INVESTIGATIONS OF ACHROMOBACTER XYLOSOXIDANS AND RALSTONIA PICKETTII IN PORCINE SEMEN EXTENSION SYSTEMS

BY

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THESIS

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ABSTRACT

Dilution and extension of semen longevity has been widely used in the swine industry to improve reproductive efficiency and genetic progress through artificial insemination. Through the dilution and extension of the life of the sperm cell, other organisms such as bacteria are also maintained and can produce a negative impact on sperm cell motility and potential reproductive capacity. Bacterial contamination in porcine semen is a widespread problem in semen collection facilities and routinely testing is essential to determine which organisms are present and the effectiveness of the antimicrobial in the semen diluents (extender). The antibiotics used in porcine semen extenders are generally chosen to be effective against the most common bacterial contaminants, gram-negative bacteria. Two isolates, *Achromobacter xylosoxidans* (AX) and *Ralstonia pickettii* (RP), were identified in the water distillation system of a boar stud facility that uses this water to extend the raw semen and found to produce pyometras in sows post-insemination. The effects of these bacteria have not been investigated in porcine semen diluents in long term storage (14 days). The objective of this study was to determine the effects of AX and RP on pH, motility, and progressive motility in culture negative semen samples over a 14 day period in 3 different diluents (BTS: Beltsville Thawing Solution; XC: X-cell; and TXC: Tri-X-cell; IMV USA; Maple Grove, MN, USA) at 16ºC. Banked isolates of AX and RP were grown on Columbia blood agar for 48 h at 37ºC. For each isolate, a single colony was selected and transferred to 10 ml of Luria broth and then incubated for 24 h at 37ºC in 5% CO_2. The broth cultures were centrifuged at 4000 rpm for 5 min. and used to make the final concentrations of approximately 2.5 x 10^7 CFUs/ml (AX) and/or 2 x 10^6 CFUs/ml (RP). There were four treatment groups per extender: AX, RP, AX + RP, and Control (no bacteria added). All samples were incubated at 16ºC and rotated once daily. Motility and morphology of all samples were

viewed using the Computer Automated Semen Analysis Program (SpermVision®, Minitube of America, Verona, WI), and pH were measured daily for each sample. Data from 6 replicates were was analyzed using PROC MIXED (SAS Institute, Inc., Cary, NC) with repeated measurements divided into 5 time periods (1-2 d; 3-5 d; 6-8 d; 9-11 d; and 12-14 d) post-inoculation. Overall, sample pH did not significantly increase over time, but was found to be the highest ($p<0.0001$) in BTS compared to XC and TXC and lower ($p=0.02$) in samples containing RP and AX+RP. Motility of BTS dropped significantly ($p<0.0001$) in the last time period (12-14 days) compared to XC and TXC. Motility did not change ($p>0.05$) drastically in the semen samples inoculated with RP+AX as compared to AX, RP or Control sample. The motility of the samples remained similar during the first week of incubations, but began dropping during period 4 (9-11 days) with the most notable decline during period 5 (12-14 days). This study showed that the presence of *A. xylosoxidans* and *R. pickettii* in water for semen extension of porcine semen does not detrimentally affect sperm motility or pH of the final solution regardless of choice of semen diluent.

For my father,

Richard D. Hurt,

for always believing in me

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CHAPTER 1

INTRODUCTION

Artificial insemination is used widely in the swine industry (Weitze, 2000). The advantages include increased availability of genetic diversity, decreased costs to feed and house large numbers of boars, and decreased disease risk. To maximize efficiency, boars can be housed at a semen collection facility out of which semen is collected, processed, and shipped to producers for use. One boar can breed 20-40 females per week via artificial insemination, versus 6 per week via natural cover (Knox et al., 2008).

Semen diluents (extenders) are used to increase the volume of diluted sperm cells, provide nutrients for metabolism, protect against cold shock, provide electrolytes for osmotic pressure, and buffer against extremes in pH (Levis, 2000). Bacteria are a normal component of the boar ejaculate (Sone et al., 1989). For this reason, semen extenders contain at least one antibiotic. Commercial extenders are formulated for different storage lengths, from short-term (1-3 days) to long-term (6-7 days).

There are a number of assays used to predict semen fertility, but most are not practical for commercial applications. The classical methods of semen evaluation include volume and concentration, total and progressive motility, and sperm cell morphology (Colenbrander and Kemp, 1990; Vazquez et al., 1998; Shipley, 1999; Gadea et al., 2004; Sutkeviciene et al., 2009). Volume and concentration reflect testicular function, which is related to fertility (Gadea, 2005); motility reflects integrity of sperm membranes and functionality (Johnson et al., 2000), which is related to farrowing rate and number of piglets born (Hirai et al., 2001; Sutkeviciene et al., 2009). Sperm morphology is also related to fertility (Gadea, 2005), and a high number of

abnormalities has an adverse effect on fertility (Bonet, 1990; Colenbrander et al., 1990; Gadea, 2005).

Bacteriospermia is a common problem, which can lead to complaints of decreased shelf life from decreased motility or sperm agglutination, as well as reproductive problems in the inseminated females (Althouse et al., 2000). Many of the common bacterial genera found in boar semen are resistant to aminoglycosides, one of the most common antibiotics found in semen extenders (Althouse et al., 2000; Reicks, 2003; Althouse and Lu, 2005). The boar itself is the primary source of bacterial contamination in the ejaculate, likely due in large part to the preputial diverticulum (Althouse and Evans, 1994). Appropriate hygiene during semen collection can decrease the bacterial contamination of the ejaculate (Sone, 1990; Levis, 2000; Althouse et al., 2005). However, other sources of contamination exist, such as human handlers, collection equipment, laboratory equipment, and the water used for semen extension (Althouse et al., 2005). To determine the source of contamination, raw and corresponding extended ejaculates should be cultured, as well as the water source and laboratory equipment (Reicks, 2003; Althouse et al., 2005). Even if there is no known bacterial problem on site, semen should be cultured regularly to monitor for contamination (Reicks, 2003).

Two common contaminants found in extended porcine semen are *Achromobacter xylosoxidans* (AX) and *Ralstonia pickettii* (RP), both Gram-negative, biofilm-forming bacteria that have been found in water systems (Reverdy et al., 1984; Morrison and Boyce, 1986; Spear et al., 1988; Anderson et al., 1990; Kendirli et al., 2004; Ryan et al., 2006; Siebor et al., 2007). These contaminants are widely considered opportunistic pathogens in the human literature, but to the author's knowledge, there is only one report citing AX as a cause of clinical disease in swine (Payne et al., 2008). In the human literature, these organisms exhibit almost uniform resistance to

multiple drugs, including aminoglycosides (Yabuuchi and Ohyama, 1971; Pien and Higa, 1978; Igra-Siegman et al., 1980; Reverdy et al., 1984; Spear et al., 1988; Maroye et al., 2000; Aisenberg et al., 2004; Gales et al., 2005).

Contamination from AX and RP was discovered in extended semen from a boar stud on routine third party analysis; however, semen quality appeared to be unaffected (third party and in-house evaluation). The contamination was sourced to the water system used for semen extension, and despite multiple attempts to clean/disinfect the system, the bacterial problem persisted. An outbreak of reproductive failure in the sow farms supplied by the boar stud was definitively linked to the AX contamination of the semen (Payne et al., 2008).

Research was initiated to evaluate both AX and RP in extended semen. In preliminary trials, semen from a culture-negative boar was extended in BTS and was inoculated with AX (2.5 x 10^7 CFU/ml) and/or RP (2 x 10^6 CFU/ml) banked from the clinical cases. These samples were stored in a manner to mimic routine swine semen storage and monitored for pH and motility for 7 days. The analysis revealed significant reductions between the AX and control groups with regard to motility, but not between the other treatment groups (Clark et al., 2008). With regard to pH, significant differences were noted between all treatment groups, and a treatment by time effect was also noted.

Expanded studies were designed, this time using three commercial extenders - Beltsville Thaw Solution (BTS), X-Cell (XC), and Tri-X-Cell (TXC) - all of which contain gentamicin. Despite the MIC data showing resistance to aminoglycosides (R>8 µg/ml), bacteria was inoculated into extender and monitored over 14 days for growth. Serial dilutions were made and

plated at 7, 10, and 14 days. During that time, AX thrived, increasing in concentration by 400%; while RP decreased as much as 98%.

Semen from a culture-negative boar was extended in three commercial semen extenders, inoculated with AX and/or RP at the same levels as the previous work, and monitored as to pH and motility (total and progressive) over fourteen days. The results were divided into 5 time periods, to facilitate analysis as repeated measurements in time. The results indicate that pH was significantly affected by extender, time, and treatment ($p<0.05$). Extender had a significant effect on total motility ($p<0.1$) and progressive motility ($p<0.05$); however, in this study, treatment did not affect either total or progressive motility.

CHAPTER 2

LITERATURE REVIEW

2.1 Artificial insemination in the swine industry

Artificial insemination (AI), the placement of spermatozoa into a female genital tract using artificial means, is a reproductive tool that has been well-utilized in the swine production industry. By the turn of the century, nearly 50% of the worldwide gilts and sows were inseminated (Weitze, 2000), and the percentages are much larger in some countries. To put it in absolute numbers, as of 2006, approximately 25 million AI breedings were registered worldwide annually (Roca et al., 2006). Natural service is still used by many, but artificial insemination carries a number of advantages over natural service. Genetic diversity and improvement become much more affordable when artificial insemination is used, as the farm does not have to purchase, feed, and house a large number of boars of varying genetic lines. The boars with higher genetic potential can be more efficiently utilized and thus are available to more producers. Additionally, limiting the number of boars on each breeding site also decreases the risk from aggressive animals to personnel and other animals in the breeding program.

To maximize efficiency and decrease costs of production, the semen can be collected, processed, and shipped from a semen collection facility. Because each animal can provide multiple breeding doses from a single ejaculate, costs are lowered and profits are maximized. One boar can often provide 20-40 breeding doses per week when utilized for AI, while boars used for natural service are unable to breed more than 6 times per week before reaching exhaustion and depleting semen reserves (Knox et al., 2008).

The swine industry is particularly sensitive to the threat of disease outbreaks. The movement of animals between or within countries, whether for food or genetic improvement, can cause disease transmission, risking the possibility of widespread outbreaks and severe financial loss (Boender et al., 2007; Maes et al., 2008). To counter this threat, regions and nations impose restrictions and health standards upon animal import (U.S.D.A., 2011; U.S.D.A., 2011). Individual farms usually employ their own strategies to control disease transmission within their herds – imposing regulations for down time and clothing worn by the human staff, restricting entrance to the herd, imposing isolation periods and health monitoring for new animals entering the herd. The use of AI has been invaluable in allowing a more rapid and less risky introduction of new genetics, as there is no direct animal contact. However, contaminated semen still poses a risk for disease transmission (Guérin and Pozzi, 2005).

Boar studs maintain high health status by vaccinations, isolation and health testing protocols, use of disposable materials during collection and processing of semen, and antibiotics in the semen diluents (extenders) to control bacterial growth and decrease the presence of environmental contaminants that may affect fertility (Althouse et al., 2005; Knox et al., 2008). Boars are isolated prior to entry and tested to ensure negative disease status prior to entry into the stud. Once the boars are acclimatized and enter the main facility, periodic serological monitoring for disease can help to assure the sow farms of the herd health status (Maes et al., 2008). The boars then never leave the boar stud unless they are culled, and entrance to the boar stud can be carefully controlled, serving to lessen the risk of disease transmission into the stud. Vigilant biosecurity and frequent monitoring of the health status of the boar herd can reduce the threat of disease transmission, but the potential for disease transmission via AI still exists.

It is nearly impossible to collect porcine semen without bacterial contaminants (Sone et al., 1989). With the typical storage temperature set between 16 and 18º C, growth of bacterial contaminants is not inhibited (Levis, 2000). For this reason, semen diluents (extenders) contain at least one antibiotic to control bacterial growth. The particular antibiotic and its concentration vary with extender, and farms should consider the bacteria typically present in their boars' ejaculates and whether the antibiotic is effective against that organism.

Another consideration when making a choice between semen diluents (extenders) is the desired storage time. Commercial semen extenders are classified as short-term (1-2 days storage), mid-term (3-5 days storage), and long-term (6+ days storage). The choice of extender is based upon the storage needs of the particular semen facility. For producers using their own boars for inseminations on their farms, a short-term extender may be considered sufficient; for semen facilities servicing client farms across the country or in other countries, long-term extenders are typically more popular.

The usefulness of AI is limited by the length of reasonable storage time. The majority (99%) of inseminations are performed with semen extended with liquid diluents. These diluents are used to increase the total volume of diluted sperm cells, provide nutrients for metabolism, provide an environment to protect the sperm cells from rapid cooling, provide buffers to protect sperm cells against extreme shifts in pH, provide electrolytes for proper osmotic pressure, and provide antibiotics to inhibit bacterial growth (Levis, 2000).

Not all boars are suitable for use in AI programs. There are criteria which should be considered when evaluating boars for use in a semen collection program for use in AI. Boars may exhibit satisfactory fertility when used via natural service on a small number of females yet

fail to meet criteria for use in AI, as the large number of spermatozoa present in the ejaculate can overcome a large proportion of anatomical or functional defects. For this reason, AI centers should carefully evaluate boars prior to entry into the program as well as each ejaculate thereafter.

2.2 Conventional semen analysis

While there are numerous tests available to predict the fertilizing ability of spermatozoa, many are impractical under commercial conditions. The classical methods of semen evaluation generally include volume and concentration, motility (gross and/or progressive), and sperm cell morphology (Colenbrander et al., 1990; Vazquez et al., 1998; Shipley, 1999; Gadea et al., 2004; Sutkeviciene et al., 2009). These assays do not predict fertilizing ability very well but are better for eliminating poor quality semen (Vazquez et al., 1998; Gadea et al., 2004). The constraints of field commercial semen evaluation are time, expense, and degree of difficulty. To be truly useful in the field, assays should not require highly technical expertise, be time-consuming, nor be expensive to run. In addition, they should be considered highly accurate.

Total sperm production by the boar reflects testicular function and can be examined by measuring sperm concentration, semen volume, and the number of sperm in the ejaculate. This is important as altered testicular function is related to decreased fertility (Gadea, 2005). Normal ejaculates can range in volume from 100-500 ml (Shipley, 1999); however, this is influenced by age of the boar, response to semen collection, and frequency of semen collection and should not be overemphasized. Ejaculate volume is of importance only when calculating total numbers of spermatozoa.

Motility is used as an indicator of the integrity of sperm membranes and functionality (Johnson et al., 2000). It is significantly related to the farrowing rate and number of piglets born (Hirai et al., 2001; Sutkeviciene et al., 2009), though the correlation coefficient in these studies is often low. When measured by the individual observer, motility is an extremely subjective assessment; however, when the mean of multiple subjective estimates is used, the sampling errors are reduced and the correlation coefficient is higher (Foote, 2003; Gadea, 2005). Sperm morphology is also related to fertility (Gadea, 2005). When the number of morphological abnormalities is high, fertility is adversely affected (Bonet, 1990; Colenbrander et al., 1990; Gadea, 2005).

Semen evaluation in the commercial semen processing laboratory usually begins with a gross examination of the fluid. Normal porcine ejaculates are gray-white to white in color and have a milky opacity. If the ejaculate is discolored (brown, yellow, red), or has a strong urine odor, contamination is likely. Many of these contaminants have spermicidal activity, and thus the ejaculate should be discarded (Shipley, 1999; Althouse, 2007).

The volume of the ejaculate is measured in a graduated cylinder or an approximation is made by weight measurement (1 g is equal to 1 ml). Sperm concentration can be determined with a dilution apparatus and a hemocytometer, but this is time-consuming and impractical for most AI laboratories. The most common method of determining concentration is by measuring the degree of sample opacity by means of a photometer (Knox et al., 2008). For accuracy, these instruments should be calibrated for the species in which they are being used (Althouse, 2007).

