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A PCR detection method for discerning *Serratia marcescens* in extended boar semen

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ABSTRACT

Serratia marcescens is a bacterial contaminant that can be spermicidal when present in extended boar semen that is typically stored prior to breeding use at 15 to 18 °C for several days. This particular contaminant appears to originate from carrier boars, where it resides in the preputial cavity, but has also been shown to then easily contaminate the semen-processing laboratory. Screening for carrier boars to date has been performed through detection of *S.marcescens* in ejaculates using traditional agar plate culture techniques. These agar growth techniques are labor and time consuming due to the need for sample °titration and temporal growth followed by isolation, leading to delays in identification. The aim of this study was to develop a rapid, sensitive traditional PCR technique that can detect the presence of *S.marcescens* in extended boar semen. Primers for the detection of *S. marcescens* 16S rRNA were designed and specificity tested. After PCR optimization, assay sensitivity was evaluated using extended boar semen that was inoculated with various physiological ratios of spermatozoa: *S.marcescens* (100:1, 50:1, 20:1, 10:1, 8:1, 6:1, 4:1, 2:1. 1:1 and 1:10). Samples, held at 16 °C, were tested every 24 h over a 96 h period, with bacterial DNA extraction performed at each time point using a commercial kit. As a final step, the developed technique was used to screen random samples of extended boar semen for *S. marcescens* contamination. Results showed that this PCR technique had a sensitivity (90%) and specificity (100%) at detecting *S.marcescens* in the different inoculated ratios as well as in random, naturally contaminated samples of extended boar semen. In conclusion, this study reports a traditional PCR technique that is effective at rapidly and accurately detecting the presence of *S.marcescens* in boar extended semen.

1. Introduction

Bacteriospermia, the presence of bacteria in semen, typically occurs as a contaminant when collecting semen from boars using the gloved hand technique (Althouse, 2008) for use in artificial insemination programs. Sources of contamination have included faecal, preputial cavity fluid, skin/hair, respiratory secretion, personnel contamination, water, plant matter, sinks/drains, air/ventilation systems and equipment (Althouse et al., 2000; Althouse and Lu, 2005; Althouse, 2008).

The presence of certain bacteria in extended boar semen over time can cause detrimental decreases to sample sperm motility, sperm viability, and reduced shelf-life of the product prior to use in breeding (Althouse et al., 2008, Bussalleu et al., 2011), along with increased premature acrosome reactions (Köhn et al., 1998). Consequently, use of contaminated semen for breeding can cause increased regular returns to estrous and/or post-insemination vulvar discharges (Althouse et al., 2000), embryonic and fetal death, endometritis, systemic infection and/or disease in recipient females (Maes et al., 2008).

The majority of bacteria that have been isolated in extended boar semen belong to the Enterobacteriaceae family. Among enterobacteria, *Serratia marcescens* is one of the more frequently identified spermicidal contaminants in extended boar semen (Althouse and Lu, 2005; Althouse et al., 2008; Maroto-Martín et al., 2010; Sone, 1990). *S.marcescens* is an aerobic, Gram-negative bacterium that has a wide host range (Kurz et al., 2003), with capacity to infect sites such as the urinary tract (Marre et al., 1989), and the prepuce (Althouse et al., 2000), both possible sources of contamination when collecting boar semen.

The detection of *S. marcescens* and other aerobic bacteria in extended boar semen is normally preformed using typical microbiological culture procedures followed by biochemical identification. These techniques, although effective, are time consuming (e.g., 48–96 h), with culture results being directly affected via the presence of sperm-safe antimicrobials in the commercial semen diluent (e.g., extender), and/or even affected by low-level interference by other biological components

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in the sample like bovine serum albumin (BSA), egg yolk-Tris, or heat homogenized and pasteurized whole milk when used in the diluent (Gradil et al. 1994).

There are certain bacteria that can be identified only by PCR because of the challenge of culturing and identification using traditional microbiological methods. To overcome these issues, PCR technology has come to the forefront for quickly and accurately determining the presence of certain types of bacteria both in livestock ejaculates and extended semen doses prepared for commercial use, even if bacteria are present at low concentrations (Gradil et al. 1994; Mermin et al. 1991).

Identification of bacteria using PCR is commonly based on the 16S rRNA subunit due to its universality among bacteria (Mignard and Flandrois, 2006; Vandamme et al., 1996). The 16S rRNA gene sequence is about 1550 bp long and contains both variable and conserved regions (Clarridge, 2004). The importance of this sequence is its number of base pairs, with enough interspecific polymorphisms to provide valid measurements to distinguish among different bacterial types (Clarridge, 2004).

The aim of this study was to develop and validate a traditional PCR technique that can rapidly detect the presence of *Serratia marcescens* in extended boar semen. Such an assay would be of great value to the industry by having the ability to more quickly and selectively remove carrier animals and contaminated semen doses before their use in swine breeding programs.

2. Material and methods

2.1. Primer design and specificity tests

To design primers for the detection of *S.marcescens*, 16 different sequences of the 16S rRNA gene were obtained from GeneBank (http://www.ncbi.nlm.nih.gov/genbank/) then aligned to the *S. marcescens* genome using the online software Multalin (Corpet, 1998). After alignment of the 16 sequences, ten primer pairs were designed and purchased (Invitrogen, NY, USA). To test primer specificity, two different strains of *S. marcescens*, one strain of *E.coli*, one strain of *Pseudomonas aeruginosa*, one strain of *Proteus vulgaris,* one strain of *Stenotrophomonas maltophilia* and one strain of *Klebsiella* spp., (all previously isolated from contaminated extended semen and banked at the Reference Andrology Laboratory (RAL), University of Pennsylvania, Kennett Square, PA), were used. DNA was extracted using a commercial kit (DNeasy, Blood and Tissue kit, Qiagen, Valencia, CA, USA), with DNA quantity and purity assessed (Nanodrop™, ThermoScientific, Wilmington, DE, USA), using only DNA with a 260/280 ratio of at least 1.8 and a 260/230 ratio of at least 2.0 was used for further experiments.

