QUALITY CONTROL OF EXTENDED BOAR SEMEN

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ABSTRACT

All boar studs should regularly monitor the extended semen doses produced and delivered to their customers by performing either internal quality control or by using a third party organization in order to make sure that all the doses have the best potential to impregnate gilts and sows inseminated artificially at the correct time in estrus. The quality control program should evaluate the number of sperm cells per dose, semen motility, semen morphology and screen for potential contamination by micro-organisms.

INTRODUCTION

The use of artificial insemination (AI) in the swine industry has grown & expanded very quickly in the last 15 years in North America. The genetic companies have boar stud centers strategically located across USA and Canada so they can promote and sell their specialized genetic lines to all the pig producers. The easiest way to introduce new genetics in a sow farm is select a genetic line and buy the extended semen from a boar stud for use in an AI program.

The contribution of a genetic company through extended semen produced in a boar stud is basically 50% of the input into a sow farmer's reproductive performance outcome (Althouse and Galligan, 2006).

The purpose of this article is to review the quality control (QC) analysis that should be done in the extended semen produced by any boar stud. The extended insemination doses will be used by pig producers and they must have the best characteristics to be capable of impregnating a gilt/sow bred at the appropriate time.

REASONS TO PERFORM QUALITY CONTROL OF EXTENDED BOAR SEMEN

To provide a product with the following characteristics for inseminating gilts and sows:

1. Absence of contagious organisms - disease
2. Maximum shelf life
3. Maximum fertility

In order to ensure these characteristics, boar studs must routinely verify the consistency of semen doses produced. They can choose either to perform quality control internally or use the services from a third party agent.

According to the industry standards, the characteristics to verify in each semen dose are:

- Accepted dose volume
- Accepted dose sperm motility
- Accepted sperm morphology parameters
- Accepted dose sperm concentration
- Accepted total sperm cells per dose (Althouse and Galligan, 2006)

The advantage of using a third party agent for this QC analysis is the neutral objectivity provided in the results.

QUALITY CONTROL TESTING STEPS

Number of Samples to be Tested and How Often

The samples to be tested (semen tubes, bottles or couchettes) must be randomly selected and the amount of testing samples depends on the quantity of semen doses (batches) produced daily by a boar stud. From a statistical perspective, in boar studs producing high quantity of batches/day, the quality control evaluation should be done based on a per 200 batch basis. For boar studs producing low number of batches/day, samples of the batches should be randomly taken and sent to the lab during the period of production of the 200 batches. The amount of batches that should be tested per 200 batches, with a 95% confidence interval and with 5 and 10% prevalence detection level is 51 and 27 respectively (Althouse and Galligan, 2006).

From the practical point of view, the ideal situation for a boar stud is to test the production of extended semen at regular intervals. Some boar studs have arrangements periodically with quality control laboratories to test batches of production every week, every 2 weeks or every 4 weeks following an annual predetermined schedule. This system allows them to monitor all the aspects of production periodically and any changes happening inside the boar stud unit could be reflected in the sample tested. For instance, changes happening in stud personnel, water purification-quality systems, hygiene & protocols for collection, equipment used for collection and semen processing, etc, can be reflected in the results observed in the semen samples tested.

Shipping

After a decision has been made about sending samples from a boar stud to a laboratory for quality control, the samples should be packaged inside a container such as double Styrofoam box system that contains cool gel packs at 17°C in order to keep the right temperature of the samples submitted. The samples should be sent to the final destination using an overnight courier. The shipping procedure should basically be the same as the one used for shipping semen from the boar stud to a regular customer.

Semen Temperature

As soon as the samples arrived to the laboratory, the arrival temperature should be taken using an appropriate instrument such as an infrared thermometer (Figure 1). The ideal arrival temperature should be between 16 and 18 °C. This evaluation will determine if the transportation/shipping system used by the boar stud is working correctly to maintain the appropriate temperature that semen requires, or if a change of protocol is required. It is important to remember that higher or lower temperatures than 16-18 °C could affect the sperm cells and cause reproductive problems when used.

Figure 1. Semen temperature evaluation using an infrared thermometer.

Dose Volume Assessment

In order to determine the volume of semen per dose, each container is weighted using a precision scale (Figure 2). It is considered that 1 gram of weight is equivalent to 1 mL of semen. The standard volumes being used in the swine industry for semen doses range between 60 and 80 mLs. Each boar stud tries to use the same volume in the doses being produced daily (i.e. 80mLs).