Sperm motility is analyzed via microscopic examination. Drops of semen are placed onto warmed glass slides and examined under magnification (100x and 400x). The percentage of

motile sperm cells can be estimated, as well as the percent progressively motile. Because sperm motility decreases during storage, ejaculates should show at least 70-80% total motility in order to be considered for further processing (Shipley, 1999; Althouse, 2007). Computer-automated sperm analysis (CASA) systems are available which measure motility more objectively – for each sample, multiple fields are analyzed and averaged to reach a measurement. The decrease in sampling errors achieved through CASA systems raises the correlation coefficient, indicating a stronger association with fertility (Foote, 2003). But as these are not inexpensive, they are not frequently found in commercial AI centers (Verstegen et al., 2002). A recent survey of North American boar studs found that only 28% of the respondents used CASA systems (Knox et al., 2008).

Morphology of the sperm cells is also examined via microscopy. Wet mounts of buffered formalin- or glutaraldehyde-fixed semen can be examined with phase-contrast microscopy to assess the morphology and acrosomal integrity. If dry mounts are preferred, there are a variety of contrast stains which can be used to accentuate the outline of the sperm cells, allowing easier visualization of the structures; some of these stains can be used with light microscopy. A minimum of 100 cells should be morphologically assessed and categorized, and normal boar ejaculates usually contain less than 15-20% abnormal sperm cells (Althouse, 1997; Shipley, 1999; Althouse, 2007). The sperm cells are examined for normality of the head, midpiece, and tail distal to the midpiece. Defects include proximal and distal cytoplasmic droplets, abnormal heads, detached heads, coiled tails, midpiece defects, and bent tails. Abaxially attached tails are not unusual in boar semen and should not be counted in the abnormalities noted (Shipley, 1999; Althouse, 2007). Cytoplasmic droplets may have an especially compromising effect on fertility,

and therefore ejaculates containing more than 15% cytoplasmic droplets (both proximal and distal) should not be used (Althouse, 1998; Johnson et al., 2000).

2.3 pH

The pH of freshly ejaculated boar semen is similar to that of other body fluids, typically 7.4 ± 0.2 (Gadea, 2003). Most commercial porcine semen extenders range from pH 6.8 to 7.2, using different buffering agents to control the pH. There are simple and complex buffers, which differ in their ability to stabilize the pH over time and over temperature ranges. When the pH of the semen is reduced, the internal pH of the spermatozoa is also reduced, leading to a decrease in the sperm's metabolism and mobility (Gatti et al., 1993; Gadea, 2003). Decreasing metabolism and mobility can be useful for prolonging the lifespan of the spermatozoa, when longer storage of semen is desired.

2.4 Bacteriospermia

Bacterial contamination in freshly extended semen is a problem that can lead to reduced shelf life and agglutination problems in the stored semen (Reicks, 2003; Althouse et al., 2005). Bacteria are typically present in ejaculate collected using the gloved hand method of collecting boar semen (Sone, 1990; Althouse et al., 2005).

A study conducted in Japan in 1990 found thirteen bacterial genera in 46 fresh semen samples (Sone, 1990). The following species were included: *Pseudomonas* spp. (80.4%), *Micrococcus* spp. (63.0%), *Staphylococcus* spp. (56.5%), *Klebsiella* spp. (52.2%), *E. coli*

(41.3%), *Citrobacter* spp. (30.4%), *Proteus* spp. (21.7%), *Actinomyces* spp. (15.2%), *Serratia* spp. (8.7%), *Enterobacter* spp. (6.5%), *Bacillus* spp. (6.5%), and *Streptococcus* spp. (4.3%).

The Japanese study also examined the survival of spermatozoa in the presence of different bacteria. Five species of enteric bacteria, including *E. coli*, resulted in decreased sperm survival, and fertility was lost entirely within 1-2 days, when the pH decreased to between 5.2 and 5.7. The presence of *Pseudomonas* spp. exerted a lesser effect on the spermatozoa. The effect of *Alcaligenes* spp., *Actinomyces* spp., *Streptococcus* spp., and *Staphylococcus* spp. was negligible, even when the bacterial concentration reached 10^{10}-10^{12} CFUs/ml and the pH averaged 6.3 to 6.5 (Sone, 1990). The effects of different antibiotics were tested on the bacteria-contaminated semen. At that time, the aminoglycosides tested (dibekacin, amikacin, and gentamicin) showed marked antimicrobial activity against all 11 species tested, with a low MIC (<6.25 µg/ml) (Sone, 1990).

Ten years after Sone's study, Althouse et al. (2000) conducted a set of field investigations into North American boar studs and farms with primary complaints of sperm agglutination and decreased sperm longevity in the extended semen. This was accompanied by an increase in return to estrus and/or vaginal discharges in sows and gilts, across parity. When the extended semen was examined microscopically, decreased gross motility (<30%), sperm agglutination, and sperm cell death within 2 days of semen collection and processing was seen, without regard for the extender used (Althouse et al., 2000). Out of 56 semen doses analyzed, over sixty percent were contaminated with a single bacteria; the rest contained two or more bacteria. The most frequently isolated bacteria were *Burkholderia cepacia*, *Enterobacter cloacae*, *E. coli*, *Stenotrophomonas maltophilia*, *Serratia marcescens*, and *Achromobacter xylosoxidans*, all of which were found to be resistant to gentamicin. The sample pH was acidic (5.7 to 6.4) in nearly

all of the samples. In this study, pure cultures of those common contaminants were inoculated into freshly extended porcine semen and monitored for 72 hours. Within 2 days, microscopic sperm agglutination and decreases in gross motility were also seen in the inoculated semen, while the control semen continued to exhibit good motility and remained free of bacterial growth for 72 hours of storage.

Several years later, a retrospective study was performed at the University of Pennsylvania (Althouse et al., 2005), examining 250 extended semen samples which were submitted for routine quality control over a period of three years. Seventy-eight samples were positive for bacterial contamination, with the six most common contaminants being *Enterococcus* spp. (20.5%), *S. maltophilia* (15.4%), *A. xylosoxidans* (10.3%), *S. marcescens* (10.3%), *Acinetobacter lwoffi* (7.7%), *E. coli* (6.4%), and *Pseudomonas spp.* (6.4%). Testing was performed to determine antimicrobial MICs for each of these bacteria, and eighty-six percent of the isolates were found to be resistant to the antimicrobial used in the semen extender. Of the remaining isolates, despite the MIC data which indicated that the bacteria should be susceptible to the levels of antimicrobials used in the extender, they were able to thrive.

The data from these studies indicate that bacterial contamination of extended porcine semen is a widespread problem which can result in reproductive inefficiency in the sow herd, leading to increased costs of production. It is for this reason that antimicrobials are used in semen extender, to decrease the bacterial load of the extended semen. However, without routine surveillance for bacterial contamination, there is no way to determine which bacterial species are present on a particular farm and which antimicrobials should be used in the semen extender. Regular culture of extended semen doses should be performed to evaluate bacterial contamination and assess whether there is a problem on a particular farm (Reicks, 2003;

Althouse et al., 2005). If bacterial growth is observed, the isolates should be identified and MIC testing should be performed. Antibiotics in the extender should be chosen based on the on-farm microbial data. While antibiotics are not a panacea for bacteriospermia, they may help mitigate the problems it causes until the source of contamination is determined.

2.5 Sources of semen contamination

The primary source of bacteria in porcine semen is the boar itself, as bacteria can be considered a normal component of the ejaculate when semen is collected using the gloved hand technique (Althouse et al., 2000; Althouse et al., 2005). A list of the bacterial genera cited in four studies which investigated bacterial contaminants in freshly collected, non-extended boar ejaculates can be found in Table 1. The majority of the bacteria found were not considered primary pathogens in swine.

The preputial diverticulum, an anatomical structure unique to the boar, contributes substantially to the potential bacterial contamination of semen. The diverticulum is capable of holding greater than 50 ml of fluid contents (urine, bacteria, semen, etc.) (Althouse et al., 1994), which can easily contaminate the shaft of the penis. This fluid may aid in lubrication for copulation, but it can serve as a source of bacterial contamination for semen collection. Appropriate hygiene on the part of the handler can help to minimize this risk – wearing two sets of latex-free gloves prior to semen collection, manually emptying the diverticulum, and then removing one set of gloves prior to grasping the penis; keeping hairs at the preputial opening trimmed to reduce contamination from debris; rinsing the penis and collector's hand prior to semen collection (Sone, 1990; Levis, 2000). If a boar has a particular problem with bacterial

infections of the diverticulum, the structure can be surgically removed, which will greatly reduce the bacterial load from the boar (Althouse et al., 1994). The penis should be held perpendicular to the boar during the collection process to decrease contamination of the collection cup by preputial fluids. The pre-sperm and gel fractions of the ejaculate contain more bacteria and should not be collected (Althouse et al., 2005).

The human handlers can also contribute to the bacterial content of the semen. Frequent hand washing or wearing protective gloves can help – not only semen collection, but in all phases of the semen processing. Hair can be a source of contamination, and caps or hairnets may be worn, much as in a restaurant during food preparation (Althouse et al., 2005). Ill personnel, especially those with upper respiratory infections, should be careful to avoid sneezing or coughing into the equipment or semen.

In the boar pens, equipment used to collect semen should be cleaned between animals or disposable. Many use thermal containers as collection receptacles but place single use collection bags (with or without filters) inside to avoid needing to clean the cups between individual animals as many detergents have been shown to be spermicidal.

In the laboratory, equipment should be properly cleaned and dried between uses. The laboratory bench should be disinfected before semen processing begins and after any spills or contamination. However, protocols must be specific to avoid leaving residues that could adversely affect semen quality (Althouse et al., 2005).

Water is essential in semen processing, as it is used to make the semen extender for fresh storage. If the water is contaminated before it comes into the laboratory, all the semen processed using that water will also be contaminated. Well or municipal water should be tested at least

quarterly and should at minimum meet local public health ordinances (Althouse et al., 2005). Purified water systems should also be tested at least quarterly, and any bacterial growth should be considered significant. If purified water is purchased, each manufactured lot should be tested (Althouse et al., 2005).

The packaged extender product should be monitored for contamination. Semen extender is often purchased in bulk, to decrease costs. On the day that a bulk container is opened, it should be immediately repackaged into more appropriate amounts and stored as per the manufacturer's instructions. Only the amount of extender needed for one day's use should be mixed, and reconstituted extender should not be kept overnight and used the next day.

There are many opportunities for bacterial contamination of semen to occur at the boar stud – whether via the boar itself, the environment, personnel, or in the semen processing laboratory. But it is equally important to examine the sources of contamination that can occur after the processed semen has been shipped. The semen must be handled in a clean manner; if a breeding dose is opened to obtain a sample for examining semen parameters, the sample should be removed with sterile pipettes, especially if the dose will not be used immediately. Equipment used in artificial insemination (pipettes, lubricant, etc.) should be kept as clean as possible or used once and discarded.

Problems due to semen contamination may not be quickly realized, if the only observations are made with regard to the sows' reproductive parameters: i.e., vulvar discharges, return to service, etc. These observations can be essential to note the degree of a problem. However, semen contamination issues can be more promptly found and addressed when the

semen collection facility is actively looking for them. Thus, extended semen should be cultured regularly to check for bacterial content (Reicks, 2003; Althouse et al., 2005).

When a problem is noted in the extended semen samples, more investigation is required to determine the source of the contamination. Raw ejaculates can be cultured with their corresponding extended samples to confirm whether the source is the boar or whether the semen was contaminated in processing. Laboratory equipment should be swabbed to look for bacterial contamination, including but not limited to pipette tips, tubing, dispensing equipment, thermometers. Surfaces in the lab – counters, slide warmers, warming boxes – and water sources should also have samples obtained for culture. If bacterial growth is found, MIC (minimum inhibitory concentration) testing of various antimicrobial agents can be performed to determine which antibiotics could be used to address the problem. Sanitation processes can also be re-evaluated in light of the identified microbe and its susceptibility data. One seven-year retrospective study (Reicks, 2003) of ten boar stud facilities revealed that nearly 14% of the more than 1500 semen samples showed bacterial growth. In that study, over half of the bacterial growth was attributed in some way to contamination of water – the water used to mix the semen extender, water in the warm water baths, etc.

2.6 Bacterial characteristics

Achromobacter xylosoxidans

Previously known as both *Alcaligenes denitrificans* subspecies *xylosoxidans* and *Alcaligenes xylosoxidans* subspecies *xylosoxidans*, *Achromobacter xylosoxidans* was first

described in 1971 by Yabuuchi and Ohyama (1971), who found it in the aural discharge of patients with chronic, purulent otitis media. The organism is frequently found in aqueous environments (Reverdy et al., 1984; Morrison et al., 1986; Spear et al., 1988). It is an aerobic, motile, oxidase- and catalase-positive, non-lactose-fermenting, Gram-negative rod, and it has a peritrichous flagella, which is useful for differentiating the organism from *Pseudomonas spp*. (Igra-Siegman et al., 1980; Duggan et al., 1996; Shie et al., 2005; Tsay et al., 2005).

This organism does not seem to be a primary pathogen in humans, and in fact *Achromobacter* species have been isolated from the normal human gastrointestinal tract and ear canal. However, it is not clear whether it may be a component of human endogenous flora (Reverdy et al., 1984; Duggan et al., 1996). Infections in humans have included many types – bacteremia, urinary tract infections, peritonitis, meningitis, etc. – in both immunocompetent and immunocompromised hosts. However, bacteremic infections due to *A. xylosoxidans* are thought to be mainly nosocomial in origin and affect mainly immunocompromised patients (Duggan et al., 1996). Cystic fibrosis patients represent an emerging class of patients at risk for *A. xylosoxidans* infection. As of 2001, it was unclear whether the organism was causing disease in these patients or merely taking advantage of an opportunity to colonize the respiratory tract (Burns et al., 1998; Saiman et al., 2001).

In a search of published reports of *A. xylosoxidans* infections in humans, the majority were found to originate from a contaminated water source; whether a well, a particular sink, or medical liquids. This bacteria is able to form a biofilm, which is an aggregate of organisms that adhere to one another and surfaces and aids in its survival in the water systems, such as sinks and pipes or tubing (Siebor et al., 2007). Intravascular items such as catheters and sterile saline can be contaminated, leading to introduction of infection (Gómez-Cerezo et al., 2003; Shie et al.,

2005). One patient became infected via untreated well water in her home; the only reported case in which a community-acquired illness due to *A. xylosoxidans* was attributed to a documented water source (Spear et al., 1988).

Infection with this organism can be especially dangerous due to its development multi-drug resistance. To the author's knowledge, every published report which cited MIC testing has listed multiple drug resistance patterns on the part of the organism, usually including some if not all penicillins, cephalosporins, and aminoglycosides, among others (Yabuuchi et al., 1971; Pien et al., 1978; Igra-Siegman et al., 1980; Reverdy et al., 1984; Spear et al., 1988; Aisenberg et al., 2004; Gales et al., 2005).

Infection due to *A. xylosoxidans* has not been cited as a clinical problem in swine. A recent search of the literature identified reports of contamination in extended semen, usually owing to a contaminated water source, and one report of the organism causing reproductive failure in sows and gilts (Payne et al., 2008). But to the author's knowledge, no other published reports have cited any clinical disease in swine resulting from this organism.

Ralstonia pickettii

Formerly known as *Pseudomonas pickettii* and *Burkholderia* pickettii, Ralstonia *pickettii* is also an aerobic Gram-negative, oxidase-positive, non-fermentative rod (Boutros et al., 2002; Ryan et al., 2006). It is found in the environment in water and soil, and it frequently contaminates water supplies, such as distilled water, water for injection, and aqueous chlorhexidine solutions (Kendirli et al., 2004; Ryan et al., 2006). The organism is able to pass

through extremely small (0.45- and 0.2-µm) filters that are used in terminal sterilization of medical products, which allows contamination to occur at the manufacturing stage (Anderson et al., 1985; Anderson et al., 1990; Ryan et al., 2006).