Extracted DNA was processed for PCR, with each reaction using the following: 25 µL of MyTaqMix™ (Bioline, MA, USA), 1 mL each of forward (Fw) and reverse (Rv) primers (both 20 µM), 200 ng of DNA, and ddH_2O q.s. to achieve a final volume of 50 µL per reaction. Reactions were performed in a thermocycler (MasterGradient, Eppendorf, NL, using a negative control without DNA), with samples, run per duplicate, incubated for 5 min at 95 °C, then 35 cycles of 95 °C for 15 s, at a primer-specific melting temperature for 15 s, 72 °C for 10 s, and finally 72 °C for 7 min. PCR products were run on a 2% agarose gel then visualised with ethidium bromide under UV light, with images acquired and analysed using the Quantity One 1-D Analysis software (BioRad, CA) package.

2.2. Optimization of PCR conditions

Once the primer set with the strongest amplification was identified (e.g., primer set 10; Table 1), the PCR was optimized via the screening of several different melting temperatures (e.g., 58°, 59°, 59.5°, 60°, 60.5°, 61° and 62 °C), primer volumes (e.g., 0.1 µM, 0.15 µM, 0.2 µM, 0.25 µM and 0.3 µM), and number of amplification cycles (e.g., 25, 30, 35 and 40). Optimal PCR conditions were determined to be a melting temperature of 59.5 °C, 20 µM of each primer and 40 cycles of amplification. With PCR conditions optimized, primer specificity was again assessed on the aforementioned bacteria in Section 2.1. Results showed that the 16S rRNA only amplified for *S.marcescens*, indicating that the primers did not cross react with the other tested enterobacteriaceae species.

Table 1
Primers used to amplify 16S rRNA gene from *S.marcescens*.

Primer	Sequence (5′-3′)	Length of amplified fragment
Forward	GGTGAGCTTAATACGTTCATCAA	107 bp
Reverse	AATTCCGATTAACGCTTGCAC	

2.3. Experiment 1: inoculation of Serratia marcescens into extended semen

A strain of *S. marcescens* (RAL20150009), identified using the Remel RapID System (Remel, KS, USA), previously isolated from contaminated extended porcine semen (Reference Andrology Laboratory, University of Pennsylvania, Kennett Square, PA) and also used in this PCR's specificity test, was grown in brain-heart-infusion (BHI) enrichment medium (BD, Franklin Lakes, NJ, USA) for about 18–24 h at 37 °C in atmosphere supplemented with 5% CO_2. Bacterial concentration was determined in reference to a 0.5 McFarland standard (Remel, KS, USA), then aliquots (40 mL) of semen extended with Beltsville Thawing Solution (BTSl IMV International, MN, USA) to a final sperm concentration of 37.5 M/mL were inoculated to achieve target ratios of spermatozoa: *S.marcescens* (100:1, 50:1, 20:1, 10:1, 8:1, 6:1, 4:1, 2:1, 1:1, 1:10). A positive control, non-inoculated extended semen sample and a negative control (without DNA) were also run in parallel. All samples were then incubated at 16 °C over a 96 h period, with subsamples obtained at 24 h, 48 h and 96 h post-inoculation for DNA extraction and PCR testing.

From each *S. marcescens* inoculated sample, a 2 mL sample was removed and bacterial DNA extracted using a commercial kit (DNeasy, Blood and Tissue kit, Qiagen, Valencia, CA). Briefly, 2 mL of each inoculated extended semen sample was centrifuged at 480 × *g* for 5 min to pellet the spermatozoa. The supernatant was harvested from the sperm pellet and then centrifuged at 7100 × *g* for 10 min, allowing for the harvesting of a secondary bacteria pellet. DNA extraction was performed on the secondary bacteria pellet, following the manufacturer's instructions. Harvested DNA was processed for PCR in 25 µL of MyTaqMixTM (Bioline, MA, USA), 1 µL (20 µM) each of forward (Fw) and reverse (Rv) primers, 200 ng of DNA, and ddH_2O q.s. to achieve a final volume of 50 µL per reaction. This procedure was performed in duplicate using different semen samples and different extractions. An Eppendorf Mastercycler (Eppendorf, NL) instrument was used for the amplification with cycling conditions set at 5 min at 95 °C, then 40 cycles of 95 °C for 15 s, 59.5 °C for 15 s, 72 °C for 10 s, and finally 72 °C for 7 min. PCR products were run on a 2% agarose gel then visualised with ethidium bromide under UV light (in test performed at 96 h post-inoculation) or in E-Gel® Precast Agarose Electrophoresis System (Invitrogen, NY, USA) (in tests performed 24 h, 48 h and 72 h post-inoculation), with images acquired and subsequently analysed using the Quantity One 1-D Analysis software (BioRad, CA) package.

DNA extracted from a pure culture of *S.marcescens* (RAL20150009) served as a positive control, with a PCR mix reaction containing purified water devoid of DNA used as a negative control.

2.4. Experiment 2: testing the PCR technique on random boar extended semen samples

Randomly selected seminal doses (N = 9) submitted to the RAL laboratory for routine semen analysis that showed reduced sperm motility upon presentation were used to test the presence of *Serratia*