen and could contribute to spread of disease between countries if precautions are not taken.

Bacteria that are potential pathogens in the horse include *klebsiella pneumonia*, *psuedomonas aeruginosa*, *Taylorella equigenitalis* (causative agent of Contageous Equine Metritis) and possibly *streptococcus zooepidemicus* and *Escherichia coli*. Swabs should be obtained from the semen, urethra, glans fossa and penile sheath prior to the start of the season and periodically throughout the season to monitor for the presence of these potential pathogens.

Stallions should also be screened for the presence of certain viruses that may be shed in semen. Of these, *equine arteritis virus* (causative agent of EVA) and *equine herpesvirus type 3* - EHV3 (causative agent of equine coital exanthema) are of greatest concern. In cases of haemospermia, blood-borne viruses (such as equine infectious anaemia virus) may also be present in semen.

**Microbiology**

Semen from stallions will often contain microorganisms. Semen is not sterile and normal bacterial flora inhabits the external genitalia and will usually contaminate collection equipment resulting in the presence of these organisms in the semen. Many of these microorganisms are not pathogens in the mare and will not cause disease. There are a number of potential pathogens, however, that can be found in semen and can have a negative impact on fertility as well as spread disease through natural mating or artificial insemination. With the advent of transported semen it is increasingly important to ensure that shipped semen does not act as a vector for the spread of disease around the country. Many bacteria and viruses can also survive cryopreservation and live in frozen-thawed semen and could contribute to spread of disease between countries if precautions are not taken.

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