Semen Processing, Extending & Storage For Artificial Insemination In Swine

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Introduction

The use of artificial insemination (AI) in swine is based on liquid extended semen. Typically, an ejaculate from a single or multiple boars is collected, and then processed individually or pooled. Pooling semen takes advantage of the improved fertility of some boars and minimizes the effects of a sub-fertile boar (or sub-fertile ejaculate). In either case the ejaculate or ejaculates are evaluated, and processed to extend its volume in order for multiple females to be inseminated from a single ejaculate. Semen fertility must also be extended over days because raw semen is only fertile for hours after collection.

Most semen extenders are classified into short (1-2 days), medium (3-4) or long-term extenders (5-7 days) from the day of collection. The proper extension of semen is a critical step to achieving success with AI. Although the procedure is not difficult, accurate extension rates are critical since too many sperm in a set extender volume may reduce sperm survival in storage. This can occur with production of greater amounts of toxic products from increased number of sperm cell metabolic by-products. Conversely, over-extension of the ejaculate allows too few sperm cells in the semen dose to achieve high fertility rates with AI. This article reports some of the procedures and important considerations when processing semen to ensure the fertility of liquid extended boar semen for AI.

Sperm Fractions

The boar ejaculates semen in fractions and does not mix the accessory sex gland fluids with the sperm before ejaculation. Therefore, the different fractions of the boar ejaculate contain drastically different amounts of sperm. The first fraction of a boar ejaculate is the pre-sperm fraction. This fraction contains fluid from the prostate gland and amounts to ~25 cc. This fraction serves to flush urine and bacteria out of the boar's urethra, and is typically discolored and foul smelling. The second phase of the ejaculate is the sperm-rich fraction. This part is usually 40-100 cc and may contain 80-90% of the total sperm. The third fraction is the largest by volume (70-400 cc) and is typically sperm poor. It is often discarded in order to prevent over dilution of the sperm rich fraction. The last fraction is the gelatinous phase and is 20-40 cc in volume. Except for the first fraction (which is discarded), all phases are collected through a filter or cheesecloth to remove the gelatinous material that originates from the boar's bulbourethral gland. Filtering the ejaculate is important since some of the gelatin material is ejaculated in all of the fractions but predominantly appears in the last fraction. During natural service, it functions to effectively seal the ejaculate volume inside the female uterus.

Gross Semen Evaluation

All ejaculates should have to meet minimal quality control standards in order to assure

that reproductive efficiency is not limited by the boar's semen fertility when using AI. In general, ejaculates from boars that are healthy and collected at proper intervals should easily meet the minimal acceptable criteria. However, since factors such as stress, disease, and environmental factors, can significantly reduce the quality of a boar ejaculate, several criteria are used to pass an ejaculate. Stringent criteria for acceptance of an ejaculate can help prevent economic losses that result from lower conception rate and reduced litter size.

In general boar ejaculates should have a sperm rich fraction of 80-100 cc. The sperm should show >70% motility. This is observed as forward progressive motion in large circles. Abnormalities should occur in less than 15% of sperm. Greater than 200 million cells per cc is the minimal concentration. Ejaculates should initially be evaluated based on weight (volume), opacity (clarity), color, odor, and turbidity (swirling) before further processing.

Semen is commonly collected in containers that allow determination of volume. This can easily be accomplished by placing the collection vessel on a scale and subtracting the weight of the collection vessel in order to determine the volume of the ejaculate. The weight, in grams, is equal to the volume in cc. Another simple method for determining volume is to transfer the ejaculate to a container with volume gradations that allow visual measurement in milliliters (cc).

Semen ejaculates should also be evaluated for color. The ejaculate should normally be milky to chalky in appearance. Pink colors indicate the presence of blood and may suggest an injury or a urinary tract infection. A yellow-tinged ejaculate may indicate that some urine was inadvertently collected, and therefore the ejaculate should be discarded. Good quality semen ejaculates should not have a strong or offensive odor. A strong odor indicates the presence of preputial fluid. This fluid originates from a pouch located near the prepuce (external opening for the penis). This fluid contains bacteria and decaying cells and is associated with a strong offensive odor. This pouch must be massaged to empty its contents prior to semen collection in order to avoid contaminating the ejaculate.