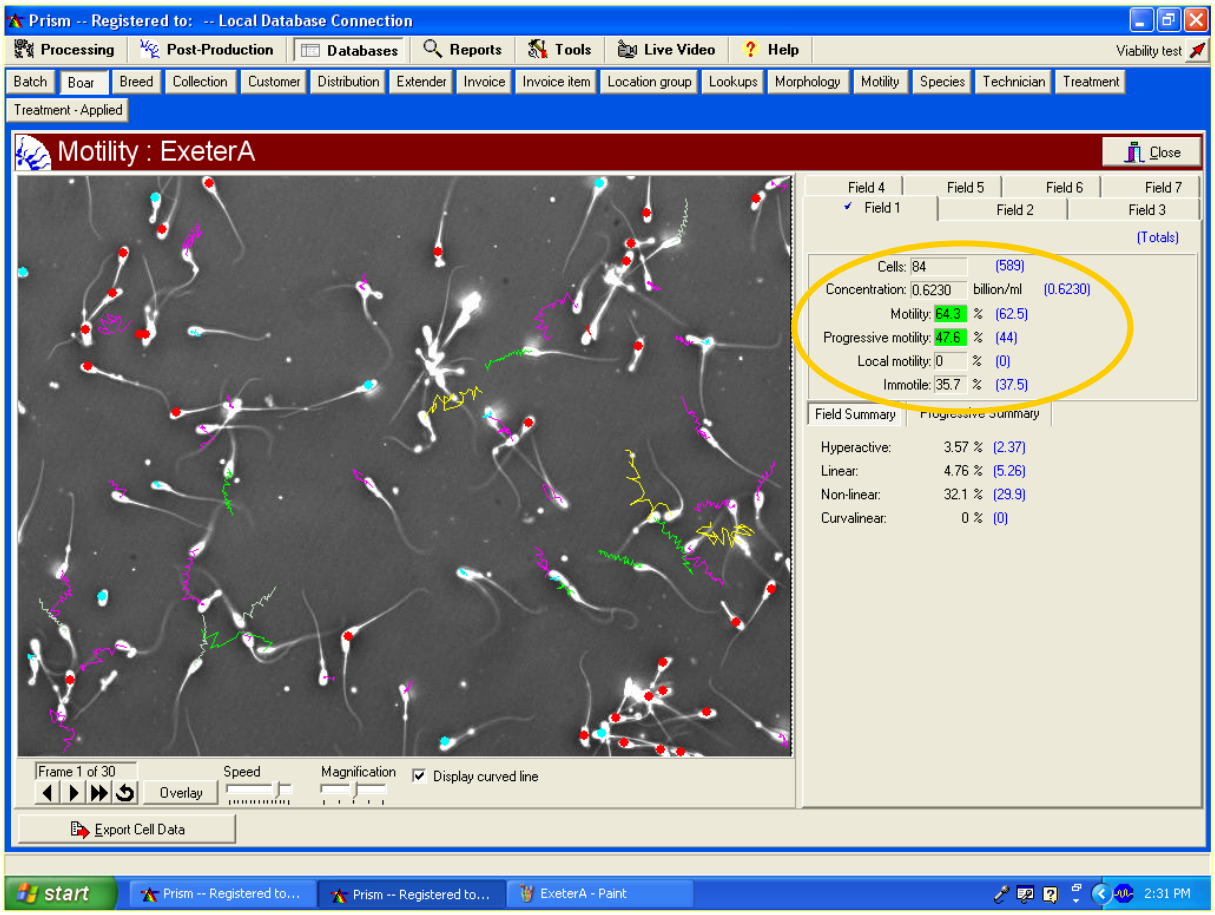
Figure 2. Dose semen volume analysis using a precision scale.

Individual Motility of Spermatozoa

The evaluation of individual sperm motility is used to determine the percentage of cells that are viable per dose of semen. The ideal way for performing this evaluation is by using a tool that in an objective way will tell us exactly how many and the percentage of sperm cells that are motile. There are presently Computer Assisted Sperm Analysis (CASA) systems available in the market such as the SpermVision. This particular CASA system has a camera connected to a computer that is placed on top of a trinocular microscope where the sample is being evaluated. The SpermVision camera is capable of taking 30 consecutive rapid photos of the sample field in 0.5 seconds, time that allows the computer to identify and capture individual sperm cells by its head size and analyze their movement pattern. Normally 7 microscope fields are evaluated per sample, to have an accurate evaluation of individual motility analyzed per sample. A sample is expected to have at least 70% motile sperm cells. It is also ideal if all the motile cells present in the sample have a straight movement, parameter known as "progressive motility" (Figure 3).

