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Research paper

A comparative study of the effects of *Escherichia coli* and *Clostridium perfringens* upon boar semen preserved in liquid storage

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ABSTRACT

The present study compares the sperm quality of boar seminal doses artificially inoculated with *Escherichia coli* and *Clostridium perfringens*, and maintained in liquid storage at 15 °C for a 9-day period. Seminal doses from 10 sexually mature Piétrain boars were diluted in a Beltsville Thawing Solution (BTS)-based extender and infected either with *E. coli* or *C. perfringens*, with bacterial loads ranging from 10^1 to 10^7 cfu mL^{-1}. During storage, the changes in sperm quality were determined by assessing pH, sperm viability, sperm motiliy, sperm morphology, sperm agglutination degree, and sperm-bacteria interaction. The infection of seminal doses led to an alkalinization of the medium, which was of higher extend in doses infected with *C. perfringens*. The effect of contamination on sperm viability and motility relied on bacterial type and load. Therefore, while *E. coli* was more harmful than *C. perfringens* in bacterial loads ranging from 10^1 to 10^6 cfu mL^{-1}, the detrimental impact of *C. perfringens* was more apparent than that of *E. coli* at a bacterial load of 10^7 cfu mL^{-1}. Despite sperm morphology not being affected by either bacterial type or load, sperm agglutination and sperm-bacteria interaction were characteristic of doses infected with *E. coli*, and increased concomintantly with bacterial load and along storage period. In conclusion, the effects of infection by *E. coli* on sperm quality were dependent of both bacterial load and storage period, whereas the effects of *C. perfringens* were mainly dependent on the bacterial load, with a threshold at 10^7 cfu mL^{-1} from which the sperm quality of seminal doses was greatly impaired.

1. Introduction

Artificial insemination (AI) using extended seminal doses has reached a very high level in global swine industry, not only in developed countries but also in emergent economies (Riesenbeck 2011). While trading of seminal doses for AI has become a key factor in the exchange of genetic potential, several hygienic precautionary measures have to be taken in order to avoid disease transmissions between farms or even countries (Riesenbeck 2011; Kutser and Althouse, 2016).

Contamination can occur as a result of the infection of male reproductive tract, but also over semen collection and processing to obtain seminal doses (Úbeda et al., 2013; Kutser and Althouse, 2016). The sources of contamination during semen collection and dilution are multiple (Yániz et al., 2010; Althouse et al., 2008; Schulze et al., 2015), and they have been classified as either mammalian (including human) or non-animal origin (Althouse et al., 2008; Kutser and Althouse, 2016). Whilst sources of mammalian origin

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include feces, fluid of preputial cavity, skin/hair, respiratory secretions, and contamination caused by the staff, those of non-animal origin are related to water, plant matter (i.e., feed, bedding), sinks/drains, and systems of ventilation (Althouse et al., 2008; Schulze et al., 2015). Although contaminants present in extended seminal doses intended to AI are not considered primary patogens in swine, they can result in a decrease of sperm viability and motility, as well as in reduced conception rates, early embryonic or fetal death, and contamination of female's reproductive tract (Yániz et al., 2010; Kutser and Althouse, 2016).

The ratio spermatozoa:bacteria at which undesirable effects appear differs significantly according to the bacterial species (Althouse et al., 2000; Yániz et al., 2010). In addition, detrimental effects of bacteria upon spermatozoa also rely on the type of extender (Althouse et al., 2008; Yániz et al., 2010). In swine, where semen is stored at 15–17 °C, antimicrobials are common components of semen extenders as to prevent bacterial growth (Althouse et al., 2008; Yániz et al., 2010). However, too much reliance is placed upon this method for bacterial growth control, as some studies have demonstrated that over 90% of bacteria isolated from extended boar semen are resistant to the most used antibiotics (Althouse et al., 2008; Bolarín Guillén, 2011; Schulze et al., 2015). Bacteria present in seminal doses are usually gram negative and belong to Enterobacteriaceae family (Althouse and Lu, 2005), despite the presence of anaerobes having also been reported (Maroto Martín et al., 2010). While most of studies have been focused on analyzing the effects of contamination by aerobic bacteria, the number of works on the effects of anaerobic bacteria is scarce. *C. perfringens* is a gram positive, rod-shaped, anaerobic aerotolerant and sporulating bacterium, which produces the largest number of toxins of any bacteria (Popoff, 2011).