A visual assessment the opacity or the amount of light that passes through an ejaculate is an indication of the number of sperm cells. Less transparent samples therefore contain more sperm cells. The sperm rich fraction is creamy in appearance and allows little light pass though. The sperm poor fractions are more watery in appearance, are more transparent, and have fewer cells. Ejaculate turbidity is another indicator for sperm cell numbers. It appears when the sample is gently rotated and a swirling motion can be seen in the liquid. For an example of this, let the sample set long enough for sperm cells to settle to the bottom of the container and then gently rotate the sample.

A gross motility evaluation can only be performed with a microscope. A warmed slide and sample will allow for fair assessment of motility. Low power is usually sufficient (100 x) to see the entire sample vigorously in motion. While performing the gross microscopic evaluation, the sperm agglutination test can also be performed. This involves a general assessment of the degree to which sperm are clumped or sticking together. A high degree of clumping may indicate the presence of gelatin or high degrees of sperm damage. Moderate to high amounts of clumping will greatly reduce the sperm available for fertilization.

Evaluation of a sample for bacterial or particle contamination is performed with higher power magnification (400 x). Bacteria are seen as tiny particles that show

rapid, vibrating motion. The effect of amount of particulate matter in a sample is not clear, but samples that have limited debris, have good fertility results.

Semen Sub-sampling

Before any further technical evaluation is performed, sub-sampling of the ejaculate should be made with a well-mixed sample. Gentle swirling is required since sperm cells settle to the bottom with time. It should be mentioned that shaking or swirling too vigorously could cause damage to the sensitive attachment region for the sperm head and tail.

Estimating Sperm Concentration

Hemacytometer

Evaluation of a semen sample with the hemacytometer (cell counting chamber) provides a method for accurate determination of sperm cell concentration. An accurate estimation of concentration will allow extension rates that optimize boar utilization and fertility of extended semen. The materials needed for this procedure should include a good quality microscope (~$1500), a hemacytometer (~$100/ea). The sample dilution system can include a red or white blood cell dilution system (Unopette, ~1.00/ea) or a hand-pipet system. The Unopette white blood cell dilution system is desirable since no additional equipment is needed, sperm cells are killed, and pipetting and dilution errors can be minimized. In this system, a sub-sample from the ejaculate is taken by mixing the ejaculate well and placing 2-3 drops on a microscope slide. The Unopette system allows a 10-20 μl sample to be obtained with a Unopette capillary pipet. The semen sample in the capillary tube is then added to the Unopette dilution chamber and mixed (Figure 1 and 2). The diluted semen sample is then applied to both chambers of hemacytometer. After 5 minutes, the sperm cells settle onto the glass and counting of sperm at 200-400 magnification can begin. Since sperm are almost transparent under light microscopy, a phase-contrast scope can facilitate sperm visualization. When using a standard light microscope, the iris diaphragm should be closed to improve sperm visualization. Sperm are counted in 5 diagonal squares. Sperm heads are <u>not</u> counted if they touch the right or bottom triple lines of the squares (Figure 3). These sperm are not included since they contribute to over-estimation of the concentration when multiplication is performed. Both chambers of the hemacytometer are counted. Sperm numbers from each side should be within 10% of each other. If not, clean the hemacytometer and repeat sample addition (it is not necessary to repeat the dilution step). When sperm counts are within 10%, average the two numbers. If a 1:100, or a 1:200 Unopette dilution was used, simply add seven zeros to get sperm concentration/cc. This is a simplified way to determine concentration. The actual formula is (*(sperm in 5 squares x 5; (or total sperm in 25 squares)) x dilution rate (100 or 200) x hemacytometer chamber volume (10,000 or 10^4) = sperm/cc)*). Example: (25 sperm average (in 5 squares) x 5) x 200 (dilution factor) x 10^4 = 250,000,000 sperm/cc. The total time required for this procedure is about 10 minutes per sample. Errors can occur with this procedure due to pipetting error and improper chamber filling (under or over-filling).

