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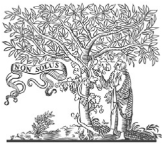

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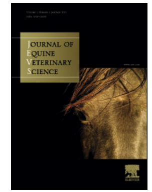

Original Research

Control Methods and Evaluation of Bacterial Growth on Fresh and Cooled Stallion Semen

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ABSTRACT

The penis and prepuce of the stallion have a high bacterial load on its surface, forming a natural microbial flora that contaminates the semen during ejaculation. Bacterial growth in semen may cause a decline on sperm quality, viability, and fertility and predisposes the occurrence of endometritis in inseminated mares. Thus, the aim of this study was to evaluate the effect of penile wash before semen collection, the addition of different commercial skim milk–based extenders containing antibiotics (BotuSemen and INRA96), and the removal of seminal plasma by filtration on the quality, viability, and bacterial proliferation on fresh and cooled stallion semen. Animals that were never submitted to penile wash before semen collection tended to have lower bacterial contamination in the ejaculate. Semen samples extended in BotuSemen showed superiority in total motility, progressive motility, average path velocity, and rapid sperm and lower bacterial contamination in relation to semen samples extended in INRA96 after 24 hours of cooling. No difference was found in these parameters between the storage temperatures (5°C and 15°C). Furthermore, the removal of seminal plasma by filtration reduced the bacterial load in semen after cooling. In conclusion, the penile wash before semen collection tended to reduce the bacterial growth in fresh semen. The use of a semen extender with appropriate antibiotics and removal of seminal plasma by filtration were effective in reducing the bacterial contamination and preserved the quality of cooled stallion semen.

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1. Introduction

The penis and prepuce have a permanent natural microbial flora with a high amount of bacteria [1] that can be transferred to the semen after collection [2]. Therefore, the equine ejaculate naturally exhibits a large bacterial contamination and is mainly composed of nonpathogenic bacteria [3].

An imbalance in the microbial environment of the external genitalia can result in the proliferation of opportunistic microorganisms [4], such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Taylorella equigenitalis*, and *Streptococcus* β-hemolytic. These microorganisms may cause endometritis in inseminated mares [1], decrease in fertility rates [5], and affect the semen quality [6].

Methods to reduce bacterial contamination and proliferation in equine semen are largely described, such as washing the stallion penis before semen collection [7], the use of an open-ended artificial vagina [4], the use of semen extenders containing antibiotics [1,4], and the reduction of semen storage temperature [4].

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Table 1
Mean values and standard deviations of total motility (TM), progressive motility (PM), velocity of trajectory (VAP), curvilinear velocity (VCL), rapid sperm (RAP) before (M0) and after (M24) cooling the semen of animals with (W) and without (NW) to wash penis before collection.

Groups	TM (%)	PM (%)	VAP (μm/s)	VCL (μm/s)	RAP (%)
W (M0)	85.2 ± 6.9	39.8 ± 5.3	123.3 ± 18.9	216.2 ± 21.6	74.8 ± 9.7
NW (M0)	75.0 ± 14.5	42.0 ± 15.1	116.4 ± 18.6	201.2 ± 22.5	65.6 ± 17.6
W (M24)	62.8 ± 15.2	29.4 ± 15.7	102.8 ± 21.3	194.7 ± 28.7	51.2 ± 22.2
NW (M24)	52.6 ± 28.3	20.4 ± 12.4	104.4 ± 22.6	193.8 ± 37.3	44.6 ± 28.4

Different letters indicate statistical difference between the groups at the same column ($P < .05$). Same letters or absence of letters indicate no statistical difference.

To achieve new methods of control of bacterial growth in equine semen, the aims of this study were (1) to determine the effect of washing the stallion penis before collection and (2) to compare different semen extenders and the seminal plasma removal using filtration on quality, viability, and bacterial growth on fresh and cooled stallion semen.

2. Materials and Methods

2.1. Semen Collection

Semen collection was performed using an artificial vagina. Initially, three ejaculates from each stallion were collected with an interval of 2 days to eliminate possible damaged cells and stabilize the sperm parameters.

2.2. Semen Cooling and Storage

After collection and processing, semen samples were stored in a container of passive cooling (BotuFlex; Botupharma, Sao Paulo, Brazil) at 15°C for 24 hours, for experiments 1, 2 and 3. For experiment 2, samples were also stored at 5°C for 24 hours.

2.3. Semen Analysis

The kinetic sperm parameters were evaluated immediately after semen collection (0 hours) and 24 hours after semen cooling. The samples were previously placed in a dry water bath at 37°C for 5 minutes and evaluated by computer-assisted sperm analysis (HTM-IVOS 12; Hamilton Thorne Research, MA). Three different fields in Makler Counting Chamber (Sefi Medical Instruments Ltd, Israel) per sample were considered for total motility (%), progressive motility (PM, %), average path velocity (μm/s), curvilinear velocity (μm/s), and rapid sperm (%).

Plasma membrane integrity (PMI, %) analysis was assessed through an epifluorescence microscopy (×400 magnification) using the fluorescent probes 6-carboxyfluorescein diacetate and propidium iodide [8], and 100 sperm cells were counted.

2.4. Bacteria Counting and Identification

Isolation of bacterial colonies was based on macroscopic and microscopic characterization (Gram stain method) and biochemical tests [9]. For the count of colony-forming units (CFUs), 0.1 mL of seminal sample was diluted in 9.9 mL of saline. Then, 0.1-mL aliquot of this dilution was plated in the following culture mediums: blood agar base with 7% sterile defibrinated sheep blood, MacConkey agar base, and Sabouraud agar base. The seeded assay plates were incubated 37°C and analyzed for 24, 48, 72, and 96 hours. The total of counted colonies was multiplied by 10^3 CFUs in accordance with the dilution used in this study. The identification and the CFU count were performed on fresh semen in experiments 1 and 3 and on cooled semen in experiments 2 and 3.

2.5. Experiments

2.5.1. Experiment 1—Effect of Penile Washing on Contamination and Quality of Stallion Semen

An ejaculate from each of 10 healthy stallions of different breeds (Mangalarga Marchador and Quarter Horse) aged 7–15 years old was used. All stallions were used in breeding programs that the semen collection was performed by artificial vagina. The animals were divided into two groups: stallions that were never submitted to penile washing before semen collection, composed by three Mangalarga Marchador and two Quarter Horse stallions (group NW; n = 5; 10 ± 3.2 years old), and stallions that are routinely (three times per week) submitted to penile washing with running water at 30°C before semen collection, composed by three Mangalarga Marchador and two Quarter Horse stallions (group W; n = 5; 10.4 ± 2.7 years old). After semen collection, the ejaculate was filtered to remove the gel fraction and diluted in a skim milk–based extender (Botusemen; Botupharma) to a sperm

Table 2
Identification of the bacteria in the culture of cooled stallion's semen with and without to wash penis before collection.

Stallions with wash penis before collection		Stallions without wash penis before collection	
1	*Staphylococcus* spp.	6	*Klebsiella oxytoca*/*Escherichia coli*
2		7	*Streptococcus* β-hemolytic/*Staphylococcus* β-hemolytic
3	*Streptococcus* spp./*Staphylococcus* spp.	8	*Bacillus* spp.
4		9	*Streptococcus* α-hemolytic
5		10	*Staphylococcus* spp.

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