The percentage of individual progressively motile sperm cells can help to predict the sperm membrane integrity and morphological integrity of the cells present in the sample (Barth, 1997).

Figure 3. Screen of a semen sample being analyzed using the Sperm Vision CASA system. Please notice in the yellow circle the concentration and motility results.

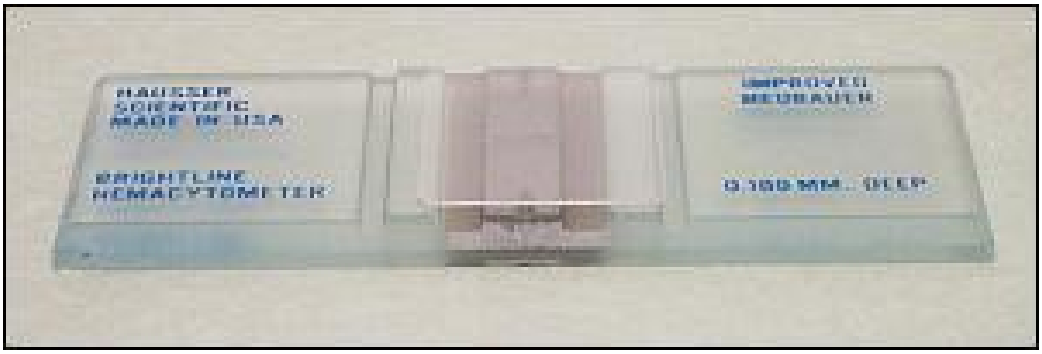


Concentration and Total Number of Spermatozoa Present per Sample

The first step is to calculate the concentration of spermatozoa per mL of extended semen. Due to the high concentration of sperm cells in a semen dose, it is necessary to dilute the semen sample to a known dilution factor to decrease the sperm concentration so that individual cells can be counted manually. The sperm concentration/mL is then estimated by filling some of the diluted sperm solution in the haemocytometer (Figure 4) and counting the individual cells with a Phase contrast microscope (Figure 5). Then a mathematic calculation is done using a known formula that requires the dilution factor used and the cells counted. This technique is considered the gold standard for calculating concentration of cells per mL of solution.

The concentration/mL obtained with the hemacytometer is then multiplied by the total volume of the dose, which will provide us the total number of sperms present in the dose.

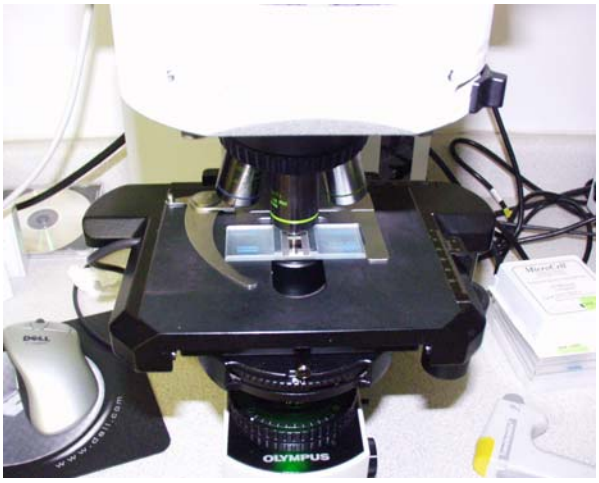
Figure 4. Picture of the improved Neubauer hemacytometer.



Other alternative available for performing this step is by using a CASA system. The SpermVision is capable of calculating the concentration of sperms per mL (Figures 3 and 5) when motility is also evaluated. In the final report of the sample, the CASA analysis gives the total number of cells present per dose of semen.

A great advantage of the SpermVision CASA system is that can also be used in combination with the Hemacytometer for performing sperm dose concentration calculations, and capturing the individual cells with help of the computer to make the respective calculation (Figure 5).

Figure 5. Left: Hemacytometer mounted on a phase contrast microscope stage ready to be used for calculating manually the sperm concentration/mL of a semen dose. Right: Screen obtained when counting sperm concentration in a sample by using both the Sperm Vision CASA system and the hemacytometer.