Photometer/Spectrophotometer

The amount of light that can pass through a semen sample and be detected on the opposite side, provides a numeric estimate of the sperm cell concentration of the sample. Photometers (single wavelength) and spectrophotometers (multiple wavelengths) are devises that are used for this application and are priced between

$1,500-$3,000. Samples are read in light absorbance units (0.0-2.0) or % light transmittance (0-100%). Whichever one is used, the readings near the upper and lower limits of detection (<10% and >90% for transmittance, or <0.2 and >1.8 absorbance) have high degrees of reading variation (low accuracy). This is because of the combination of too few or too many cells, and other factors that can alter the passage of light through a semen sample. Many of the commercially available photometers have predetermined curves that calculate the sperm/cc while others provide a reading that is used to convert the reading to sperm/cc. The accuracy for each machine is based on a standard curve. Taking multiple semen samples and diluting them (1:10, 1:25, 1:50, 1:100, and 1:200) generates the standard curve (Figure 4). The transmittance or absorbance is then measured for each sample. A sperm cell count can then be performed on the original semen sample to determine the actual number of sperm cells/cc. A linear curve can be generated by dividing the actual known cell concentration by each of the dilutions (i.e. 5 .0 x 10^8/cc divided by 200 (e.g. 1:200 dilution) = 2.5 x 10^6/cc. This is repeated for each dilution of each ejaculate sample. Each sample dilution is associated with an absorbance reading and establishes a relationship with sperm cell numbers (linear regression). A regression equation is then used to "predict" sperm cell numbers based on the absorbance or transmittance reading. There is inherent variation around any reading and only repeated sampling of different ejaculates around a photometer reading will improve confidence in the estimate of the sperm cell numbers. Unfortunately, light scattering is affected by seminal plasma within and between boar ejaculates which may account for up to
-19% to +30% errors in photometer estimations of actual sperm concentrations. Some units require dilution prior to reading while others do not. Manufacturer instructions should be followed and calibration and accuracy of the equipment should be performed.

Computerized
New computerized semen analysis software is available from commercial suppliers. Unfortunately, the equipment is very expensive. However, these systems appear to be quick and reliable for concentration, motility, and abnormality assessment. They, like many of the photometers, can also be programmed to determine extension rates.

Semen Extension

Extender

Extenders function to allow multiple inseminations from a single ejaculate. In addition, the extender must provide temperature protection for sperm while reducing the metabolic rate of sperm cells in cool storage. The extender functions to provide membrane stabilization in cool temperatures, energy sources for sperm metabolism, pH buffering from sperm cell waste, ions for membrane and cell balance, and antibiotics to prevent growth of microbes that can cause disease and compete for nutrients. Various extenders can be used. Some formulas are published and can be made up in the lab while others must be purchased from a commercial supplier since they offer unique advantages and their formulas are not proprietary.

Semen Extension

In gross semen extension, criteria such as volume, opacity, turbidity, and color are used to evaluate whether to extend the sample. In this system, no microscope or photometer is used to determine sperm concentration. Extension rates instead, are based on previous knowledge of the normal ranges for total sperm output in an ejaculate.

Since most boars that are healthy and have not been overused, ejaculate in the range of 20-60 billion sperm, dilution rates between 1:4 and 1:10 can be used. For example, a 1:4 dilution rate, would in theory, provide semen doses with 5-15 billion cells per dose, while a 1:10 dilution would produce doses containing 2-6 billion sperm. Obviously, there are inherent flaws in this system, but "blind" dilutions within this range can generally produce acceptable fertility results.

Extender Preparation

To prepare the extender, weigh out the specified amount to make 1 liter. Using a graduated cylinder or a scale, pour one liter of distilled water into a mixing container. Add the extender powder to the water and mix well until powdered or particulate material is dissolved. The extender should be made up 1-2 h in advance in order to let the pH and ions achieve equilibrium (Figure 5). The extender can be made up in advance and stored frozen. However, if it will not be frozen immediately, it should be utilized within 24 h of mixing. This is to prevent the antibiotics, which are effective for a limited amount of time, from losing their potency. Once the semen sample has been collected and evaluated, and the extender has equilibrated at room temperature, the semen temperature should be measured and the extender temperature adjusted to within 1° C or 2° F of the semen sample.

