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Effect of extender supplementation with various antimicrobial agents on viability of *Brucella ovis* and *Actinobacillus seminis* in cryopreserved ovine semen

V.S. Moustacas, M.N. Xavier, C.A. Carvalho-Júnior, E.A. Costa, M. Henry, R.L. Santos*

Departamento de Clínica e Cirurgia Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901 Belo Horizonte, MG, Brazil

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Abstract

The objective was to determine the effectiveness of various antimicrobial agents added to semen extender for inactivation of *B. ovis* or *A. seminis* in ovine semen after cryopreservation. In Experiment 1, 20 ejaculates from a crossbred ram infected with *B. ovis* were cryopreserved in Tris-based extenders with various antimicrobial agents: (I) control without antibiotics, (II) with penicillin and streptomycin (1000 IU/mL and 1 mg/mL, respectively), (III) lincomycin (0.15 mg/mL), (IV) sulphadiazine (0.60 mg/mL), and (V) gentamicin sulphate (0.25 mg/mL). Semen was stored in 0.25 mL straws at a final concentration of 150×10^6 spermatozoa/mL. After thawing (37 °C for 30 s), sperm total motility (TM), sperm morphology, integrity of sperm membranes, and bacterial growth were assessed. In Experiment 2, six *B. ovis* isolates were separately inoculated into aliquots of a fresh ejaculate from a *B. ovis*-free ram. Mock inoculated semen was processed for cryopreservation using the five extenders described above, and bacteriologically evaluated after thawing. In Experiment 3, sensitivity of *A. seminis* to the same antimicrobial agents was evaluated by inoculating an ejaculate from an *A. seminis* and *B. ovis*-free ram. There were no significant differences among treatments in post-thawing sperm parameters. *B. ovis* was isolated from 100% (20/20), 0% (0/20), 95% (19/20), 100% (20/20), and 5% (1/20) of semen samples diluted in tris-based extender of untreated (I) and treated semen samples with antimicrobial agents II, III, IV, and V, respectively. Frequencies of isolation from samples treated with antimicrobial agent II and V were significantly lower than untreated ones ($P < 0.05$). There were no significant differences in the profile of antimicrobial resistance of different *B. ovis* isolates. *A. seminis* had a similar sensitivity to the antimicrobial agents. We concluded that addition of a combination of penicillin and streptomycin or gentamicin alone to ram semen cryo-extenders inactivated *B. ovis* and *A. seminis*.

Keywords: *Brucella ovis*; *Actinobacillus seminis*; Sheep; Semen; Cryopreservation

1. Introduction

Artificial insemination (AI) is currently the most common and widespread biotechnology of reproduction. However, intensive use of this technique increases the risk of spreading diseases or infectious agents within and between herds worldwide, since a wide range of pathogens may be shed in genital secretions, including *Brucella ovis*, *Actinobacillus seminis*, *Actinobacillus actinomycetemcomitans, Histophilus somni*, *Arcanobacterium pyogenes*, and Maedi-visna virus [1–4]. Moreover, fecal bacteria, preputial flora, respiratory secretions, and skin/hair may also act as potential sources of contamination for diluted semen, especially

* Corresponding author. Tel.: 55-31-3409-2239; fax: 55-31-3409-2230.

E-mail address: rsantos@vet.ufmg.br (R.L. Santos).

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when semen samples are obtained without satisfactory sanitary conditions [3]. Additionally, pathogen-contaminated semen samples may result in substantial economic losses due to low fertility rates, abortion, and premature culling of breeding animals. Therefore, in order to diminish microbial contamination, various antibiotics are routinely added to semen extenders. Several earlier studies assessed the effectiveness of antimicrobial agents to inhibit bacterial growth in frozen-thawed and chilled semen samples [5]. Penicillin, streptomycin, sulfas, lincomycin, and gentamicin were among the products most frequently used [6,7]. Penicillin is a β-lactam antibiotic, which inhibits the bacterial cell wall synthesis in multiplying bacteria. Streptomycin and gentamicin bind to the 30S ribosomal subunit and interfere with mRNA translation. Sulphonamides and their derivatives act by inhibiting the synthesis of folic acid and consequently DNA synthesis. Lincomycin is an antibiotic from the lincosamides group that binds to the 50S subunit of bacterial ribosome and inhibits protein synthesis [8]. Nevertheless, the actual effectiveness of these antibiotics in preventing growth of pathogenic bacteria has been poorly investigated in cryopreserved ovine semen.