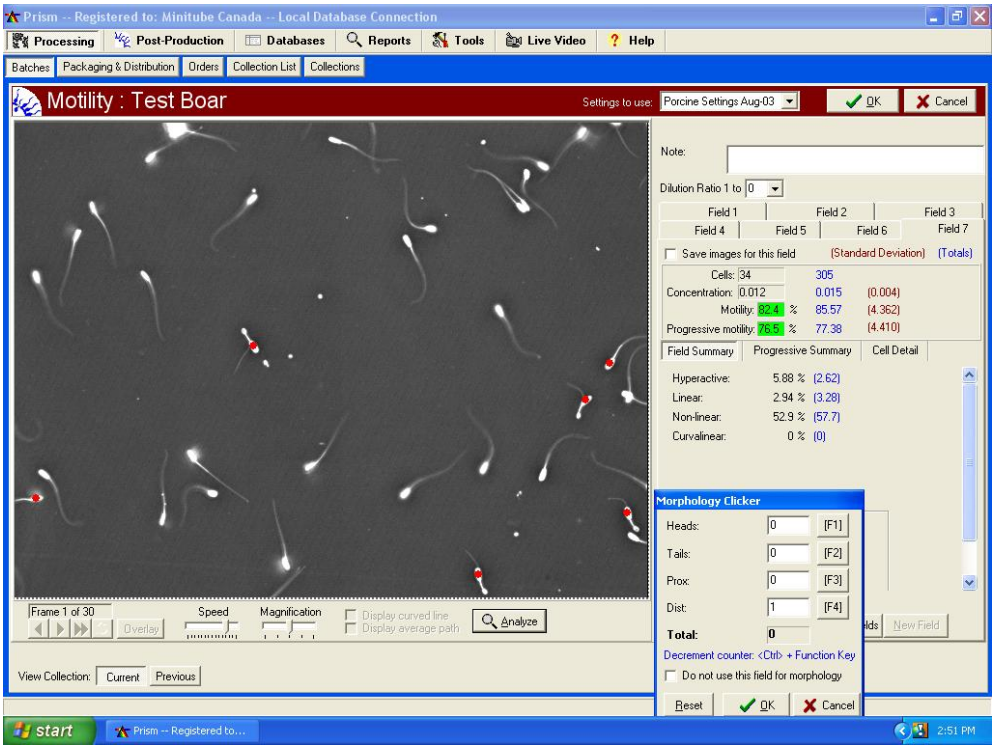
Sperm Morphology Evaluation

1) Gross morphology

This evaluation is performed to have a general idea of the sperm morphology present in the sample analysed and search for any evident sperm abnormality. This method is used especially when several semen ejaculates of different boars have been pooled and extended together (pooled doses). Observation of the sperm morphology is done with a microscope using a low magnification objective (20X) and counting normal and abnormal cells. Defects normally found with this technique are abnormal heads, abnormal tails, cytoplasmic droplets, and detached heads. This type of evaluation does not allow detecting acrosome, DNA, vacuoles, and other sperm morphological abnormalities that require a higher microscopic magnification and staining of the cells.

Gross morphology evaluation can be done with a CASA system such as the SpermVision (Figure 6).

Figure 6. Gross morphology evaluation using the Sperm Vision.



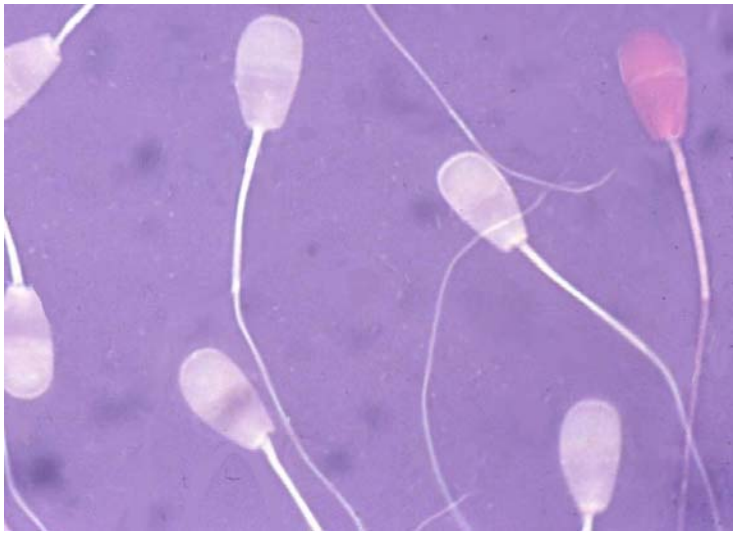
2) Detailed differential morphology

Sperm cells are translucent when observed with bright field microscopy reason to require the use of either special microscopy techniques or sperm staining techniques in order to perform a thorough and detailed morphological evaluation of the sample sperms, especially when reproductive sub-fertility is suspected. When using wet mounts, a drop of extended semen is