The organism was identified in 1990 in biofilm formation in plastic water piping (Anderson et al., 1990). During laboratory testing of the organism's ability to form biofilms, there was strong evidence that it could survive within colonized PVC pipes after seven days' exposure to a variety of chemical treatments (Anderson et al., 1990). The thickness of the material in the biofilm can apparently protect the organism from the action of the germicidal chemicals and serve as a continuous source of contamination in test pipes and of water flowing through the pipes (Anderson et al., 1990). The organism has even been described as ubiquitous in ultrapure water, considered to be an extreme environment with very low nutrients, and it is speculated that it may be able to utilize the lysed products from dead biomass in the biofilm to survive (Kulakov et al., 2002; McAlister et al., 2002).

While *R. pickettii* has been isolated from human secretions, it is not typically pathogenic (Maroye et al., 2000; Ryan et al., 2006). However, it has been identified as an opportunistic pathogen in nosocomial infections, most typically in immunosuppressed patients (Ryan et al., 2006). Most of the nosocomial outbreaks with *R. pickettii* have been reported in connection with contamination of hospital supplies, especially involving water. There has even been a case of *R. pickettii* noted with contaminated chlorhexidine skin-cleansing solutions (Maroye et al., 2000), an incident traced back to contaminated distilled water used to produce the chlorhexidine solutions.

Much like *A. xylosoxidans*, reports of *R. pickettii* in the literature demonstrate multi-drug resistance. In much of the available literature, resistance to some or all of the penicillins and aminoglycosides has been reported (Maroye et al., 2000; Gales et al., 2005).

A literature search did not identify any clinical disease noted in swine attributable to *R. pickettii*. It has been noted as a contaminant in extended porcine semen, but to the author's knowledge, no published reports exist of disease or reproductive problems resulting from use of the contaminated semen.

2.7 Boar stud field investigation

System description

In the fall of 2005, the Porcine Andrology Laboratory (PAL) at the University of Illinois College of Veterinary Medicine (UI-CVM) became involved in a field investigation involving bacterial contamination of porcine semen (Payne et al., 2008). In that field investigation, a 180-head boar stud located in the Midwest United States supplied a 21,000-sow system as well as several other smaller sow herds in northern Illinois. The sow system consisted of two genetic nucleus farms, two gilt multipliers, five commercial sow centers, two gilt breeding-gestation sites, and one commercial farrow-to-finish sow farm .

Case introduction

The herd veterinarian submitted extended semen doses for routine third-party analysis to the PAL at UI-CVM. As part of the analysis, samples were submitted for culturing, and bacterial

contamination was discovered. The primary contaminant, *Achromobacter xylosoxidans* (AX), was consistently recovered. Another related organism, *Ralstonia pickettii* (RP), was recovered sporadically. Despite this contamination, the concurrent semen analysis found no negative effects on semen characteristics such as motility and morphology, and there were no apparent clinical effects on the female reproductive tracts.

Microbial susceptibility

However, the herd veterinarian considered the contamination to be unacceptable, and a field investigation was begun to further assess the clinical isolates and locate the source of the contamination. The organisms were identified by the Clinical Microbiology Laboratory at the University of Illinois Veterinary Diagnostic Laboratory (UI-VDL) using a 96-well substrate system (Biolog MicroID; Biolog, Hayward, CA). The identification of AX was confirmed by forward and reverse genetic sequencing of the 16s-23s rRNA genes, performed by the Lloyd Carver Biotechnology Institute (University of Illinois, Urbana-Champaign, IL) on a typical isolate. The percent genetic similarity in these genes, compared with sequences deposited in the National Center for Biotechnology Information database was 99% (forward) and 100% (reverse). Further samples were tested for antimicrobial susceptibility, based on minimum inhibitory concentration (MIC) testing using Clinical and Laboratory Standards Institute M31-A2 Guidelines (C.L.S.I., 2008). Both organisms were found to be resistant to ceftiofur, clindamycin, erythromycin, florfenicol, gentamicin, neomycin, penicillin, spectinomycin, sulfachlorpyridazine, sulfadimethoxine, sulphathiazole, tiamulin, tilmicosin, and trimethoprim-sulfamethoxazole. They were both susceptible to ampicillin, chlortetracycline, enrofloxacin, and oxytetracycline. There were no interpretative guidelines for tylosin and danofloxacin. Susceptibility to amoxicillin was not tested.

The boar stud laboratory used the Beltsville Thaw Solution semen extender (BTS; IMV USA; Maple Grove, MN, USA), which contains gentamicin at 200 mg/L. Ceftiofur sodium (Naxcel®; Pfizer Animal Health, New York, NY) was added to the extender to reach a final concentration of 300 mg/L. Tri-X-cell™ semen extender (TXC; IMV USA; Maple Grove, MN, USA), which contains gentamicin, amoxicillin, and tylosin, was also sometimes used in the laboratory.

Source of the contamination

Multiple samples of raw semen and the corresponding single-sire extended semen doses were cultured on Columbia agar with 5% sheep blood (Remel, Lenexa, KS). The consistent lack of growth of AX and RP from raw semen samples and repeatable growth from the extended semen samples confirmed that the extension process was the source of the bacterial contamination.

The water used in semen extension was well water, which was exposed to multiple treatments (Figure 1) prior to entry into the laboratory as well as within the laboratory. Prior to the discovery of the contamination, the water treatment flow was as follows:

> Water had been pumped from a well and treated with hydrogen peroxide (1 mg per L of water from an 11% hydrogen peroxide stock solution), had flowed through a carbon filter, entered a water softener, and passed through an in-line ultraviolet light (Sterilight Silver, Model SQ-PA; R-Can Environmental Inc., Guelph, Ontario, Canada) into a single distiller at the rate of 5.6 L per minute. The distilled water dripped into a holding vat with a submersible ultraviolet light (253.7 nm, 30 mJ per cm^2, 10W bulb). It was then pumped through a single flexible 0.64-cm opaque tube with a drop line entering an extension vat where powdered extender was added to the warm water. A stir rod aided in reconstituting and mixing the extender solution, which was added to raw semen via a peristaltic pump (Masterflex; Cole-Parmer Instrument Co, Vernon Hills, IL) and disposable 0.64-cm transparent tubing. A second peristaltic pump and

> disposable tubing pumped the extended semen into cochette bags. (Payne et al., 2008)

Twenty-eight water samples and culturette swabs were taken from various points in the water-treatment flow. Eleven of the twenty-eight (39%) grew AX, with the flexible tubing downstream from the water softener and all critical control points thereafter culture-positive for AX throughout the investigation. All critical control points upstream from the water softener were culture negative.

Attempted removal of bacteria

Use of the well water was discontinued in late 2005 as the first control measure. Bottled distilled water from a commercial water supply company was purchased for use during the downtime of the on-site water system. The distilled water was cultured several times (n=5) and remained culture-negative for AX, although it was sporadically culture-positive for RP. During this time, the on-site water system was emptied and a 50% bleach solution was added to the distiller tank and drained through the system, remaining in the lines overnight. Commercially purchased and culture-negative distilled water was used to rinse the lines several times, before the lines were completely drained. They were allowed to dry for 2 weeks, after which time the system was returned to use, and weekly samples were collected for culture from the end of the treatment line. For three weeks, the water remained culture-negative for AX and was used for semen extension. After the third week, AX was again cultured from water samples. The system was shut down and the cleaning process repeated. Additionally, internal and external surfaces, connectors, and valves were sprayed with 70% ethanol. However, within two weeks, AX was again cultured from the water samples.

In early 2006, a new distiller, distiller tank, second in-line UV light, and new disposable lines were purchased and installed. The following week, the new system's water was immediately used for semen extension. During the next two weeks, the water cultured negative, but in the third week, the samples once again cultured positive for AX. However, at that time, the decision was made to continue using the water from the on-site distillation system for semen extension, as there had been no problems reported from the sow system, and both in-house and third-party analysis had shown no ill effect on semen quality.

Sow herd case description

During the fall of 2005 and early 2006 (Figure 2), there had been no complaints from the sow system. All reproductive health parameters – conception rates, farrowing rates, pigs born alive, and numbers of mummies and stillborns – had remained stable for the normal parameters of that sow system. However, in week 14 of 2006 (six weeks after the new parts of the water system were installed), one farm reported white purulent vulvar discharges from sows and gilts within 3 to 19 days of breeding. Upon further inquiry, it became apparent that all sow farms in the system were affected, and the discharges had started up to four weeks earlier, but the herd veterinarian had not been notified. The prevalence of the vulvar discharges ranged from 8 to 15% for breeding groups within 3 weeks of breeding. It was determined that the contaminated semen was the source of the discharges.

Over 50% of sows and gilts in which the vulvar discharges were noted were found not to be pregnant at pregnancy check with real-time ultrasound 4 to 5 weeks post artificial insemination. From weeks 10 to 17 in 2006, the average conception rate for the sow farms within the 21,000-head sow system dropped by 10-15% (Figure 3). Beginning at week 16,

purchased distilled water was again used in semen extension, and conception rates returned to normal (85-90%) at week 18.

Seven sows from four different sow farms within the system were submitted live for necropsy and diagnostic testing between 5 and 21 days post-breeding. Five of the sows had vulvar discharges, while the other two appeared normal with no evidence of vulvar discharge. Endometritis was diagnosed in each of the five sows with discharges, and AX was isolated from the uterus of four of the five. No uterine inflammation was noted in the two control sows, and AX was not cultured from the tissues. Within the seven sows, *Escherichia coli* was isolated from two sows with vulvar discharges and one sow without discharge. Based on this, *E. coli* was not implicated as the etiological agent of the endometritis.

Bacterial growth was monitored with the use of intrauterine swabs (Double Guarded Culture Swabs; Jorgenson Laboratories, Inc., Loveland, CO). The uterine swabs were collected during the summer of 2006 from sows with vulvar discharges (n=9) and sows without discharges (n=4). Upon culture of these swabs, AX was grown from six of the nine sows with discharges but not from any of the sows without discharges.

Other potential causes of endometritis could be ruled out based upon the widespread discharge and conception-rate problem within the system. The affected herds were located in three separate counties, and feed was provided from five separate feed mills with widely different feed-ingredient sources. Because the clinical signs occurred in the sow herds nearly simultaneously, it was unlikely that management or nutrition was the cause. Breeding technique and semen handling were also examined in several herds when the clinical signs were first noted, with no indication of issues in technique or timing of AI.

Predisposing factors causing immune-system dysfunction in the breeding herd were evaluated. Routine mycotoxin testing results from the feed mills were examined with no cause for concern found. Feed samples were submitted monthly from five sow herds, with no abnormalities found in the diet composition. All sow herds were positive for PRRS (Porcine Reproductive and Respiratory Syndrome) virus, including the genetic and multiplier herds. The PRRS virus may cause immune-suppression (Benfield et al., 1999).Endemic PRRS may be a predisposing factor for bacterial endometritis.

During the outbreak in the sow herds, attempted treatments were based on the susceptibility profiles of both the semen and uterine isolates, which were identical. Parenteral (oxytetracycline), intrauterine (oxytetracycline), and oral (chlortetracycline) antibiotic protocols were used to treat affected sows but were found to be ineffective. Because of this, parenteral ceftiofur, penicillin, and tylosin were also used, despite the isolates demonstrating resistance to ceftiofur. This protocol also demonstrated lack of efficacy. Cull rates increased up to 30% as sows and gilts with discharges were found to be not pregnant and culled based on "uterine infection." There were no reports of rejected carcasses or significant cut-outs from the slaughter facilities.

Implications

This field investigation was the first reported instance of AX as the cause of reproductive failure in sows, though endemic PRRS was most likely a predisposing factor for the endometritis. The clinical signs and negative effect on conception rate could have been insignificant in a PRRS-negative sow system. The thorough diagnostic workup on females with and without discharges gave strong evidence that AX was the etiological agent of the

endometritis in this sow system, although further work would need to be done to satisfy Koch's postulates.

2.8 Laboratory follow-up to the boar stud field investigation

As the field investigation described in the previous section was concluding, follow-up studies were initiated in the PAL at UI-CVM. Based on the review of the literature on bacteriospermia, a loss of semen quality over time would have been expected in the contaminated extended semen doses. However, based upon in-house and third party evaluations, this was not the case (Payne et al., 2008). Research projects in the PAL were initiated to repeat the contamination under laboratory conditions and further evaluate and monitor the contaminated extended semen.

Although AX was determined to be the etiological agent of the endometritis, RP was also cultured consistently from both the water and extended semen samples. Therefore, the project was designed to examine the effects of both bacteria on extended semen.

Both strains were cultured from pure isolates banked from the clinical case at UI-VDL, on Columbia agar with 5% sheep blood (Remel, Lenexa, KS) or Luria broth at 37º C in 5% CO_2 using standard microbiological techniques.

Growth characteristics

The first study objective was to determine the fundamental growth patterns of the organisms up to 48 hours. Banked isolates were revived on Columbia agar with 5% sheep blood.

Following incubation for 48 hours at 37º C in 5% CO_2, a single colony of each strain was inoculated into 25 ml of Luria broth in a 50 ml conical tube. Broth cultures were held at 37º C in 5% CO_2 without shaking for 24 hours. Following this initial 24 hour incubation, aliquots were taken every 12 hours for measurement of the optical density (600 nm) using a spectrophotometer. Since neither strain developed significant growth during that period, as determined by unmeasurable OD_{600}, producing a growth curve for these slow growing organisms was abandoned and instead, a final concentration was determined following 24 and 48 hour incubation. To determine the final concentration for each bacterium, serial dilutions were performed, plated in triplicate, and counted after 24 hours of incubation to determine colony-forming units (CFUs)/ml. The experiment determined that AX grew to approximately $1x10^8$ CFUs/ml after 24 hours incubation in Luria broth, and RP grew to approximately $1x10^7$ CFUs/ml (Clark et al., 2007).

Inoculation of extended semen

The next study examined the effects, if any, of the two bacteria on semen quality over time. Culture-negative (AX & RP) semen from a single boar was used to eliminate the possibility of variability in the semen source. The semen was processed within 30 minutes of collection, according to standard protocol, and extended in BTS extender, as used by the boar stud laboratory. One ml was removed from each tube of cultured Luria broth and collected via centrifugation at 4000 rpm for 5 minutes The Luria broth was removed without disturbing the pellet and extended semen was added to dilute the bacteria to the final concentration desired: 2.5×10^7 CFU/ml (AX) and 2×10^6 CFU/ml (RP). The treatment groups were AX, RP, and control (no bacteria). The samples were stored at 16º C and rotated daily, as is standard for swine semen storage. Motility and morphology, viewed using the Computer Automated Semen Analysis

Program (Spermvision®, Minitube of America, Verona, WI), and pH were measured daily for each sample. Data from 4 replicates were used in the analysis. (Clark et al., 2008).

Results

For motility, an ANOVA revealed no significant differences ($p<0.05$) between the control and inoculated samples. A PROC MIXED analysis (SAS, SAS Institute, Inc., Cary, NC) revealed no treatment by time interaction with sperm motility after inoculation. For sample pH, statistically significant differences ($p<0.05$) were seen between all the treatment groups, primarily contributed by a treatment by time effect. The pH of the control samples became more basic (from 6.94 to 7.32) over the study duration. This was seen in all samples, regardless of treatment group, but the AX samples remained closer to a pH of 7.0 than the RP samples (Clark et al., 2008). RP inoculation decreased the final pH of extended semen more than AX.

When these results were presented at a national scientific meeting, one of the criticisms of the study was that the extended semen was monitored only to 7 days. It is recommended that extended semen be used quickly, rather than being stored for many days. However, it is known that breeders still hold semen doses for a number of days rather than discarding them and using fresh semen. Also, some extenders are now marketed for long-term semen storage (up to 10 days). Because of this, the PAL felt that the study should be expanded, evaluating different types of extenders and monitoring semen parameters for a longer period of time.

CHAPTER 3

MATERIALS AND METHODS

Objectives

The study was designed to evaluate the effect of *Achromobacter xylosoxidans* and *Ralstonia pickettii* on semen quality – specifically pH, motility, and progressive motility. The intent was to inoculate culture-negative semen, under laboratory conditions, and monitor the extended semen samples over a period of 14 days. Three different commercial semen extenders were used to determine whether the particular extender used would be detrimental to the contaminated bacteria that was introduced into the extended semen sample. We hypothesized that the bacterial contamination would increase the pH, as seen in the previous PAL work, but that it would have no effect on the sperm cell motility. We also hypothesized that there would be differences between the extenders with regard to motilities over the study period and that there would be no detrimental effect of extender on either bacteria that was inoculated into the extended semen samples.

