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How do different concentrations of *Clostridium perfringens* affect the quality of extended boar spermatozoa?

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ABSTRACT

Bacteriospermia in boar fresh and extended semen is a frequent finding that produces alterations on sperm quality and, consequently, causes economic losses in artificial insemination (AI) centres. The present study sought to evaluate the effect of different infective concentrations of *Clostridium perfringens* on boar sperm quality, assessed as sperm motility (CASA), morphology and viability, through 11 days of storage at 15 °C (experiment 1), and after 96 h of incubation at 37 °C (experiment 2). With this purpose, different seminal doses were artificially inoculated with different infective concentrations of *C. perfringens*, ranging from 10^2 to 10^8 cfu mL^{-1}. The negative controls were non-inoculated doses. Sperm quality was checked after 0, 1, 2, 3, 4, 7, 8, 9, 10 and 11 days of storage at 15 °C in experiment 1, and after 0, 24, 48, 72 and 96 h at 37 °C in the second experiment. Moreover, the presence/absence of bacteria was detected by PCR analyses during both experiments at different time points.

In both experiments, sperm morphology of inoculated samples did not differ from the negative control. Conversely, detrimental effects on sperm viability and motility were observed after 24 h of incubation/storage at the highest infective concentrations in both experiments. The deleterious effects observed because of the presence of *C. perfringens* in semen emphasise the relevance of detecting bacteria in extended doses destined to AI. So, this study suggests that the evaluation of bacterial contamination in semen is a procedure that should be routinely applied while assessing sperm quality in AI centres to avoid the use of doses with low sperm quality and the possible spread of bacterial contaminants.

1. Introduction

Nowadays, artificial insemination (AI) is widely used in swine industry. Unlike natural mating, AI increases the genetic diversity in sow herds and decreases the costs to feed and house large number of boars, all with a minimal risk of disease transmission (Althouse and Rossow, 2011; Clements, 2011). The presence of bacteria in semen is a frequent finding in fresh and extended boar ejaculates (Althouse and Lu, 2005). Bacteria adhere to the sperm surface producing ultrastructural changes at the level of the midpiece, plasma membrane and acrosome (Diemer et al., 1996) that can reduce sperm motility and viability and provoke a premature acrosome reaction (Diemer et al., 1996; Kohn et al., 1998; Zan Bar et al., 2008). Therefore, sperm function is compromised by bacterial contamination (Villegas et al., 2005). Moreover, the contamination of extended semen with high bacterial loads can produce reduced conception rates, shorter lifespan of semen doses, early embryonic or foetal death due to the direct invasion of the embryo by the pathogen, endometritis, and systemic

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infection and/or disease in the recipient female resulting from the transmission of the pathogen by semen (De Grau et al., 2006; Maes et al., 2008). So, although the risk of disease transmission using AI may be minimal, the economic looses that semen contaminated with pathogens causes to swine industry can be enormous (Maes et al., 2008).

Related to this, it is worth mentioning that contamination of semen can be due to systemic and/or urogenital tract infections in the boar (Althouse and Lu, 2005) or also due to the hands of the technician performing the collection. The equipment used during semen collection and processing also plays an important role in contamination (De Grau et al., 2006).

The majority of contaminants found in extended seminal doses destined to artificial insemination (AI) are not considered primary pathogens in swine. Most of them are gram negative bacteria, especially from the Enterobacteriaceae family (Althouse and Lu, 2005). Traditionally, given that anaerobes are sensitive to semen preservation conditions, their presence in semen samples has not been widely studied (Eggert - Kruse et al., 1995). However, the presence of anaerobes in semen of horse, human and boar is not exceptional (Corona and Cherchi, 2009; Damirayakhian et al., 2006; Maroto Martín et al., 2010). In this study we have worked with the aerotolerant anaerobe gram positive bacterium *Clostridium perfringens*, which can survive and proliferate in oxygen tensions that are inhibitory for other clostridia (Joclik and Willett, 1991). *C. perfringens* has a wide distribution in the environment, as well as in the intestinal flora of both animals and humans. This bacterium can act as an opportunistic pathogen causing different diseases, such as diarrhoea or necrotic enteritis in young piglets (Morris and Fernández-Miyakawa, 2009), and is highly prevalent throughout the swine industry (Baker et al., 2010), so semen contamination during its collection or processing can occur.