placed on a glass slide, the sperm cells are immobilized with a little drop of glutaraldehyde and then a cover slip is placed on top of the semen drop. To evaluate detailed differential sperm morphology in wet mounts, observation at x 1000 magnification under immersion oil is required in combination with specialized microscopic optical techniques such as Phase Contrast or Differential Interference Contrast (DIC) (Barth and Oko, 1989).

An alternative for detailed differential morphology is to make an extended semen smear stained with Eosin-Nigrosin observed at x 1000 magnification under immersion oil using bright or phase contrast microscopy (Figure 7) (Barth and Oko, 1989).

Figure 7. Detailed differential sperm morphology evaluation in a semen sample stained with Eosin-nigrosin observed at x 1000 magnification/oil immersion with bright microscopy.



With any of the techniques described it is necessary to count at least 100 sperm cells per sample which will be classified in morphological categories. The number of categories used will depend on the training received by the evaluator and how confident he/she feels about performing the evaluation. In general, the basic categories used for sperm differential morphology evaluation are normal cells, head defects, tail defects, and cytoplasmic droplets. However the classification can be extended to categories such as acrosome defects, detached heads, midpiece defects, proximal droplets, distal droplets, teratoid cells, other cells present, etc.

SEMEN CULTURE FOR BACTERIOLOGY

Bacteriospermia or contamination of semen with bacteria is a very common finding in collected boar ejaculates (Althouse and Lu, 2005).

Although the boar reproductive tract is free of bacteria, boar ejaculates post-collection are heavily contaminated with bacteria containing 10^2-10^6 microorganisms/mL (De Grau et al.,

2006). The primary origin of semen bacterial contamination is the boar, but other contaminant sources contributing are the barn environment, personnel working in the barns and laboratories, and the quality of water used to dilute the semen extender (Althouse and Lu, 2005). The semen contamination normally happens during the collection process due to the proximity of preputial fluids, manure, hair and skin. The hands of the technician performing the collection and equipment used during semen collection, processing and extension play a very important role in contamination (De Grau et al., 2006).

The majority of the bacteria species found as contaminants in boar semen are from the family Enterobacteriaceae. The most popular species of bacteria reported in the literature as contaminants are *Enterococcus* spp (20.5%), *Stenotrophomonas maltophilia* (15.4%), *Alcaligenes xylosoxidans* (10.3%), *Serratia marcescens* (10.3%), *Acinetobacter lwoffi* (7.7%), *Escherichia coli* (6.4%), *Pseudomonas* spp (6.4%) and other species (23.0%) (Althouse and Lu, 2005). In a recent retrospective study (Table 1) regarding bacteria found in boar semen samples collected at 12 boar studs across Canada and submitted to 3 different laboratories, the most common grown isolates were *Pseudomonas* spp (25%), *Acinetobacter* spp (9.7%), *Escherichia coli* (6.4%), *Staphylococcus* spp (6.4%), *Citrobacter* spp (6.4%), and *Shewanella putrefaciens* (3.2%) (De Grau et al., 2006).

Contamination of extended semen with high concentrations of bacteria can produce reduced fertility, lower conception rates and short shelf life of semen doses. Bacteriospermia could reduce semen quality by reducing sperm motility, causing sperm cell death, and damage to the acrosome. Sows inseminated with semen contaminated with bacteria can show vulvar discharges and endometritis. For these reasons it is important to emphasize to the boar stud personnel the need of using hygienic semen collection and processing procedures. Excellent cleaning and disinfection of the laboratory equipment and premises is also required, and the addition of antibiotics to the semen extenders has been implemented to protect the sperm cells (De Grau et al., 2006).

Due to the risk of bacteriospermia, it is ideal that boar studs should request quality control of extended ejaculates to detect contaminant bacteria. Each extended semen sample is streak out on a 5% blood agar culture plate using a microbiology culture loop that will be incubated at 37°C for at least 24 hours to detect any contaminant micro-organism present.

The ideal scenario is to have no micro-organisms growing post-culture (Figure 8) (Reicks, 2003).