Bacterial source

Isolates of *A. xylosoxidans* (AX) and *R. pickettii* (RP) derived from the clinical cases from the field investigation (Payne et al., 2008) described in the introduction were banked frozen at -80º C following the addition of 25% glycerol to overnight growth in trypticase soy broth. Frozen stocks were maintained by the Microbiology Laboratory of the University of Illinois Veterinary Diagnostic Laboratory (UI-VDL). These stocks were used for all inoculations for our project.

Water source

All water used in these experiments was double-distilled water from a Millipore water purification system. Glass bottles and Erlenmeyer flasks were half-filled with the water, covered loosely with lids (bottles) or foil (flasks), and then autoclaved at 121º C for 1 hour. The containers and water were allowed to cool and then stored until use.

Growth characteristics

In previous work performed in the Porcine Andrology Laboratory, these particular isolates were grown in multiple media and found to grow best in Luria broth and on Columbia agar with 5% sheep blood (Remel, Lenexa, KS) plates (Ness, personal communication). A portion of that work to evaluate the growth characteristics was repeated to confirm the previous results.

The Luria broth used in the experiments was made from stock powder (Sigma Life Science, St. Louis, MO) and sterilized water according to the label directions. On the day it was made, the broth was also autoclaved as described for the water.

All microbiological work was performed in a certified biosafety cabinet, using standard aseptic technique. The banked isolates were removed from the -80º C freezer and kept on ice for the brief time they were out of the freezer. Sterilized wooden applicator sticks were used to remove a few crystals from the surface of frozen stock cultures, which were then streaked for isolation on Columbia blood agar plates. The plates were incubated in a 37º C 5% CO_2 incubator for approximately 48 hours, at which time a single, well-isolated colony of each bacteria was placed into separate 15 milliliter (ml) centrifuge tubes (Corning, Inc., Corning, NY), each

containing 10 ml of sterilized Luria broth. The tubes were then cultured in a 37º C 5% CO_2 incubator for 24 hours.

A UV/visible spectrophotometer was used to measure the optical density of the samples when they were removed from the incubator. For each sample, the tube was gently inverted several times to thoroughly resuspend the bacteria within the broth, and 1 ml broth solution was removed and placed into a cuvette. The absorbance in each cuvette was measured at 600 nm, with sterilized, non-inoculated Luria broth used as a blank. Serial dilutions were made out to 10^{-9} from each cultured tube, using 100 microliters (µl) of each sample and 900 µl of the sterilized, non-inoculated LB. These serial dilutions were also measured at 600 nm. The original samples and the dilutions were plated (100 µl) in triplicate on Columbia blood agar, using T-shaped spreaders (Copan Diagnostics, Inc., Murrieta, CA) and spin-plating technique. These plates were then cultured in a 37º C CO_2 incubator for 48 hours, at which time the plates were removed from the incubators and the colonies counted on each plate. Only plates containing between 30 and 300 colonies were averaged to determine the final concentration of each bacterium as cultured in the broth for twenty-four hours.

Semen source

Semen used in the experiments was obtained from two purebred Yorkshire boars, both approximately 18 months of age, housed at one of the University of Illinois at Urbana-Champaign (UIUC) swine farms. All procedures involving the boars were followed in accordance with the Institutional Animal Care and Use Committee at the University of Illinois. Both boars were housed singly in pens with other boars and female swine nearby. They were fed 5-6 lbs of a 14% protein corn/soybean meal complete ration as needed to maintain adequate body

condition. Both boars were trained to mount dummy sows, and the semen was obtained by farm personnel using the gloved hand technique. The raw ejaculate was processed within 30 minutes of collection.

Extenders

Three different extenders were used: Beltsville Thaw Solution (BTS; IMV USA; Maple Grove, MN, USA) and X-Cell® (XC; IMV USA; Maple Grove, MN, USA), both of which contain gentamicin at 200 mg/L; and Tri-X-cell™ (TXC; IMV USA; Maple Grove, MN, USA), which contains gentamicin, amoxicillin, and tylosin. The extenders came in packets of powder intended to make 1 liter (L) total volume. Each packet was divided into four equal parts (by weight), which were kept refrigerated (4º C) in sterile plastic bags (Whirl-Pak®, Nasco, Fort Atkinson, WI) for future use, to be reconstituted with 250 ml water each. An appropriate amount of each extender was mixed for that day's use, using sterilized water, and the extenders were placed into glass bottles in a 37º C water bath (Isotemp 220; Fisher Scientific, Pittsburgh, PA) for at least 1 hour prior to use.

Semen processing

The raw ejaculate was measured in a graduated cylinder and then divided into three equal parts by volume. The semen was poured into a glass bottle to which an equal volume of a warmed extender was added. The three semen volumes were each extended with a different extender. The bottles were labeled and stored in a semen storage unit (Minitube of America, Verona, WI) at 16º C until use. Each extended sample was processed separately.

Motility and progressive motility were measured using a commercially available computer automated semen analysis (SpermVision® CASA, Minitube of America, Verona, WI) system validated for porcine use (B. Day, personal communication). At the time of analysis, the semen bottles were inverted several times to gently mix them, before approximately 1 ml of each extended sample was transferred to a labeled 1.5 ml microcentrifuge tube (Fisherbrand microcentrifuge tube, Fisher Scientific, Pittsburgh, PA) and warmed at 37º C for 10-15 minutes. Pre-warmed four-chambered glass slides (Conception Technologies, San Diego, CA) were used for evaluation on a heated (37º C) microscope stage. Each sample was gently mixed immediately prior to evaluation. Seven random fields were analyzed for each extender group and measurements averaged with a frame rate of 58/second. Motility was defined as the percent spermatozoa displaying any motion while progressive motility was the percent spermatozoa displaying forward motion.

Concentration was calculated manually using a Unopette™ (BD, Franklin Lakes, NJ) and hemocytometer. The Unopette™ system provides a 1:100 dilution using ammonium oxalate as the diluent, and the semen is evaluated according to the protocols for assessing white blood cell concentration. Sperm cell morphology was assessed manually by examining 300 cells under phase-contrast microscopy (Olympus BX41; Olympus America, Inc., Center Valley, PA) and averaging the results. These concentration, motility, and morphology results were used to determine the volume of extended semen (1:1 dilution) needed to reach a breeding dose (3 billion live, normal, progressively motile cells per 80 ml) in each sample volume.

Bacterial preparation

For each study replicate, isolates were streaked onto Columbia blood agar plates three days prior to semen inoculation. The plates were incubated in a 37º C CO_2 incubator for approximately 48 hours, at which time separate, labeled 15 ml centrifuge tubes containing 10 ml Luria broth were inoculated with a single, well-isolated colony of a bacterium. These tubes were then incubated in a 37º C 5% CO_2 incubator for 24 hours.

Upon removal from the incubator, the tubes containing the broth cultures were inverted several times to thoroughly resuspend the samples, before 1 ml was removed from each and placed into separate, labeled 15 ml centrifuge tubes. The tubes were then centrifuged at 4000 rpm for 5 minutes (5810R, Eppendorf, Hauppauge, NY). The excess liquid was poured off, and the bacterial pellet was resuspended in 10 ml of warmed extender.

There were four treatment groups per extender: AX, RP, AX plus RP, and a control (no bacteria added). Based on the bacterial growth characteristics, the appropriate amount of bacterial solution was calculated to reach a final concentration of approximately 2.5×10^7 CFUs/ml (AX) and/or 2×10^6 CFUs/ml (RP). (These concentrations followed those of the previous PAL work and were based upon the concentrations found in contaminated semen from the clinical case report (Payne et al., 2008).) The resuspended bacterial solution was pipetted into a sterile 50 ml centrifuge tube (Corning, Inc., Corning, NY). The appropriate volume of semen was added to obtain a breeding dose (as described previously), and enough warmed extender was added to complete the necessary volume (40-50 ml total volume in the centrifuge tube). The labeled tubes were then placed into the semen storage unit to mimic routine swine semen storage.

A second method was also used to determine bacterial concentration. For this, plates were streaked and cultured as described previously. After removal of the plates from the incubator at approximately 48 hours of growth, the bacteria was used to inoculate separate, labeled sterile tubes of distilled, deionized water to reach a concentration of $1x10^8$ CFUs/ml as determined by optical density using a nephelometer (Duggan et al., 1996; Zbinden et al., 2002; Donay et al., 2004; Althouse et al., 2008). The solution was used within two hours to inoculate samples to the desired concentration.

Sample evaluation

On a daily basis, the tubes of inoculated semen were rotated gently, as is standard practice for stored swine semen. Each day, a 2 ml volume was obtained from each sample using standard sterile technique and placed into a labeled 15 ml centrifuge tube. These samples were then allowed to equilibrate to ambient temperature, and pH readings were taken with a benchtop pH meter (Accumet Basic AB15, Fisher Scientific, Pittsburgh, PA). The samples were also evaluated for motility and progressive motility as during semen processing, using the SpermVision® program. The inoculated semen samples were evaluated in this manner over 14 days.

Bacterial growth in extender

A separate set of experiments was conducted to evaluate the bacterial growth in the extenders. Each of the three extenders was inoculated with bacteria to reach the same final concentrations as the inoculated semen samples. The inoculated extender samples were placed into 50 ml sterile centrifuge tubes and stored at 16° C in the semen storage unit. The tubes were

rotated daily, as with the inoculated semen, and serial dilutions were made and plated at days 7, 10, and 14, following the same protocol as in the growth curves.

Statistical Analysis

Data analysis was performed using SAS (Statistical Analysis System; SAS Institute, Inc., Cary, NC). The data was analyzed as repeated measurements in time; however, it was first divided into 5 groups of time, as the software could not compare so many repeated data points. A normality check was performed first, and the data was found to be non-normal, therefore requiring transformation prior to analysis. The pH values were transformed by taking the log of the measurements, and the motility values were transformed as the arcsine of their measurements. All least squares means (LSM) estimates were back-transformed for purposes of demonstration; however, the results of the F-test (*p*-value) are reported for the transformed data. Significance was set at $p<0.05$.

CHAPTER 4

RESULTS

Growth dynamics

After analysis, the growth of AX after 48 hours of culture on Columbia agar with 5% sheep blood and a subsequent 24 hours culture in Luria broth was found to be 1 x 10^8 CFUs/ml. The growth of RP under the same conditions was found to be 1 x 10^7 CFUs/ml.

The bacterial broth was also analyzed with the mass spectrophotometer after 24 hours of incubation, and the results are shown in Table 2. Four broth cultures were made for each bacterium, and each were measured at OD_{600} for the original culture and the first two serial dilutions. As the second dilution resulted in a reading of 0.001 on the mass spectrophotometer, it was determined that further dilutions would not yield informative data. The original broth cultures and the first two serial dilutions were each plated on Columbia agar with 5% sheep blood as previously described. At 24 hours, the growth on all plates was found to be too numerous to count (TNTC).

Bacterial growth in extender

The experiments involving inoculation of extender with bacteria revealed that both bacteria survived in the extender, despite the presence of antibiotics. However, their survival dynamics were very different. While RP survived in the extenders on the order of at least 10^3 CFUs/ml, bacterial growth was substantially inhibited, decreasing by 85-92% in the first week

and by a total of 92-98% by the end of the second week. In contrast, AX thrived in the extenders, increasing by a minimum of 100% in the first week and at least 400% by the end of the second week. The results are summarized in Figure 4.

Semen characteristics

Three separate ejaculates were used in the six study replicates. The mean ejaculate volume was 196 ml (range 168 to 230 ml). Analysis of each ejaculate revealed at least 92% morphologically normal cells, and the overall motility of each ejaculate when extended in the three extenders was greater than 80%. Specific data for each ejaculate are listed in Table 3.

Daily sample assessments

Statistically relevant data from the daily sample assessments are summarized in Tables 4-7. Contrast statements were used to further examine differences within the ejaculates and extenders. Those results are summarized in Tables 8-9.

pH

When the inoculated semen samples were evaluated for pH values in each of the 3 extenders (Figure 5A), it was noted that BTS had a higher ($p<0.0001$) values over the entire 5 time periods (range 7.45 to 7.75) compared to XC (range 7.4 to 7.52) and TXC (range 7.21 to 7.32) (Table 6). The values remained similar during the initial time period of 1-2 days post-inoculation, but rapidly deviated during the next 4 time periods. The pH of XC started to increase ($p=0.04$) during the final time period of 12-14 days post-inoculation, but the TXC did not change.

Additionally, the pH of semen samples inoculated with *Ralstonia pickettii* (RP and AX+RP) were reported to be lower (p=0.02) than those with AX or control samples (Table 5). As time from inoculation increased, the pH of the samples remained lower for the first 2 time periods (days 1-2 and 3-5), but began to rise and stay relatively constant up to 14 days.

Motility

Total spermatozoal motility began similarly across each extender (all approximately 90% in the first time period, dropping to between 86-89% in the fourth time period) and remained as such until the final time period (days 12-14 post-inoculation). At that time, the motility in BTS (76.41%) dropped significantly (p<0.0001) as compared to XC (85.43%) and TXC (80.7%; Figure 5B, Table 6). TXC also appeared to experience a decline in motility (an 8.6% decline) during that final time period.

The motility of semen samples inoculated with both bacteria (AX+RP) did not decrease over time as significantly as when they were added separately or compared to the control samples. This was the case across all extenders (Figure 6A). The motility of all samples remained fairly constant through the first 3 time periods but began to drop during period 4 (days 9-11) and experienced the most precipitous decline in period 5 (12-14 days) (Table 7).

Progressive motility

Examination of progressive spermatozoal motility revealed a similar pattern between extenders as seen with total motility. The progressive motility was similar across each extender for the first three time periods (between 78-81% in the initial time period), with BTS slightly higher than the others for the first two time periods. Over the next three time periods, however,

the progressive motility within BTS declined considerably, and in the final time period, progressive motility was greatest in XC (68.29%), followed by TXC (63.01%; $p<0.0001$, Figure 5C, Table 6).

When examining the progressive motility by treatment, the same pattern was seen. The progressive motilities were similar across treatments for the first two time periods (between 78-81% in the initial time period) but separated as the decline began in the third time period (days 6-8) and became more pronounced in the fourth and fifth time periods ($p=0.0585$, Figure 6B, Table 6). The semen samples inoculated with RP (both RP and AX+RP) did not have the highest progressive motility in the first time period but remained fairly consistent into the second time period (days 3-5) before beginning a gradual decline into a more dramatic decline in the fourth and fifth time periods (Figure 6B, Table 7). In the final time period (days 12-14), the semen samples containing RP (RP and AX+RP) retained the highest progressive motilities (RP=63.32%, AX+RP=66.74%, $p=0.0585$).

CHAPTER 5

DISCUSSION

Growth dynamics

The growth study was repeated to confirm the previous results as obtained by researchers in the PAL. As the same banked isolates and the same media and culture conditions were used for the growth study, it was an expected finding that the results would be approximately the same.

The spectrophotometry analysis was performed in an attempt to generate a growth curve (optical density at 600 nm versus CFUs/ml) to more accurately quantify the bacterial growth in the liquid media. The previous attempts by PAL researchers had been abandoned due to the growth dynamics of the bacteria – they were slow-growing, resulting in little to no change in optical density between measurements (at half-hour or hourly measurements) and still continuing to grow at 48 hours in media (L. Borst, personal communication). When the author repeated the experiment and examined broth cultures at 24 hours, dilutions that resulted in no readable change in optical density (10^{-2}) also resulted in colonies too numerous to count when plated and incubated. Because of this, the attempt to develop a growth curve with spectrophotometry was discarded.