To minimize the effects of bacterial load on extended semen, antimicrobials with bactericidal or bacteriostatic activity are usually included in the semen extender formulation (Althouse et al., 2000; Yániz et al., 2010). However, too much reliance is placed on this method of bacterial control, because some studies have demonstrated that over the 90% of bacteria isolated from extended semen are resistant to the most used antibiotics in extended semen (Bolarín Guillén, 2011).

Against this background, the aim of this study was to determine how different bacterial loads of *C. perfringens* can affect the sperm quality of extended boar semen when incubated at 37 °C and 15 °C. With this aim, two experiments were performed simultaneously, one at 15 °C for 11 days and the other at 37 °C for 96 h.

2. Material and methods

2.1. Semen collection

Thirteen seminal doses coming from thirteen healthy and sexually mature Piétrain boars, aged from 9 to 12 months and submitted to a collection frequency of twice a week, were used in the study. All the samples were manually collected using the gloved-hand technique. After removing the gelatinous fraction of the semen using a gauze filter under high hygienic measures, the sperm rich-fraction was immediately diluted 1:6 (v:v) in a long-term extender (Duragen®, Magapor, Zaragoza, Spain) to a final concentration of 3×10^7 spermatozoa mL^{-1}, divided into 90 mL semen doses and cooled at 15–17 °C. Finally, two seminal doses were transported within 24 h post-collection to the laboratory inside an insulated container held at 15–17 °C.

Upon arrival, all the seminal doses were assessed for semen quality parameters (performed as described below) and, according to the boar semen quality criteria (total motile spermatozoa > 80%; morphologically normal spermatozoa > 85%; spermatozoa with intact acrosome and mitochondrial sheath > 80%), all thirteen seminal doses were qualified for the present study.

2.2. Bacterial inoculation of extended semen

Different pathogenic *C. perfringens* strains purchased from Spanish collection of microbiological type-cultures (CECT, Valencia, Spain) and identified as CECT 4110, CECT 4647, CECT 822, CECT 376 and CECT 486, were used in this study. Strains were cultured in liquid Liver Broth medium (Conda/Pronadisa, Madrid, Spain) for 36–48 h in a universal oven (MEMMERT UNB 200, Schwabach, Germany) at 37 °C and under anaerobic conditions. After assessing the cell concentration with a spectrophotometer (SmartSpec™ Plus, Bio-Rad, California, USA) at a wavelength of 600 nm (optical density, OD_{600}), the culture was diluted with Ringer solution (Scharlau, Barcelona, Spain) up to 10^3 cfu mL^{-1}. Then, 9 mL of semen were taken and inoculated with 1 mL of each bacterial dilution. Thus, the final infective concentrations, as reported by Bussalleu et al., 2011, were: tube A (inoculated with a bacterial concentration of 10^8 cfu mL^{-1}), tube B (with 10^7 cfu mL^{-1}), tube C (with 10^6 cfu mL^{-1}), tube D (with 10^5 cfu mL^{-1}), tube E (with 10^4 cfu mL^{-1}), tube F (with 10^3 cfu mL^{-1}), tube G (with 10^2 cfu mL^{-1}) and tube H (with 1 mL of Ringer solution; this was the negative control tube). The tubes were stored at 15 °C for 11 days in a Z-15 storage chamber (Magapor®, Zaragoza, Spain) in experiment 1, and at 37 °C for 96 h in a universal oven (previously mentioned) in experiment 2. All this procedure was repeated 13 times ($n = 13$) inoculating only one strain in each replicate.

2.3. Sperm quality analyses

After inoculation, an aliquot of each tube (A, B, C, D, E, F, G and H) containing semen inoculated with *C. perfringens* was taken on days 0, 1, 2, 3, 4, 7, 8, 9, 10 and 11 in the experiment at 15 °C, and at 0 h and every 24 h over a 96 h time period in the experiment carried out at 37 °C. Sperm motility and sperm morphology were assessed using a computer-assisted sperm analysis system (SCA® 2002 Production, Microptic, Barcelona, version 2002). Sperm motility, evaluated after 20 min of incubation at 37 °C, was established as counts of motile/immotile spermatozoa and results expressed as the percentage of motile spermatozoa. For each infective concentration, three counts/replicates, of minimum 1000 spermatozoa, were performed.