Although *Brucella ovis* is genetically very closely related to other organisms of the genus [9], it is one of the two classical *Brucella* species that does not have zoonotic potential [2]. *B. ovis* infection is recognized as the main cause of epididymitis in rams, and consequently subfertility in sheep flocks in several regions of the world. In addition, *B. ovis* can also cause abortion in ewes [2]. Infection with *Actinobacillus seminis* is also associated with epididymitis and reduced fertility, particularly in sexually immature rams [10]. These infections lead to a decrease in total number of spermatozoa per ejaculate, a decrease in spermatozoa motility, and an increased rate of sperm abnormalities [9]. Cases of ovine epididymitis due to *B. ovis* and *A. seminis* infections have been reported in most major sheep-producing regions in the world, e.g. New Zealand, Australia, South Africa, several European countries, North America, and in various regions of South America, including Brazil [1,2,11]. These microorganisms can easily spread in flocks subjected to AI, since the bacteria may be shed in high numbers in semen [2]. Importantly, although chronically affected rams may have regression of lesions and clinical signs of epididymis, they often continue to shed the pathogen in the semen for prolonged intervals. In fact, only 30 to 50% of infected rams developed clinical signs of epididymitis [1]. Therefore, asymptomatic rams that shed *B. ovis* or *A. seminis* in the semen play a key role in the maintenance of infection in a flock. *B. ovis* infection may also result in reduction of pregnancy and lambing rates in ewes, in which vaginitis and salpingitis, as well as persistent mammary infection may also be observed [1,11]. Although usually infected ewes do not exhibit any clinical sign, they may carry *B. ovis* in the vagina for at least 2 mo, and therefore they may play a significant role in the epidemiology of the disease [12,13]. Abortions may also result from *A. seminis* infection [14,15]. Clinical infections are usually poorly responsive to antibiotic treatment [16].

It is noteworthy that *B. ovis* is highly resistant to low temperatures [17], another fact that favors transmission by AI of frozen-thawed semen. Considering the lack of information regarding inactivation of *B. ovis* and *A. seminis* in cryopreserved ovine semen, as well as the importance of these agents as a cause of infertility, this study aimed at evaluating the effectiveness of various antimicrobial agents commonly used in semen extenders for inhibiting the growth of *B. ovis* and *A. seminis* in frozen-thawed ram semen.

2. Materials and methods

2.1. Experiment 1

The goal of this experiment was to assess the efficacy of various antimicrobial agents to inhibit *B. ovis* viability in frozen-thawed semen samples.

2.1.1. Ram treatment and semen collection

Twenty culturally positive ejaculates were collected by an artificial vagina from a 2-y-old crossbred ram positive for *B. ovis* by serology, bacterial isolation and PCR with DNA samples extracted from semen. A ewe treated with 2 mg of estradiol cypionate (ECP, Rhodia-Mérieux Veterinária, São Paulo, SP, Brazil) was used to stimulate rams prior to semen sampling. Prior to sampling, the prepuce was profusely wiped with disposable wipes with 70% ethanol and a sterile ethylene-vinyl acetate inner sheath covered the lumen of the artificial vagina. The ram had been experimentally inoculated intraconjunctivally and intrapreputially with a total of 3.6×10^9 CFU (colony forming units) of *B. ovis* (strain ATCC 25840), with 2 mL of a suspension containing 1.2×10^9 CFU instilled intrapreputially, and 50 μL of a suspension containing 1.2×10^{10} CFU in each eye. Semen samples were collected between 2 and 5 mo postinfection. The experimental infection was approved by the Universidade Federal de Minas