Bacterial growth in extender

During the course of the clinical case that prompted this research project, the AX and RP strains which were isolated and cultured were repeatedly found to be resistant to gentamicin at >8 µg/ml when assayed using CLSI methods. All three of the extenders used in this study contain gentamicin at ≥ 200 µg/ml. However, as it is impractical to test seemingly resistant bacteria out to indefinite antimicrobial concentrations, the antimicrobial testing is based on clinically relevant serum concentrations, perhaps going out one to two endpoints beyond the therapeutic concentration. In the food animal antimicrobial panel used at UI-VDL, gentamicin susceptibility is tested from 1 µg/ml to16 µg/ml. Because of this, one cannot simply assume that an organism which is considered resistant at >8 µg/ml is still able to survive at 200-250 µg/ml. Data from the UI-VDL past cases shows that, of the AX isolates recovered from porcine submissions (semen, tissues, blood, etc.) over the past three years, 81% had MICs greater than 8 µg/ml for gentamicin (n=21). For RP isolates during the same time period, 70% had MICs greater than 8 µg/ml for gentamicin (n=13). (UI-VDL, 2010) The breakpoint for gentamicin is 4 µg/ml, and the MIC90's for both organisms was ≥16 µg/ml. It is difficult to compare the MIC in extenders to the MIC derived using Muller-Hinton Broth as specified by CLSI, but it has been well established that very nutrient rich media tend to increase the MICs of most organisms.

Of the organisms identified (from the case in Payne et al., 2008) and for which MIC data was available (5 RP, 5 AX), all showed very similar susceptibility patterns. As pertinent to this experiment, all were resistant to gentamicin at >8 µg/ml, and all were listed at > 20 µg/ml for tylosin, which has no CLSI interpretive criteria for these organisms. Susceptibility to amoxicillin was not tested; however, the organisms did exhibit resistance to other beta lactams (at >8-16 µg/ml).

For this experiment, the organisms were inoculated into extender separately, for ease of examination of the plated dilutions. This approach unfortunately does not address the possibility of any interaction between the two strains, whether one may inhibit the other when co-cultured. Nor does it address any questions regarding whether the strains would suffer any deleterious effects from other bacteria typically found in boar ejaculates, but it does verify survival or growth of the bacteria at the concentrations of antibiotic found in the extenders. To the author's knowledge, almost every published case report regarding AX and RP in extended porcine semen has listed both organisms as being resistant to gentamicin (Althouse et al., 2000; Althouse et al., 2005; Althouse et al., 2008; Payne et al., 2008); however, none have specifically mentioned whether growth testing in the extenders was performed. In the lone study which demonstrated differing susceptibility patterns (Sone, 1990), *Alcaligenes* spp. and *Pseudomonas* spp. were found to be susceptible to aminoglycosides at less than 6.25 µg/ml. As AX was previously classified as *Alcaligenes xylosoxidans* and RP as *Pseudomonas pickettii*, these data show that it is possible for the organisms under study to exhibit sensitivity to gentamicin.

The results of the growth extender study show that AX continued to thrive in the extender, increasing to more than 400% of the inoculated count by the end of the second week. However, RP decreased to between 92-98% in the same time. The organism was still present on the order of at least 10^3 CFU/ml; still, that represented only 2-8% of the original inoculated dose.

Further work would need to be performed to determine the minimum inhibitory level of gentamicin for RP. This study can only conclude that the level is less than 200 µg/ml.

Semen characteristics

The project was originally designed with only one boar to be used as a semen donor, in order to eliminate the possibility of a "boar effect." Unfortunately, due to lameness and semen quality issues, Boar A (ejaculates 1 and 2) was culled from the herd prior to the final replicates of the project, and boar B was utilized instead (ejaculate 3). The gel-free volumes of the ejaculates were within the reported ranges (Shipley, 1999), and the sperm morphology and motility data were above the recommended guidelines for use in AI .

pH

The effect of ejaculate upon pH approached statistical significance (p=0.0557). Ejaculates 1 and 2 (boar A) showed no statistically significant differences, but ejaculate 2 and ejaculate 3 were somewhat different, with overall pH of 7.37 versus 7.53 (SEM = 0.029,p=0.025). All handling and storage of semen was similar between ejaculates and replicates, although the standardizing solutions for the pH meter were replaced between ejaculates 2 and 3. Though this effect was not significant at the level set, and the difference in pH was not large, these results indicated the possibility of a "boar effect" which should be considered when designing future studies.

The effect of time upon pH was statistically significant (p=0.0018), as was expected, based on the preliminary work done by the PAL (Clark et al., 2008). The pH increased gradually across extenders, becoming more basic over time, although the differences between the five time periods was less than 0.2 units (7.35 in the first time period, 7.53 in the final time period). This was in agreement with a study (Vyt et al., 2004), which compared five commercial extenders (2 short-term, 3 long-term) and found an increase in pH across all extenders over the study period

of 7 days. The pH measurements of the individual extenders were not all statistically significant from one another, but all increased over time.

Another possible explanation for the rise in pH is found in a separate study (Vyt et al., 2007) in which the authors examined the rise in pH during storage of extended porcine semen. The rise in pH was attributed to CO_2 loss from the buffering system of the extender (BTS) but was also found to be more pronounced when the air volume of the storage container increased. In this study, the 50 ml centrifuge tubes used as semen storage containers were opened and closed in ambient air when taking the daily samples for assessment. While the tubes were opened just before taking the sample and closed immediately afterward, that would be an opportunity for increasing the air content of the tube. And as 2 ml were removed daily, the available space within the tube increased quickly, allowing increasing air contact over time. This would contribute to the effect attributed to time.

Unlike the Vyt *et al*. (2004) study, however, the effect of extender upon pH in this study was statistically significant ($p<0.0001$), which is not surprising. While the extenders all claim to maintain pH over time, the extenders studied in this experiment claim to do so over different time periods. BTS is classically considered a short-term extender, up to 3-4 days. XC and TXC are both considered long-term extenders, lasting greater than 6-7 days. If the label claims are correct, one would expect a statistical difference in the effect of extender upon pH over 14 days. This is not the case in the study of Vyt *et al*. (2004), as in that study, BTS was not statistically different from one of the long-term extenders studied. However, that study lasted 7 days, not 14 days. In our study, BTS was significantly different from the two longer-term extenders ($p<0.0001$ for both). XC and TXC, were also significantly different from each other ($p=0.0424$).

Still, the difference between the pH of the extenders was less than 0.4 units (BTS=7.67, TXC=7.31).

The interaction between extender and time hinted at statistical significance with regard to pH (p=0.0731). This is somewhat to be expected, as both extender and time exerted statistically significant effects upon pH. When examined over the course of the study, BTS increased from 7.45 to 7.75; XC increased from 7.4 to 7.52, despite dropping in the second time period; and TXC increased from 7.21 to 7.39 in the third period, before dropping back to 7.32 in the final period. These were not large differences. Still, there was some agreement with Vyt *et al*, (2004), as within each extender, pH tended to increase over the given time period.

There was a significant effect of treatment upon pH (p=0.0205), as was seen in the previous work done in the PAL. However, in contrast to the previous work (Clark et al., 2008), the pH of the samples containing RP remained closer to 7.0 than either the AX alone or the control sample. This could be due to the longer study time (7 days versus 14 days) for this study. Althouse *et al*. (2000), in conducting field investigations of complaints of decreased sperm longevity, sperm clumping, and/or increased returns to estrus, found that 52 of the 56 semen samples analyzed were acidic (pH 5.7 to 6.4), and all were contaminated with bacteria. However, that study looked at pH on the day of arrival at the referral laboratory, not over time.

The differences between the treatments were examined, and it is interesting to note that with regard to pH, AX (7.53) was statistically higher than both RP (7.39, p=0.0037) and AX+RP (7.43, p=0.0302), while RP was not different from AX+RP (p=0.4581). The difference between RP and the control (7.48) approached statistical significance (p=0.0575), but neither AX nor AX+RP was statistically different from the control (p=0.3113 and 0.2456, respectively). This

raises the question of what caused the difference between AX and RP. As the growth in extender data showed, RP growth was inhibited over the course of the experiment and a very large percentage of the organism died. It is possible that the dying bacteria in some way influenced the pH of the extended semen. It is known that growing cells can release byproducts which can change the pH of their environment; however, a search of the literature did not reveal any specific reports of RP changing the pH of its environment during cell death. Also, while there is statistical significance between the two treatments with regard to pH, the difference between the two was less than 0.2 units.

Motility and progressive motility

There was a statistically significant effect of ejaculate upon gross motility, which ranged from 85-92%, and a trend towards statistical significance upon progressive motility, which ranged from 71-75%. With regard to gross motility, ejaculate 3 (boar B, 91.49%) was statistically different from both ejaculates 1 and 2 (both boar A; 85.58 and 86.42%, respectively). For progressive motility, as with pH, the only significant difference was between ejaculates 2 and 3 (75.15 and 71.71%, respectively). This appeared to be a simple "boar effect" and may have been avoided by using pooled semen, a single donor (as originally intended), or a larger ejaculate sample size.

The effect of extender upon gross motility trended towards significance, but its effect upon progressive motility was clearly significant. Still, the overall differences were small: less than 4% between highest and lowest extender. BTS was statistically different from XC and trended towards a difference from TXC with regard to gross motility but was clearly different from both with regard to progressive motility (Table 9). XC and TXC showed no statistical

difference with regard to either variable (Table 9). This was not unexpected, as XC and TXC are similar long-term extenders, and BTS is a short-term extender. The Vyt *et al.* (2004) study found a higher motility in porcine semen extended with BTS than with a particular long-term extender; however, that study examined motility over 7 days. Also, in that study, a different long-term extender outperformed BTS in terms of motility. (Neither long-term extender used in the Vyt *et al.* (2004) study was used in this study.)

Motility and progressive motility were reduced over time as expected (motility decreased nearly 10% over the study period, while progressive motility decreased nearly 20% - Table 5). The amount of nutrients in semen extender is finite, and over time, as those nutrients are exhausted, the remaining viable cells decrease in motility until they die. As shown in Table 5, the change in motility (LSM) over the first three time periods does not appear large but does steadily increase. The Vyt *et al.* (2007) study also noted decreased motility in extended semen with higher air volume and increased pH. As noted earlier, time has the effect of increasing pH in this study, and as pH increases, motility is expected to decrease. Also expected is the significant interaction between extender and time (Table 6). BTS decreased by approximately 25% with regard to both motility and progressive motility over the study time; while both XC and TXC decreased by less than 15% with regard to each. When each treatment type exerts such a strong effect, it would be surprising if there was no significant interaction between the two.

However, there was no significant effect of treatment upon motility or progressive motility, with less than 3% difference among the treatment groups with regard to both variables. This is in contrast to several published reports of bacteriospermia resulting in decreased sperm motility (Althouse et al., 2000; Althouse et al., 2005; Althouse et al., 2008). These findings are, however, partly in agreement with the earlier work performed in the PAL (Clark et al., 2008). In

that study, a significant reduction in motility was noted for AX versus the control group, but no other significant differences were seen between treatments.

Sone's study (1990) examined the survival of spermatozoa in the presence of several species of bacteria. Though the published report does not address the specifics of motility measurements, it mentioned that the survival of spermatozoa was "remarkably affected" by 5 species of enteric bacteria, but that it was affected "to a lesser extent" by the presence of *Pseudomonas* spp. The report does not specify which *Pseudomonads* were examined, but RP was at one time classified as *Pseudomonas pickettii* and could very well have similar results. Also, the report states that 4 other species of bacteria, including *Alcaligenes* spp., had "almost no effect" upon spermatozoal survival, even at concentrations of 10^{10}-10^{12} CFU/ml. Again, the specific strain was not indicated, but AX was previously classified as *Alcaligenes xylosoxidans*. This would seem to further agree with the results of the Payne *et al.* (2008) case report, in which no deleterious effects of AX were seen on semen quality during the routine laboratory and third-party analysis.

CHAPTER 6

CONCLUSIONS

The present study allowed us to conclude:

- *A. xylosoxidans* can survive and in fact thrive in porcine semen extended in commercial semen extenders containing gentamicin sulfate (200 µg/ml).
- *R. pickettii*, despite similar MIC data to *A. xylosoxidans*, is severely inhibited by the presence of gentamicin sulfate (200 µg/ml).
- Seminal pH and spermatozoal motility characteristics may vary between boars.
- The pH of extended porcine semen increases during cooled storage regardless of commercial extender used.
- Total and progressive motility of porcine spermatozoa decrease over time with cooled storage in commercial semen extenders.
- The choice of commercial semen extender has a significant effect on progressive motility of porcine spermatozoa.
- *R. pickettii* can exert a significant, though small, effect upon the pH of extended porcine semen.
- These particular strains of *A. xylosoxidans* and *R. pickettii* do not exert a significant effect on total or progressive motility of extended porcine spermatozoa.

Both *A. xylosoxidans* and *R. pickettii* are documented bacterial contaminants in extended porcine semen, usually due to a contaminated water source. Previous reports of these organisms

in semen indicated that both consistently exhibit resistance to gentamicin, one of the most common antibiotics found in commercial porcine semen extenders. These particular strains were tested in porcine semen extenders containing gentamicin (200 mg/L), and while *A. xylosoxidans* thrived, *R. pickettii* declined greatly, indicating that despite resistance exhibited to gentamicin in CLSI-specified broth testing, inhibition by gentamicin is possible in a field situation.

While previous reports of these semen contaminants have indicated that semen quality is greatly diminished, these particular strains did not exert a detrimental effect on semen quality. The pH in all of the samples increased over the time period of the study, regardless of the commercial extender used, but the increases themselves were minimal. And while the choice of commercial extender had a significant effect on progressive motility of the spermatozoa (with differences between the extenders of up to 15% in the final time period), the bacterial contamination did not. This is important to note, as semen collection facilities which rely on semen quality assurance checks that do not include semen culture may fail to observe a problem with bacterial contamination, until the contaminated semen results in problems with the inseminated females, such as vulvar discharges and drops in conception rate.

CHAPTER 7

FIGURES AND TABLES

Figure 1. Schematic of the laboratory closet and critical control points to eliminate contamination of water used to make semen extender for a 180-head boar stud (From Payne et al., 2008).

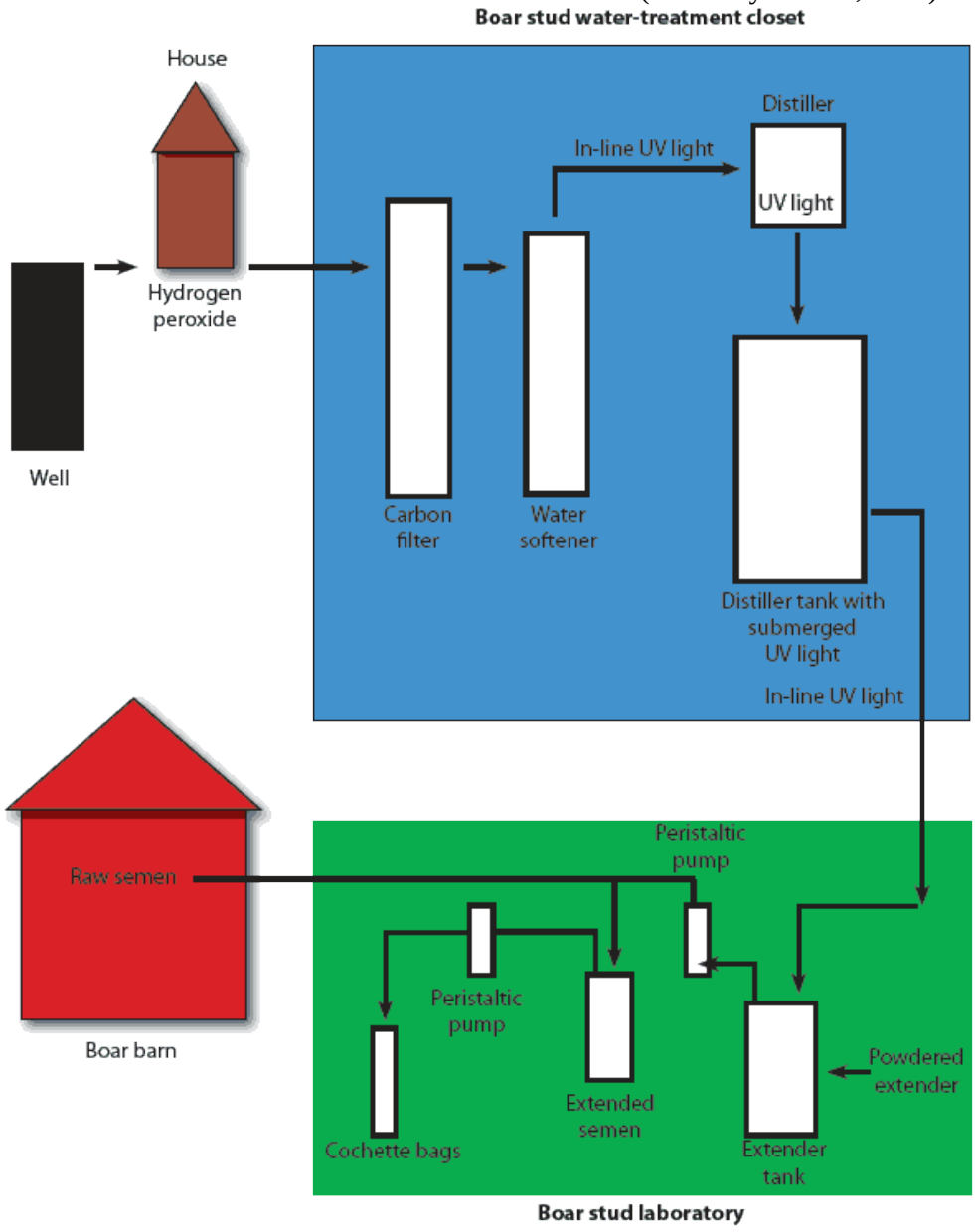


Figure 2. Timeline of identification of *A. xylosoxidans* (Ax) in the water used to make semen extender at a 180-head boar stud and clinical signs in the 21,000-sow system supplied with semen from the stud (From Payne et al., 2008).