Our hypothesis is that the effects of contamination on sperm quality as well as sperm-bacteria interaction depend on the bacterial type. Therefore, in the present study we sought to compare the response of extended boar semen to infection by an aerobic (*E. coli*) and an aerotolerant, anaerobic bacteria (*C. perfringens*). Because both bacteria are present across swine industry (Baker et al., 2010), contamination during semen collection and processing may occur. In different works, our research group has reported a reduced sperm quality of extended seminal doses contamined with *E. coli* (Bussalleu et al., 2011) and *C. perfringens* (Sepúlveda et al., 2013). However, neither a comparative study involving these two species has been conducted nor whether the attachment of bacteria to sperm occurs during liquid storage has been addressed. Therefore, and in order to achieve reliable comparisons and reduce experimental variability, we used seminal doses from 10 boars, which were infected with *E. coli* and *C. perfringens* and stored at 15 °C for nine days.

2. Material and methods

2.1. Material

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich Química, S.A. (Madrid, Spain) and fluorochromes were purchased from Molecular Probes (Life Technologies, Leiden, The Netherlands).

2.2. Seminal doses

The study was performed using 10 semen samples coming from 10 different sexually mature Piétrain boars of the same genetic line (Semen Cardona, Cardona, Spain), aged two years, and included in AI programs without fertility problems. Boars were kept in the same husbandry conditions, i.e. lodged in the same pens, fed under standard protocols and provided with water ad libitum, and collected twice a week.

Ejaculates were obtained by the same expert technician with the gloved-hand technique. The sperm-rich fraction of each ejaculate was filtered to remove the gel, diluted with Beltsville Thawing Sotution (BTS)-based extender (Cidosa SL, Tarragona Spain), and split into doses of 90 mL each (final concentration: 3×10^9 spermatozoa/dose). Commercial doses were stored at 15 °C, and two doses per ejaculate and boar were sent to our laboratory in a heat-insulating recipient at 15 °C. All ejaculates were first checked for semen quality, and all were confirmed to present parameter values over the thresholds: 80% morphologically normal spermatozoa, 80% total motile spermatozoa, 50% progressive motile spermatozoa, and 80% viable spermatozoa. These quality parameters were determined as described below.

Once in the lab, one seminal dose per male was infected with *Escherichia coli* (*E. coli*), whereas the other was infected with *Clostridium perfringens* (*C. perfringens*).

2.3. Infection of seminal doses

Two pathological, commercial strains of *Escherichia coli* (*E. coli*) and *Clostridium perfringens* (*C. perfringens*) were purchased from the Spanish collection of microbiological type-cultures (CECT, Valencia, Spain).

E. coli strain, identified as CECT 4783, was cultured in Luria Bertani (LB) medium (Conda/Pronadisa, Madrid, Spain) for 48 h in a shaking water bath at 37 °C. All *E. coli* isolates were collected by centrifugation at 4800 × *g* for 10 min *E. coli* concentration was assessed with a spectrophotometer (SmartSpect™ Plus, BioRad, Hercules, CA, USA) at a wavelength of 600 nm (optical density, OD_{600}). All isolates averaged $41.07 \cdot 10^7 \pm 6.13 \cdot 10^7$ cfu mL^{-1} (mean ± standard deviation, SD) and they were diluted with BTS from 10^7 to 10^1 cfu mL^{-1} (Sepúlveda et al., 2013).

C. perfringens strain, identified as CECT 822, was cultured in liquid Liver Broth medium (Conda/Pronadisa, Madrid, Spain) for 48 h in an universal oven (MEMMERT UNB 200, Schwabach, Germany) at 37 °C under anaerobic conditions. *C. perfringens* isolates were also collected by centrifugation at 4800 × *g* for 10 min *E. coli* concentration was assessed with a spectrophotometer (SmartSpect™ Plus, BioRad, Hercules, CA, USA) at a wavelength of 600 nm (optical density, OD_{600}). Isolates averaged $26.91 \cdot 10^7 \pm 4.42 \cdot 10^7$ cfu mL^{-1} (mean ± standard deviation, SD) and they were diluted with BTS from 10^7 to 10^1 cfu mL^{-1} (Sepúlveda et al., 2013).