If there is growth of bacteria in any extended semen sample (Figure 9), the bacterium species needs to be identified and sensitivity/minimum inhibitory concentration (MIC) testing must be done to determine which antibiotics will be able to control the micro-organism (Reicks, 2003). The identification and antibiotic sensitivity/(MIC) can be done by a specialized veterinary microbiology laboratory such as the Animal Health Laboratory located at the Ontario Veterinary College.

Table 1. **Results from 181 semen samples collected at 12 Canadian boar studs submitted to three different diagnostic labs across Canada during 2004 - 2005 (adapted from (De Grau et al., 2006).**

Bacterium species	Percentage of isolates	Possible source
ALCALIGINES	3.23	Water
Bacillus	3.23	Tubing/ extending system
Candida guilliermondi	3.23	Skin, feces
Clostridium perfringens	3.23	Environment, feces
Enterobacter cloacae	3.23	Skin, feces
Enterobacter sp	3.23	Skin, feces
Enterococcus	3.23	Feces
Lactobacillus	3.23	Feces
Micrococcus	3.23	Skin, environment
Moraxella	3.23	Skin
Providencia rettgeri	3.23	Feces
Pseudomonas aeruginosa	3.23	Soil, water
Shewanella putrefaciens	3.23	Water, soil
Stenotrophomonas maltophilia	3.23	Water
Citrobacter	6.45	Feces
E coli	6.45	Feces
Staphylococcus sp	6.45	Skin
Acinetobacter	9.68	Water baths/warming box
Pseudomonas sp	25.81	Environment

Figure 8. **Blood agar plate (below left) with no bacterial contamination post-incubation at 37° C.**

Figure 9. **Culture plate (above on right) of an extended semen sample contaminated with different micro-organisms.**

Extended semen quality control needs to be also implemented in the boar stud laboratory. It is important that specific laboratory areas in any boar stud could be screened and cultured in a regular basis to monitor the presence of micro-organisms (at least once/month). The areas to be cultured should be those ones getting in contact with semen or extender plus those that are normally warm and moist. Some examples of these areas are the water system, tubing used for transport of water or extender, pipette tips, extender vats, collection cups, incubators, water baths, warming boxes, slide warmers, etc. (Reicks, 2003).

When a bacterium is found and identified in a semen sample, it is sometimes possible to predict the potential source of contamination where it is coming from (Table 1).

Minitube Canada received in 2007 semen samples from several boar studs located across Canada for third party quality control evaluation. Of all the samples received, 449 samples were requested for bacteriology culture to monitor potential micro-organism contamination. Out of these specific 449 samples received, 157 (34.96%) showed contamination by growing 1 or more Colony Forming Units (CFU) of bacteria per plate after using a sterile culture loop with a capacity volume of 10 microliter. Out of the 449 samples, 45 (10.02%) grew $\geq$5 (CFU) of bacteria per plate which represents $\geq$500 bacteria per mL of extended semen. Due to this high level of contamination, these 45 semen samples were sent to the Animal Health Laboratory – Ontario Veterinary College (OVC) for bacterial identification and antibiotic sensitivity. In 38 samples out of the 45 submitted to the OVC laboratory were identified bacterial contaminants. Fifty eight isolations of bacteria species were found in these 38 samples and more than 1 species of bacteria were found in some of these bacteriospermic samples. Table 2 summarizes the species of the bacteria identified and their frequency of isolation.

Table 2. Species of bacteria isolated in 38 samples submitted by Minitube Canada to the Animal Health Laboratory – Ontario Veterinary College due to high level of bacteriospermia.

Bacterium species	Times isolated in 38 positive samples	Frequency of isolation
Klebsiella oxytoca	6	15.79%
Enterobacter agglomerans	1	2.63%
Enterobacter cloacae	3	7.89%
Serratia marcescens	9	23.68%
Acinetobacter spp.	1	2.63%
Klebsiella pneumoniae	7	18.42%
Stenotrophomonas maltophilia	7	18.42%
Stenotrophomonas spp.	3	7.89%
Pseudomonas spp.	16	42.11%
Moraxella spp.	2	5.26%
Proteus mirabilis	1	2.63%
Bacillus spp.	1	2.63%
Streptococcus sp. Alpha hem.	1	2.63%

CONCLUSIONS

Performing regular quality control evaluation of the semen doses produced by a boar stud is an excellent practice that serves to monitor and improve the techniques used by stud personnel to collect, evaluate, process, package, and transport boar semen. At the same time it provides assurance to the sow farmers that the final product received is of excellent quality for their AI programs.

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