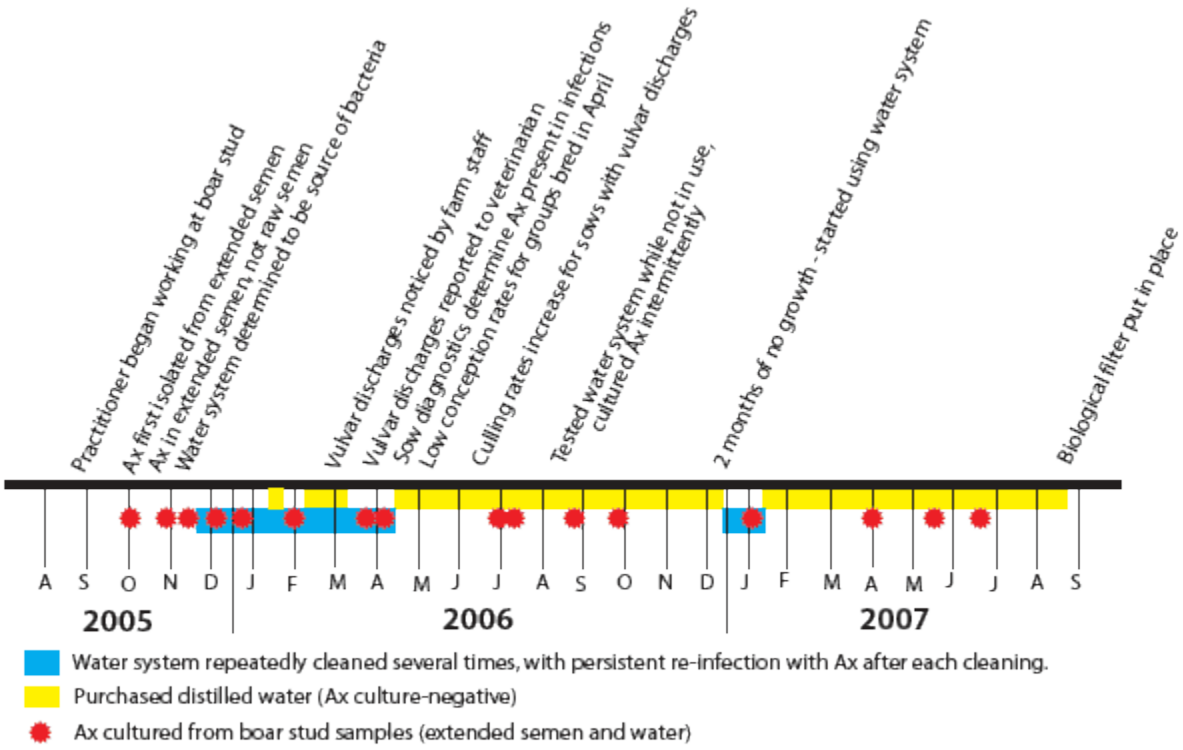


Figure 3. Average conception rates across a 21,000-sow system in which semen doses used for artificial insemination were contaminated with *A. xylosoxidans*. Semen extender made using water from the distillation system in the boar-stud laboratory (shown in Figure 1) was the source of contamination. Samples from the distillation system became culture-negative for *A. xylosoxidans* after extensive cleaning and disinfection of the system. During treatment of the distillation system, purchased distilled water that was culture-negative for *A. xylosoxidans* was used in semen extender (From Payne et al., 2008).

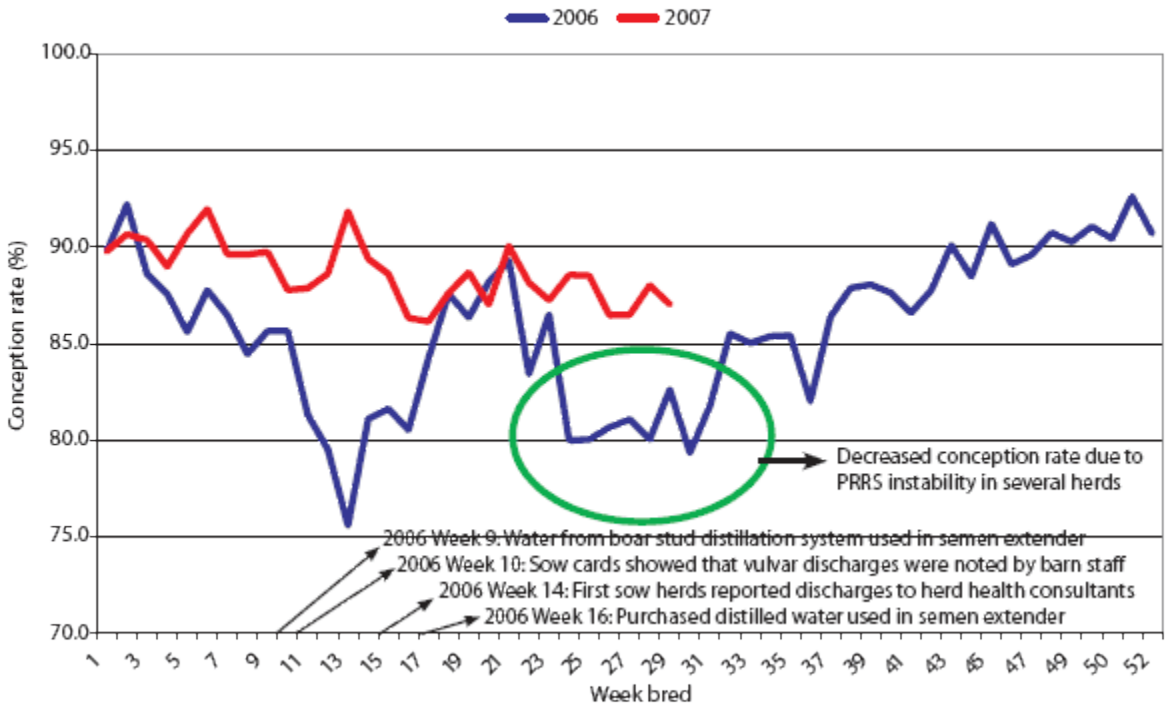
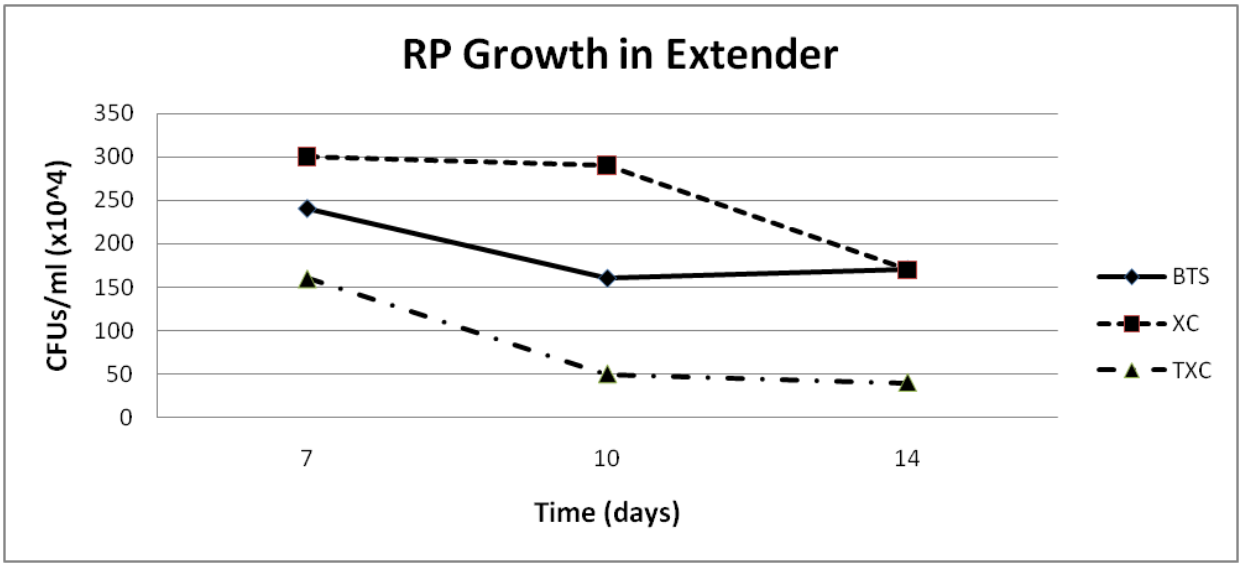
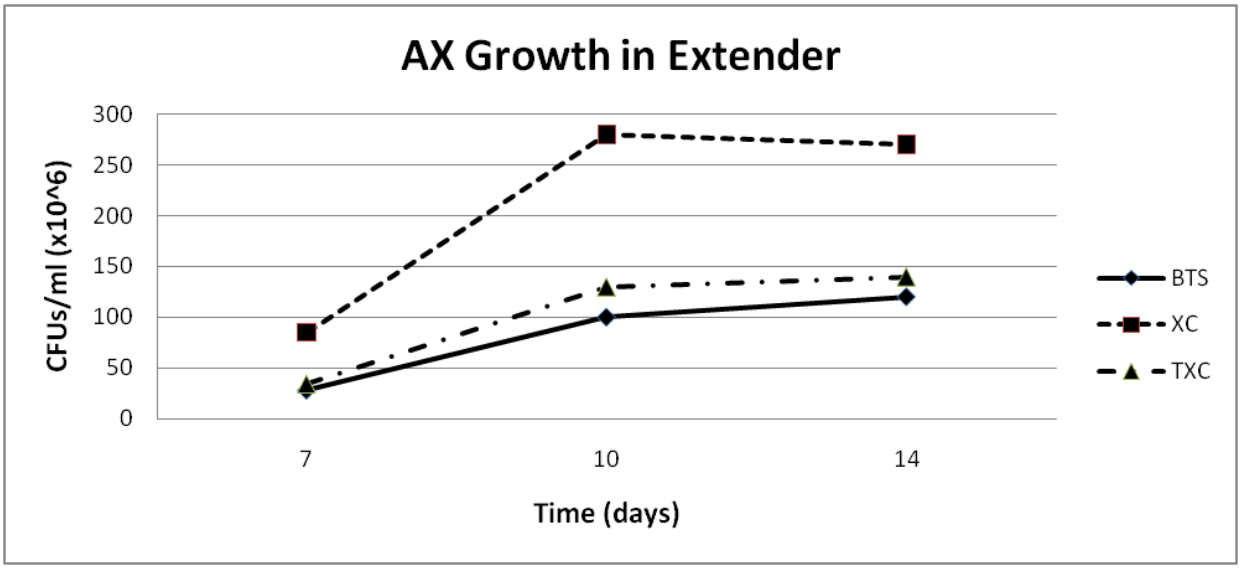


Figure 4. Growth of A) *Ralstonia pickettii* (RP) and B) *Achromobacter xylosoxidans* (AX) in 3 different semen extenders (BTS, X-Cell, and Tri-X-Cell) over 14 days, based upon serial dilutions at time points 7, 10, and 14 days.

A).



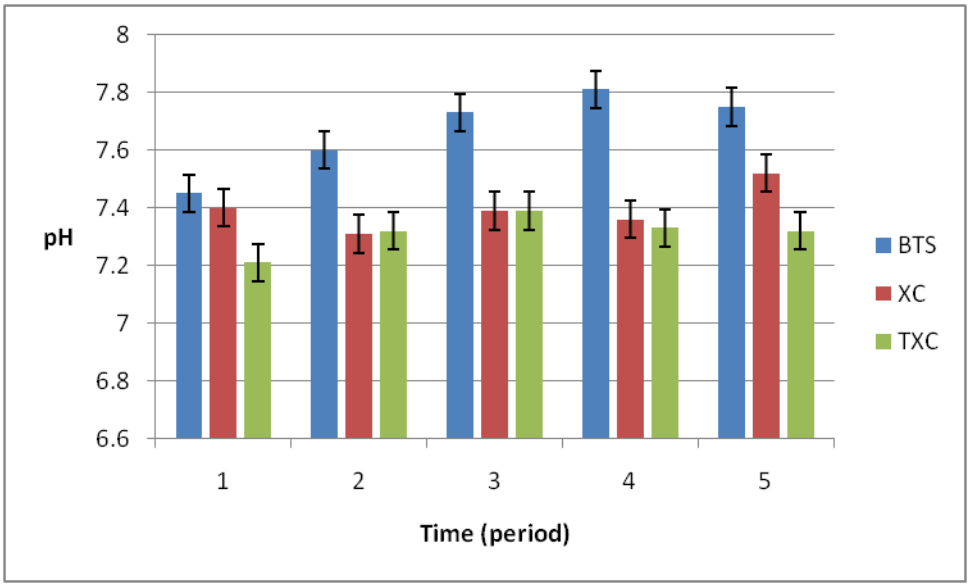
B).



*Extender: BTS = Beltsville Thaw Solution
XC = X-Cell
TXC = Tri-X-Cell
All extenders produced by IMV USA; Maple Grove, MN, USA.

Figure 5. Bar graphs of the overall interaction by extender (BTS, X-Cell, and Tri-X-Cell) and time on the least square means (±SE) of pH, motility, and progressive motility of boar semen from all treatment groups (AX: *A. xylosoxidans*, RP: *R.pickettii*, AX+RP, and Control). Analysis of repeated measures was performed on various time periods (1: days 1-2, 2: days 3-5, 3: days 6-8, 4: days 9-11, 5: days 12-14) after preparation of the extended semen samples. Statistical significance was set at $p<0.05$.

A: Effect of interaction by extender and time on pH ($p=0.0731$), as analyzed by a pH meter (Accumet Basic AB15, Fisher Scientific, Pittsburg, PA).



B: Effect of interaction by extender and time on motility ($p<0.0001$), as analyzed by a commercially available computerized spermatozoal-motility analyzer (SpermVision® CASA, Minitube of America, Verona, WI).