Determine Extension Rate

1. Sperm cells/cc x % motile x % normal x ejaculate volume = total sperm.
2. e.g. 60 billion sperm x 90% motile x 95% normal x 150 cc = 51 billion sperm.
3. To get a desired 3 billion sperm/80cc insemination dose:
   340×10^6 sperm/cc (determined sperm concentration) x 150 cc (ejaculate volume) =
   51×10^9 total sperm.
4. 51×10^9 sperm ÷ 3.0×10^9 sperm/dose = 17 doses
5. Multiply doses (17) x volume of semen dose (80 cc) = 1360 cc final volume.
6. 1360 cc - 150 cc = 1210 cc
7. Add 1210 cc of extender to 150 cc semen sample.
8. Gently aliquot 80 cc of extended semen into 17 bottles, tubes, or bags for 3.0×10^9 sperm/dose.

Semen Collection and Cooling Notes

Collection vessels for the ejaculate should be between 20° C but not above 37° C (68-98° F). When diluting semen, make sure all materials (and even room) is as close to 20-25° C as possible (or 68- 72° F, room temperature, RT). Gently add extender to semen (although the opposite procedure does not appear to cause any apparent harm, limited information is available on the effects of this methodology. Mix the semen and extender gently. Aliquot the semen into storage dose sizes by pouring slowly at a slight angle down the sides of the container. Avoid bubbling and excessive amounts of air in the sealed dose. Label with sire identification, collection date and expiration date (determined by type of extender). Allow the extended semen to reach RT (20-25° C) over the next 2 hours at the desired cooling rate of ~1° C per hour. When at RT, move the semen to a 12-18° C cooler (54-64 °F). The exact temperature should be matched to the recommendations for the specific extender used.

Semen Storage

Semen doses placed in a 12-18° C cooler will reach storage temperature over the next 4-8 hours. Methods to slow semen cooling, such as placing doses in styrofoam boxes, do not appear to greatly influence sperm characteristics but this extra step to slow the rate of cooling may be beneficial with certain extenders (Figure 6). It is important not to pack semen packages in the cooler so that they are prevented from exposure to cool air. Packages should not be stacked so that some have cool air exposure and others do not. Prevent fluctuation in cooler temperatures. Cooler temperature can be monitored with inexpensive portable tracking devices. Limit the opening and closing of the storage unit especially during hot weather. Check to make sure the door seals well. Rotate semen once to twice daily. Rotation is necessary since sperm metabolize energy sources and return lactic acid into the extender. If the acid builds up, it could lower the pH and kill sperm. Do not use semen that is past its expiration date. Even with high quality ejaculates, a 5-10% drop in semen motility is expected within the first 24 h of storage. After this time, few observable changes are expected in the semen quality within the limits of the extender. Semen quality should periodically be checked in storage for quality assurance.

Critical Steps

In order to be successful when collecting and extending boar semen, there are several areas that deserve attention to ensure reproductive efficiency is achieved. For maximal fertility when using AI, the starting quality of the ejaculate must meet or exceed the minimal criteria, and therefore semen evaluation is a necessary part of this procedure. When collecting, processing and storing semen, temperature fluctuations should be avoided. If temperature extremes are expected, anticipate these and ensure materials or temperatures that the ejaculate is exposed to, fall within safe limits. Avoid poor quality ingredients that include water, extender (or ingredients), and equipment (microscope and cooler). Investment in good quality, reliable semen extension equipment will help prevent the inefficiencies of low farrowing rates, and poor litter sizes that result from matings with poor semen. Sanitation and disease prevention are more critical now than ever with the increased use of AI. It is essential to insure high health status of boars through frequent testing and elimination of carrier animals. Risk of contamination of semen during the collection and processing steps should be minimized and proper handling of extender to help maintain antibiotic effectiveness will help maintain semen quality. This can also be aided by the use of disposable equipment. An important part of a quality assurance program is to evaluate the product at different stages of processing. This should involve both a gross and technical evaluation of semen quality before, and during, processing and storage. Finally, errors from improper extension rates should be minimized to allow maximum boar utilization, and to utilize labor, and expendables more effectively. Proper extension rate will also optimize the fertility of the semen in storage for the end user. Extension rates should be based on the number of sperm cells, the percent motile and percent normal cells, to provide semen with the greatest fertility for breeders.