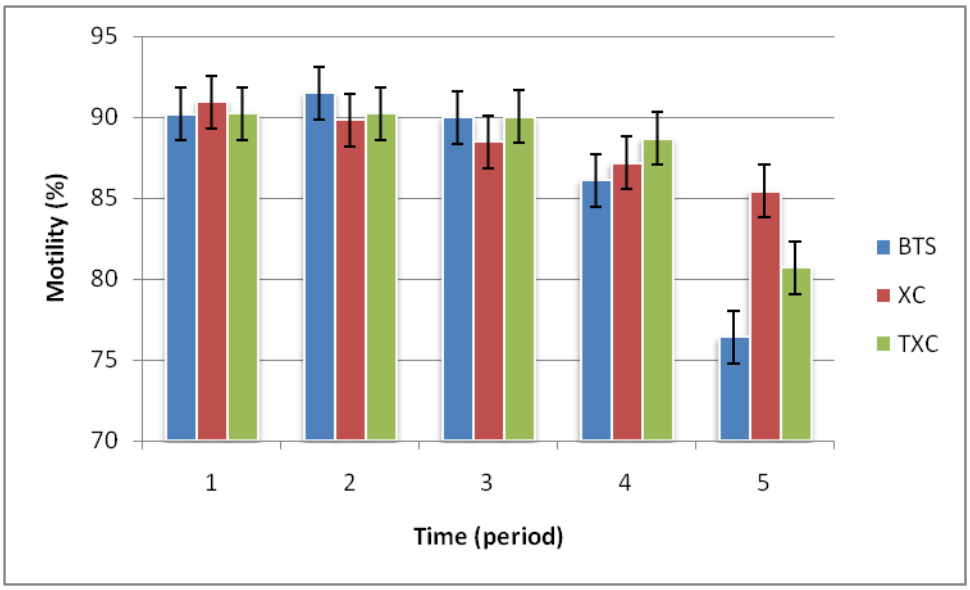
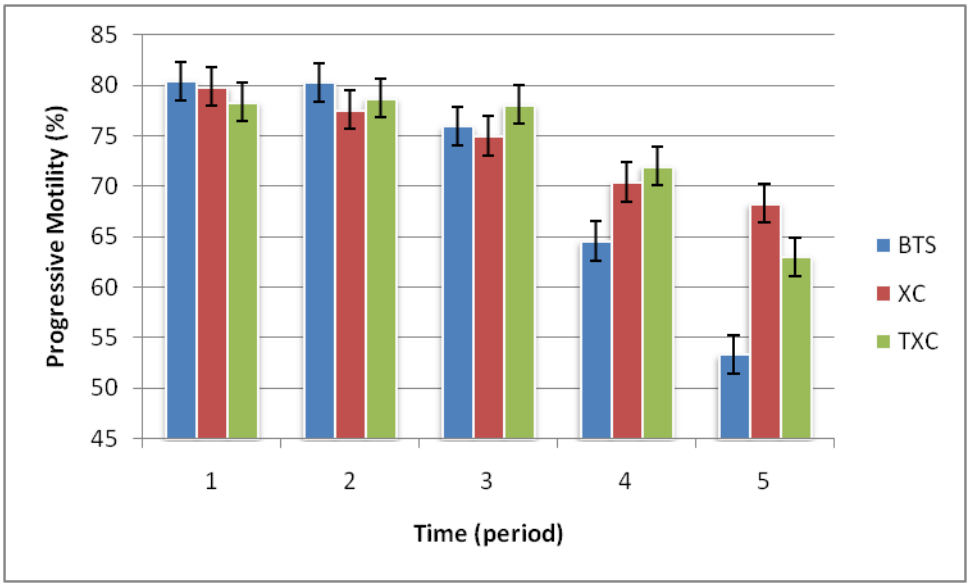


Figure 5 (cont.)

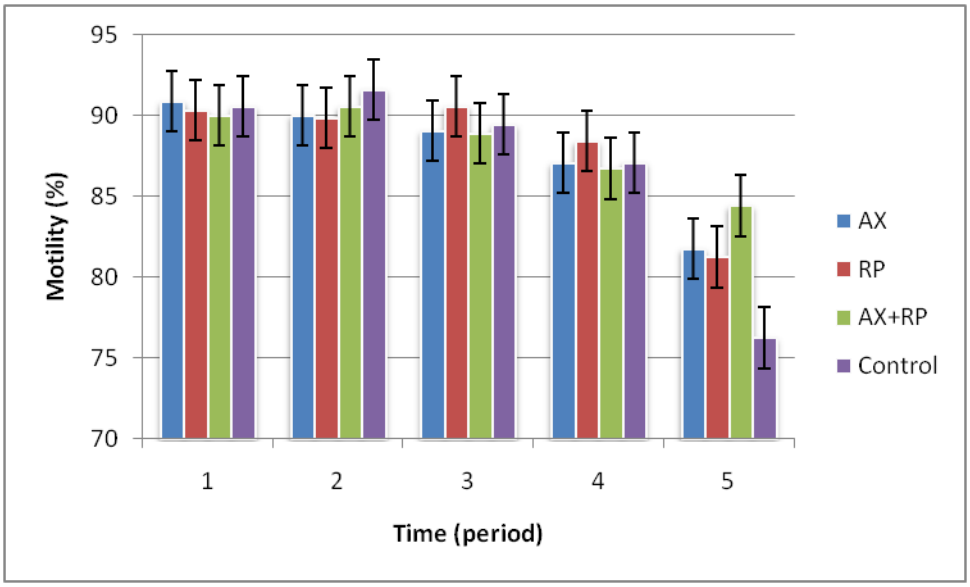
C: Effect of interaction by extender and time on progressive motility ($p<0.0001$), as analyzed by a commercially available computerized spermatozoal-motility analyzer (SpermVision® CASA, Minitube of America, Verona, WI).



All extenders produced by IMV USA; Maple Grove, MN, USA.

Figure 6. Bar graphs of the overall interaction by treatment (AX: *A. xylosoxidans*, RP: *R.pickettii*, AX+RP, and Control) and time on the least square means (±SE) of motility, and progressive motility of boar semen from all extenders (BTS, X-Cell, and Tri-X-Cell). Analysis of repeated measures was performed on various time periods (1: days 1-2, 2: days 3-5, 3: days 6-8, 4: days 9-11, 5: days 12-14) after preparation of the extended semen samples. Statistical significance was set at $p<0.05$.

A. Effect of interaction by treatment and time on motility ($p=0.0098$) as analyzed by a commercially available computerized spermatozoal-motility analyzer (SpermVision® CASA, Minitube of America, Verona, WI).



B. Effect of interaction by treatment and time on progressive motility ($p=0.0585$) as analyzed by a commercially available computerized spermatozoal-motility analyzer (SpermVision® CASA, Minitube of America, Verona, WI).

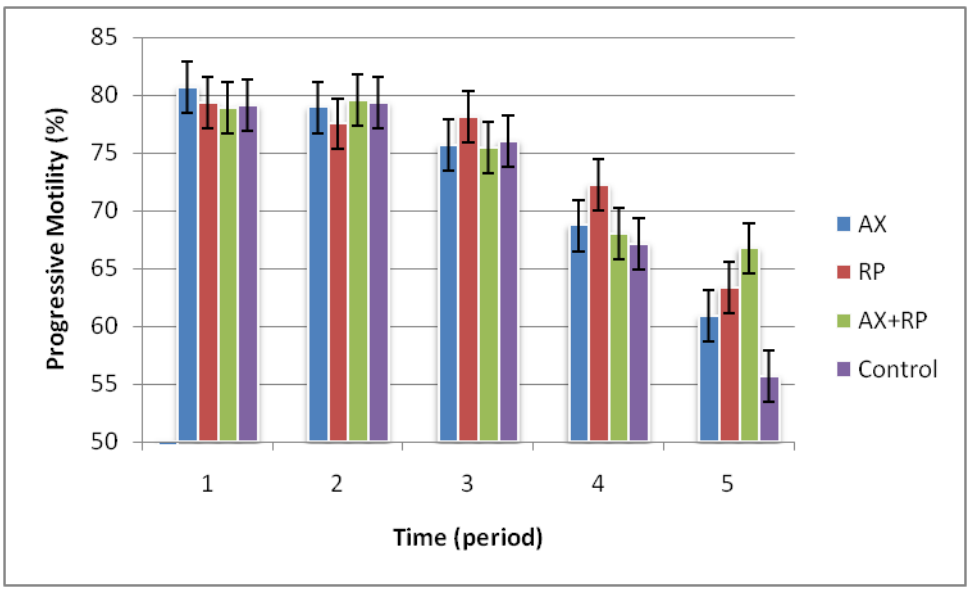


Table 1. Common bacterial flora isolated from the neat and extended boar ejaculate (adapted from Althouse and Lu, 2005).

Neat boar ejaculate	Extended boar semen
E. coli	*Enterococcus* spp.
Pseudomonas spp.	*Sternotrophomonas maltophilia*
Bacillus spp.	*A. xylosoxidans*
Staphylococcus spp.	*Serratia marcescens*
Klebsiella spp.	*Acinetobacter lwoffi*
Proteus spp.	*E. coli*
Enterobacter spp.	*Pseudomonas* spp.
Pasteurella spp.	*Comamonas testosteroni*
Citrobacter spp.	*Klebsiella* spp.
Providencia spp.	*Providencia rettgeri*
Neisseria spp.	*Burkholderia cepacia*
Corynebacterium spp.	*Enterobacter cloacae*
Streptococcus spp.	*Corynebacterium* spp.
Actinomyces-like spp.	*Pasteurella multocida*
Bacteroides spp.	*Proteus mirabilis*
Lactobacillus spp.	*Streptococcus suis*
Acinetobacter spp.	
Actinobacillus spp.	
Flavobacterium spp.	
Micrococcus spp.	
Serratia spp.	

Table 2. Spectrophotometric (OD 600) analysis of bacterial broth cultures and their serial dilutions.

	Dilutions		
RP	1	10^{-1}	10^{-2}
1	0.098	0.012	0.001
2	0.107	0.011	
3	0.119	0.012	
4	0.11	0.011	

	Dilutions		
AX	1	10^{-1}	10^{-2}
1	0.284	0.016	0.001
2	0.288	0.022	
3	0.313	0.023	
4	0.317	0.021	

All dilutions were plated (100 µl with spin-plating technique) and examined at 24 hours.
All plates read as TNTC.

*AX = *A. xylosoxidans*; RP = *R. pickettii*

Table 3. Summary of ejaculate (n=3) characteristics from 2 Yorkshire boars after processing with 3 different extenders (BTS, XC, and TXC).

Ejaculate	Extender	Ejaculate volume	Normal cells (%)	Motility (%)	Progressive motility (%)
1		230	92		
	BTS			83.47	77.97
	XC			89.25	83.18
	TXC			89.87	79.73
2		191	95		
	BTS			86.47	67.78
	XC			98.75	83.75
	TXC			88.51	73.31
3		168	97		
	BTS			90.94	75.5
	XC			91.29	87.46
	TXC			92.53	83.82

*Extender: BTS = Beltsville Thaw Solution
XC = X-Cell
TXC = Tri-X-Cell
All extenders produced by IMV USA; Maple Grove, MN, USA.

Table 4. Effects of individual ejaculates and each of 3 extenders (BTS, X-Cell, and Tri-X-Cell) on least squares mean (±SEM) of pH, motility, and progressive motility of boar semen from all treatment groups (AX: *A. xylosoxidans*, RP: *R.pickettii*, AX+RP, and Control) and across all time periods (samples were assessed daily for 14 days after preparation). pH was analyzed by a pH meter (Accumet Basic AB15, Fisher Scientific, Pittsburg, PA), and motility and progressive motility were analyzed by a commercially available computerized spermatozoal-motility analyzer (SpermVision® CASA, Minitube of America, Verona, WI). Statistical significance was set at $p<0.05$.

	Ejaculate			SEM	*p*-value
	1	2	3		
pH	7.46	7.37	7.53	0.029	0.0557
Mot	85.58	86.42	91.49	0.747	0.0037
Prog Mot	73.42	75.15	71.71	0.907	0.0789
	Extender			SEM	*p*-value
	BTS	XC	TXC		
pH	7.67	7.39	7.31	0.029	<0.0001
Mot	87.28	88.44	88.19	0.732	0.0845
Prog Mot	71.63	74.37	74.28	0.873	0.0029

*Extender: BTS = Beltsville Thaw Solution
XC = X-Cell
TXC = Tri-X-Cell
All extenders produced by IMV USA; Maple Grove, MN, USA.
*Mot = motility
*Prog Mot = progressive motility

Table 5. Effects of treatment (AX: *A. xylosoxidans*, RP: *R.pickettii*, AX+RP, and Control) and time
on least squares mean (±SEM) of pH, motility, and progressive motility of boar semen from all extenders (BTS, X-Cell, and Tri-X-Cell). Analysis of repeated measures was performed on various time periods (1: days 1-2, 2: days 3-5, 3: days 6-8, 4: days 9-11, 5: days 12-14) after preparation of the extended semen samples. pH was analyzed by a pH meter (Accumet Basic AB15, Fisher Scientific, Pittsburg, PA), and motility and progressive motility were analyzed by a commercially available computerized spermatozoal-motility analyzer (SpermVision® CASA, Minitube of America, Verona, WI). Statistical significance was set at $p<0.05$.

	Treatment				SEM	p-value
	AX	RP	AX+RP	Control		
pH	7.53	7.39	7.43	7.48	0.034	0.0205
Mot	87.94	88.29	88.22	87.45	0.844	0.5413
Prog Mot	73.39	74.38	73.97	72	1.003	0.1164

	Time (periods)					SEM	p-value
	1	2	3	4	5		
pH	7.35	7.41	7.5	7.49	7.53	0.038	0.0018
Mot	90.45	90.52	89.5	87.33	81.01	0.942	<0.0001
Prog Mot	79.53	78.85	76.34	69.06	61.73	1.118	<0.0001

*Mot = motility
*Prog Mot = progressive motility

Table 6. Effect of the interaction between extender (BTS, X-Cell, and Tri-X-Cell) and time on least squares mean (±SEM) of pH, motility, and progressive motility of boar semen from all treatment groups (AX: *A. xylosoxidans*, RP: *R.pickettii*, AX+RP, and Control). Analysis of repeated measures was performed on various time periods (1: days 1-2, 2: days 3-5, 3: days 6-8, 4: days 9-11, 5: days 12-14) after preparation of the extended semen samples. pH was analyzed by a pH meter (Accumet Basic AB15, Fisher Scientific, Pittsburg, PA), and motility and progressive motility were analyzed by a commercially available computerized spermatozoal-motility analyzer (SpermVision® CASA, Minitube of America, Verona, WI). Statistical significance was set at $p<0.05$.

	Extender x Time					SEM	p-value
	BTS x 1	BTS x 2	BTS x 3	BTS x 4	BTS x 5		
pH	7.45	7.6	7.73	7.81	7.75	0.065	0.0731
Mot	90.18	91.49	89.97	86.09	76.41	1.625	<0.0001
Prog Mot	80.4	80.26	75.93	64.57	53.32	1.921	<0.0001
	XC x 1	XC x 2	XC x 3	XC x 4	XC x 5		
pH	7.4	7.31	7.39	7.36	7.52	0.065	0.0731
Mot	90.92	89.84	88.48	87.16	85.43	1.625	<0.0001
Prog Mot	79.85	77.55	74.97	70.43	68.29	1.921	<0.0001
	TXC x 1	TXC x 2	TXC x 3	TXC x 4	TXC x 5		
pH	7.21	7.32	7.39	7.33	7.32	0.065	0.0731
Mot	90.23	90.2	90.03	88.67	80.7	1.625	<0.0001
Prog Mot	78.31	78.71	78.07	71.97	63.01	1.921	<0.0001

*Extender: BTS = Beltsville Thaw Solution
XC = X-Cell
TXC = Tri-X-Cell
All extenders produced by IMV USA; Maple Grove, MN, USA.
*Mot = motility
*Prog Mot = progressive motility

Table 7. Effects of the interaction between treatment (AX: *A. xylosoxidans*, RP: *R.pickettii*, AX+RP, and Control) and time on least squares mean (±SEM) of pH, motility, and progressive motility of boar semen from all extenders (BTS, X-Cell, and Tri-X-Cell). Analysis of repeated measures was performed on various time periods (1: days 1-2, 2: days 3-5, 3: days 6-8, 4: days 9-11, 5: days 12-14) after preparation of the extended semen samples. pH was analyzed by a pH meter (Accumet Basic AB15, Fisher Scientific, Pittsburg, PA), and motility and progressive motility were analyzed by a commercially available computerized spermatozoal-motility analyzer (SpermVision® CASA, Minitube of America, Verona, WI). Statistical significance was set at $p<0.05$.

	Treatment x Time					SEM	p-value
	AX x 1	AX x 2	AX x 3	AX x 4	AX x 5		
pH	7.44	7.44	7.53	7.55	7.68	0.075	0.6948
Mot	90.86	90.04	89.05	87.07	81.75	1.875	0.0098
Prog Mot	80.7	78.95	75.69	68.73	60.88	2.216	0.0585
	RP x 1	RP x 2	RP x 3	RP x 4	RP x 5		
pH	7.27	7.38	7.46	7.44	7.43	0.075	0.6948
Mot	90.33	89.85	90.58	88.4	81.25	1.875	0.0098
Prog Mot	79.38	77.52	78.16	72.21	63.32	2.216	0.0585
	AX+RP x 1	AX+RP x 2	AX+RP x 3	AX+RP x 4	AX+RP x 5		
pH	7.27	7.39	7.46	7.44	7.59	0.075	0.6948
Mot	90.02	90.54	88.91	86.72	84.41	1.875	0.0098
Prog Mot	78.91	79.58	75.43	68.05	66.74	2.216	0.0585
	C x 1	C x 2	C x 3	C x 4	C x 5		
pH	7.44	7.43	7.55	7.55	7.43	0.075	0.6948
Mot	90.57	91.61	89.44	87.09	76.25	1.875	0.0098
Prog Mot	79.1	79.34	76.04	67.15	55.66	2.216	0.0585

*Mot = motility
*Prog Mot = progressive motility

Table 8. Statistical differences between 3 ejaculates provided by 2 Yorkshire boars with regard to least squares mean (±SEM) of pH, motility, and progressive motility of boar semen across all extenders, treatment groups, and time periods. pH was analyzed by a pH meter (Accumet Basic AB15, Fisher Scientific, Pittsburg, PA), and motility and progressive motility were analyzed by a commercially available computerized spermatozoal-motility analyzer (SpermVision® CASA, Minitube of America, Verona, WI). Statistical significance was set at $p<0.05$.

	p-values		
	1 vs 2	1 vs 3	2 vs 3
pH	0.0982	0.1688	0.025
Mot	0.2794	0.002	0.0031
Prog Mot	0.1609	0.1707	0.0356

Table 9. Statistical differences between extenders with regard to least squares mean (±SEM) of pH, motility, and progressive motility of boar semen across all treatment groups, and time periods. pH was analyzed by a pH meter (Accumet Basic AB15, Fisher Scientific, Pittsburg, PA), and motility and progressive motility were analyzed by a commercially available computerized spermatozoal-motility analyzer (SpermVision® CASA, Minitube of America, Verona, WI). Statistical significance was set at $p<0.05$.

	p-values		
	BTS vs XC	BTS vs TXC	XC vs TXC
pH	<0.0001	<0.0001	0.0424
Mot	0.0343	0.0996	0.6358
Prog Mot	0.0026	0.0036	0.9212

*Extender: BTS = Beltsville Thaw Solution
XC = X-Cell
TXC = Tri-X-Cell
All extenders produced by IMV USA; Maple Grove, MN, USA.
*Mot = motility
*Prog Mot = progressive motility

REFERENCES

Aisenberg, G., K. V. Rolston, et al. (2004). "Bacteremia caused by Achromobacter and Alcaligenes species in 46 patients with cancer (1989–2003)." Cancer **101**(9): 2134-2140.

Althouse, G. C. (1997). "Evaluating porcine semen for artificial insemination. I. Standard tests." Compendium's Food Animal Medicine and Management: S30-S35.

Althouse, G. C. (1998). "Cytoplasmic droplets on boar sperm cells." Swine Health and Production **6**(3): 128.

Althouse, G. C. (2007). Artificial insemination in swine: Boar stud management. Current Therapy in Large Animal Theriogenology. R. S. Youngquist and W. R. Threlfall. St. Louis, MO, Saunders Elsevier**:** 731-738.

Althouse, G. C. and L. E. Evans (1994). "Closed resection of the preputial diverticulum in the boar." Agri-Practice **15**(9): 18-21.

Althouse, G. C., C. E. Kuster, et al. (2000). "Field investigations of bacterial contaminants and their effects on extended porcine semen." Theriogenology **53**(5): 1167-1176.

Althouse, G. C. and K. G. Lu (2005). "Bacteriospermia in extended porcine semen." Theriogenology **63**(2): 573-584.

Althouse, G. C., M. S. Pierdon, et al. (2008). "Thermotemporal dynamics of contaminant bacteria and antimicrobials in extended porcine semen." Theriogenology **70**(8): 1317-1323.

Anderson, R. L., L. A. Bland, et al. (1985). "Factors associated with Pseudomonas pickettii intrinsic contamination of commercial respiratory therapy solutions marketed as sterile." Applied and Environmental Microbiology **50**(6): 1343-1348.

Anderson, R. L., B. W. Holland, et al. (1990). "Effect of disinfectants on pseudomonads colonized on the interior surface of PVC pipes." American Journal of Public Health **80**(1): 17-21.

Benfield, D. A., J. E. Collins, et al. (1999). Porcine reproductive and respiratory syndrome. Diseases of Swine. B. E. Straw, S. D'Allaire, W. L. Mengeling and D. J. Taylor. Ames, Iowa, Iowa State University Press**:** 201-232.

Boender, G. J., R. Meester, et al. (2007). "The local threshold for geographical spread of infectious diseases between farms." Preventive Veterinary Medicine **82**(1-2): 90-101.

Bonet, S. (1990). "Immature and aberrant spermatozoa in the ejaculate of Sus domesticus." Animal Reproduction Science **22**(1): 67-80.

Boutros, N., N. Gonullu, et al. (2002). "Ralstonia pickettii Traced in Blood Culture Bottles." Journal of Clinical Microbiology **40**(7): 2666-2667.

Burns, J. L., J. Emerson, et al. (1998). "Microbiology of Sputum from Patients at Cystic Fibrosis Centers in the United States." Clinical Infectious Diseases **27**(1): 158-163.

C.L.S.I. (2008). Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacterial Isolated from Animals; Approved Standard - Third Edition., Clinical and Laboratory Standards Institute. **M31-A3**.

Clark, S., A. Ness, et al. (2008). "Inoculation of culture-negative porcine semen with novel biofilm-forming bacteria." Reproduction, Fertility and Development **20**(1): 85-85.

Clark, S., A. Ness, et al. (2007). "Description of growth dynamics of biofilm bacteria found in extended porcine semen." Biology of Reproduction **77**(1 Supplement): 84-85.

Colenbrander, B. and B. Kemp (1990). "Factors influencing semen quality in pigs." Journal of Reproduction and Fertility. Supplement **40**: 105-115.

Donay, J.-L., D. Mathieu, et al. (2004). "Evaluation of the Automated Phoenix System for Potential Routine Use in the Clinical Microbiology Laboratory." Journal of Clinical Microbiology **42**(4): 1542-1546.

Duggan, J. M., S. J. Goldstein, et al. (1996). "Achromobacter xylosoxidans Bacteremia: Report of Four Cases and Review of the Literature." Clinical Infectious Diseases **23**(3): 569-576.

Foote, R. H. (2003). "Fertility estimation: a review of past experience and future prospects." Animal Reproduction Science **75**(1-2): 119-139.

Gadea, J. (2003). "Review: Semen extenders used in the artificial insemination of swine." Spanish Journal of Agricultural Research **1**(2): 17-27.

Gadea, J. (2005). "Sperm factors related to in vitro and in vivo porcine fertility." Theriogenology **63**(2): 431-444.

Gadea, J., E. Sellés, et al. (2004). "The Predictive Value of Porcine Seminal Parameters on Fertility Outcome under Commercial Conditions." Reproduction in Domestic Animals **39**(5): 303-308.

Gales, A. C., R. N. Jones, et al. (2005). "Antimicrobial susceptibility patterns of unusual nonfermentative gram-negative bacilli isolated from Latin America: report from the SENTRY Antimicrobial Surveillance Program (1997-2002)." Memórias do Instituto Oswaldo Cruz **100**: 671-677.

Gatti, J.-L., C. Chevrier, et al. (1993). "External ionic conditions, internal pH and motility of ram and boar spermatozoa." Journal of Reproduction and Fertility **98**(2): 439-449.

Gómez-Cerezo, J., I. Suárez, et al. (2003). "Achromobacter xylosoxidans Bacteremia: A 10-Year Analysis of 54 Cases." European Journal of Clinical Microbiology & Infectious Diseases **22**(6): 360-363.

Guérin, B. and N. Pozzi (2005). "Viruses in boar semen: detection and clinical as well as epidemiological consequences regarding disease transmission by artificial insemination." Theriogenology **63**(2): 556-572.

Hirai, M., A. Boersma, et al. (2001). "Objectively measured sperm motility and sperm head morphometry in boars (Sus scrofa): relation to fertility and seminal plasma growth factors." Journal of Andrology **22**(1): 104-110.

Igra-Siegman, Y., H. Chmel, et al. (1980). "Clinical and laboratory characteristics of Achromobacter xylosoxidans infection." Journal of Clinical Microbiology **11**(2): 141-145.

Johnson, L. A., K. F. Weitze, et al. (2000). "Storage of boar semen." Animal Reproduction Science **62**(1-3): 143-172.

Kendirli, T., E. Çiftçi, et al. (2004). "Ralstonia pickettii outbreak associated with contaminated distilled water used for respiratory care in a paediatric intensive care unit." Journal of Hospital Infection **56**(1): 77-78.

Knox, R., D. Levis, et al. (2008). "An update on North American boar stud practices." Theriogenology **70**(8): 1202-1208.

Kulakov, L. A., M. B. McAlister, et al. (2002). "Analysis of Bacteria Contaminating Ultrapure Water in Industrial Systems." Applied and Environmental Microbiology **68**(4): 1548-1555.

Levis, D. G. (2000). Liquid boar semen production: Current extender technology and where do we go from here. International Conference on Boar Semen Preservation IV: Proceedings, IV International Conference on Boar Semen Preservation held at Beltsville, Maryland USA, August 1999. Lawrence, KS, Allen Press. **IV:** 121-128.

Maes, D., H. Nauwynck, et al. (2008). "Diseases in swine transmitted by artificial insemination: An overview." Theriogenology **70**(8): 1337-1345.

Maroye, P., H. P. Doermann, et al. (2000). "Investigation of an outbreak of Ralstonia pickettii in a paediatric hospital by RAPD." Journal of Hospital Infection **44**(4): 267-272.

McAlister, M. B., L. A. Kulakov, et al. (2002). "Survival and nutritional requirements of three bacteria isolated from ultrapure water." Journal of Industrial Microbiology & Biotechnology **29**(2): 75-82.

Morrison, A. J., Jr and K. Boyce, 4th (1986). "Peritonitis caused by Alcaligenes denitrificans subsp. xylosoxydans: case report and review of the literature." Journal of Clinical Microbiology **24**(5): 879-881.

Payne, B. J., S. Clark, et al. (2008). "Achromobacter xylosoxidans in extended semen causes reproductive failure in artificially inseminated sows and gilts." Journal of Swine Health and Production **16**(6): 316-322.

Pien, F. D. and H. Y. Higa (1978). "Achromobacter xylosoxidans isolates in Hawaii." Journal of Clinical Microbiology **7**(2): 239-241.

Reicks, D. L. (2003). "Bacterial contamination and semen quality." Proceedings of the Allen D. Leman Swine Conference: 169-170.

Reverdy, M. E., J. Freney, et al. (1984). "Nosocomial colonization and infection by Achromobacter xylosoxidans." Journal of Clinical Microbiology **19**(2): 140-143.

Roca, J., J. M. Vázquez, et al. (2006). "Challenges in Pig Artificial Insemination." Reproduction in Domestic Animals **41**: 43-53.

Ryan, M. P., J. T. Pembroke, et al. (2006). "Ralstonia pickettii: a persistent Gram-negative nosocomial infectious organism." Journal of Hospital Infection **62**(3): 278-284.

Saiman, L., Y. Chen, et al. (2001). "Identification and Antimicrobial Susceptibility of Alcaligenes xylosoxidans Isolated from Patients with Cystic Fibrosis." Journal of Clinical Microbiology **39**(11): 3942-3945.

Shie, S.-S., C.-T. Huang, et al. (2005). "Characteristics of Achromobacter xylosoxidans bacteremia in northern Taiwan." Journal of Microbiology, Immunology, and Infection **38**(4): 277-282.

Shipley, C. (1999). "Breeding soundness examination of the boar." Swine Health and Production **7**(3): 117-120.

Siebor, E., C. Llanes, et al. (2007). "Presumed pseudobacteremia outbreak resulting from contamination of proportional disinfectant dispenser." European Journal of Clinical Microbiology & Infectious Diseases **26**(3): 195-198.

Sone, M. (1990). "Investigations on the control of bacteria in boar semen." Japanese Journal of Animal Reproduction **36**: 23-29.

Sone, M., T. Kawarasaki, et al. (1989). "Effects of bacteria-contaminated boar semen on the reproductive performance." Japanese Journal of Animal Reproduction **35**(3): 159-164.

Spear, J. B., J. Fuhrer, et al. (1988). "Achromobacter xylosoxidans (Alcaligenes xylosoxidans subsp. xylosoxidans) bacteremia associated with a well-water source: case report and review of the literature." Journal of Clinical Microbiology **26**(3): 598-599.

Sutkeviciene, N., V. Riskeviciene, et al. (2009). "Assessment of sperm quality traits in relation to fertility in boar semen." Acta Veterinaria Scandinavica **51**(1): 53.

Tsay, R.-W., L.-C. Lin, et al. (2005). "Alcaligenes xylosoxidans bacteremia: clinical features and microbiological characteristics of isolates " Journal of Microbiology, Immunology, and Infection **38**(3): 194-199.

U.S.D.A. (2011). "International Animal Export Regulations." Retrieved March 17, 2011, from http://www.aphis.usda.gov/regulations/vs/iregs/animals/.

U.S.D.A. (2011). "International Animal Product Export Regulations." Retrieved March 17, 2011, from http://www.aphis.usda.gov/regulations/vs/iregs/products/.

UI-VDL. (2010). "MIC90 panel query." Retrieved March 3, 2011, from https://vetmed.illinois.edu/webvad/mic90/MIC90.php.

Vazquez, J. M., E. A. Martinez, et al. (1998). "The Fertilizing Ability Assessment of Fresh and Stored Boar Semen." Reproduction in Domestic Animals **33**(3-4): 267-270.

Verstegen, J., M. Iguer-Ouada, et al. (2002). "Computer assisted semen analyzers in andrology research and veterinary practice." Theriogenology **57**(1): 149-179.

Vyt, P., D. Maes, et al. (2004). "Comparative Study on Five Different Commercial Extenders for Boar Semen." Reproduction in Domestic Animals **39**(1): 8-12.

Vyt, P., D. Maes, et al. (2007). "Air Contact Influences the pH of Extended Porcine Semen." Reproduction in Domestic Animals **42**(2): 218-220.

Weitze, K. F. (2000). Update on the worldwide application of swine AI. Boar semen preservation IV: Proceedings, IV International Conference on Boar Semen Preservation held at Beltsville, Maryland USA, August 1999. Lawrence, KS, Allen Press. **IV:** 141-145.

Yabuuchi, E. and A. Ohyama (1971). "Achromobacter xylosoxidans n. sp. from human ear discharge." Japanese Journal of Microbiology **15**(5): 477-481.

Zbinden, R., V. Punter, et al. (2002). "In Vitro Activities of BAL9141, a Novel Broad-Spectrum Pyrrolidinone Cephalosporin, against Gram-Negative Nonfermenters." Antimicrobial Agents and Chemotherapy **46**(3): 871-874.