Figure 1. Unopette System

Figure 2. Sample Addition

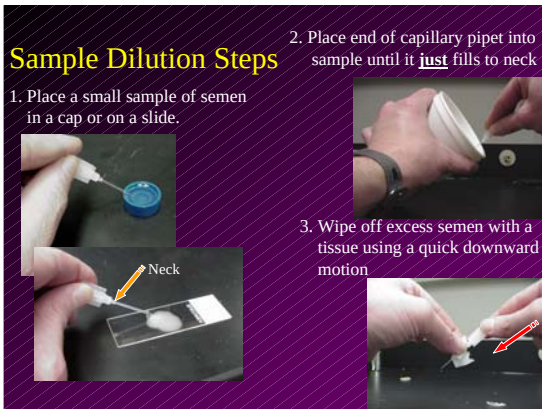

Figure 3. Hemacytometer Grid

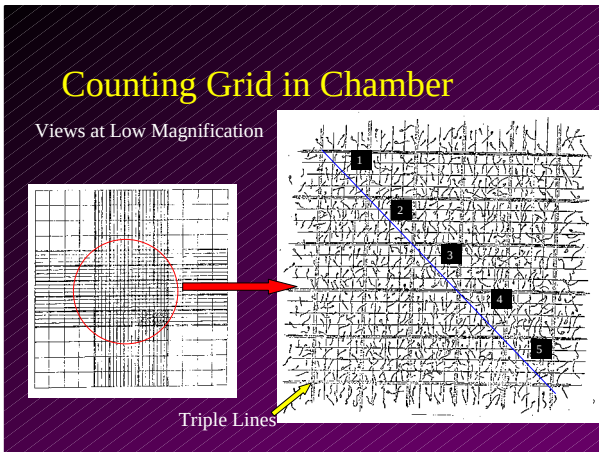

Figure 4. Photometer Standard Curve

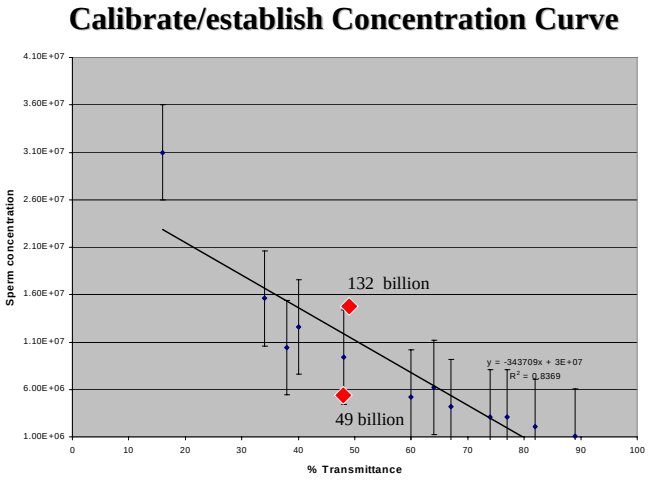

Figure 5. Extender Equilibration

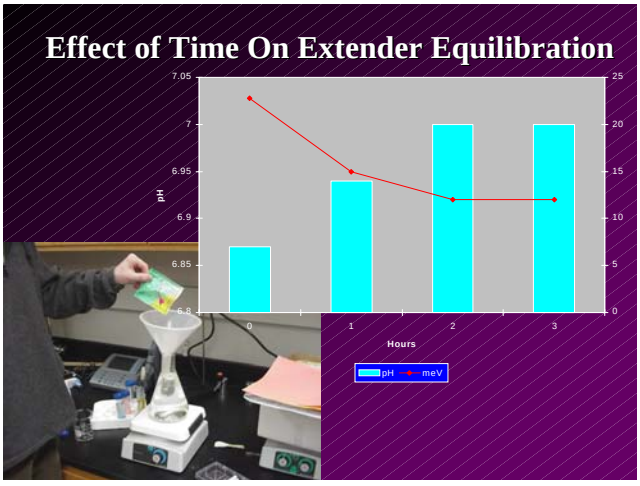

Figure 6. Effect of Temperature on Semen Cooling rate