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Bacteriology of Frozen-Thawed Semen of Tunisian Arab Stallions

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Abstract: The experience was taken to study the bacteriology of frozen-thawed semen of Tunisian Arab stallions. A sample of one straw from each ejaculate frozen (total ejaculates = 21) was thawed and analysed quantitatively and qualitatively for microbial contamination. Bacterial flora concentration was determined in fresh semen before his freezing, in diluted semen in skim milk extender and Penicillin and Gentamicin during the process of freezing and after freeze-thawing semen. Results showed that bacterial flora concentration decreased in the diluted semen and in the semen after frozen-thaw ($p < 0.05$) comparing to the one in the fresh semen. Two pathogenic bacteria were detected in fresh semen before the process of freezing *Pseudomonas aeruginosa* at 33 % of ejaculates and *Staphylococcus aureus* at 10 % of ejaculates. The influence of the process of semen dilution and the frozen-thawing semen was marked on these pathogenic bacteria. *Staphylococcus aureus* completely disappeared just after dilution in the diluted semen. However, *Pseudomonas aeruginosa* increased in the semen after dilution and after freezing - thawing semen respectively at 48 % and 62 % of ejaculates. This study showed that the freezing process decreased the bacteria flora concentration. But, it has any influence on *Pseudomonas aeruginosa* which is responsible of endometritis in mare.

Key words: Bacteriology · Frozen-thawed semen · Arab stallions · Pathogenic bacteria

INTRODUCTION

Nowadays, equine frozen semen is more employed in artificial insemination (AI) program [1]. Before the freezing process, collected semen is usually evaluated for percentage of motile spermatozoa [2, 3]. But, bacteriological studies on fresh, cooled or frozen semen are little mentioned in bibliography. In fact, the stallion penis and prepuce are inhabited by a great variety of bacteria including potentionally pathogenic bacteria that contaminate sperm at ejaculation [4-6]. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus zooepidemicus* and *Klebsiella pneimoniae* are the most pathogenic bacteria that can carry with AI and cause infection in genital tracts of the mare, specially the endometrial aging leading to infertility [5, 6]. Moreover, in the case of AI these bacteria are harmful for spermatozoa survival. They compete with them for the use of nutrients and secrete metabolic substances sometimes harmful to spermatozoa [7].

The purpose of this work was to study quantitative and qualitative bacterial contamination during the process of freezing semen and after frozen-thawed semen.

MATERIALS AND METHODS

General: Experience took place in the National Stud Farm of Sidi Thabet, situated in the north of Tunisia, between January and February 2010. Semen was collected from 5 Arab stallions named A, B, C, D and E (total ejaculates = 21; 4 ejaculates for each of the 4 stallions and 5 ejaculates for the 5th stallion) aged between 11 to 15 years. The bacteriology analysis was carried in the department of microbiology-immunologie of the veterinary medicine school of Sidi Thabet.

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Semen Collection: Ejaculates were collected from stallions using a Missouri artificial vagina (AV) (IMV, France) and an estrous mare. After collection, the ejaculate was immediately filtered using a suitable filter as gauzes with hidrophilics to remove the gel fraction [8]. After filtration, a sample of 1ml of fresh semen was taken to study microbial quality.

Semen Freezing Process: Semen was frozen as described by Haras Nationaux [8]. Collected semen was firstly diluted in skim milk and antibiotics Penicillin and Gentamicin at +37°C (¼ semen, ¾ extender) and put for 10 minutes in a water bath at +22°C. Then, semen was centrifuged at 600 g for 10 minutes. The supernatant was eliminated and the sperm pellet was diluted again in INRA 96® (200 ml, IMV, L'aigle, France) supplemented of 2.5 % glycerol and 2 % egg yolk. The diluted semen was kept at +4°C about 80 minutes. Then, semen was identified and packaged in straws of 0.5 ml. Before packaging straws, a sample of 1ml of diluted semen was taken to study microbial quality. After that, straws were frozen with a cool mini digit which ensured the temperature decrease from +4°C to - 140°C at the rate of 60°C /minute during 10 minutes. At the end of this stage, straws were immediately immersed in liquid nitrogen at - 196°C. A sample of one straw from each ejaculate was analyzed for microbial quality after one month of storage.

Microbial Quality of Semen: We focus on the research of 4 pathogenic bacteria which are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus zooepidemicus* and *Klebsiella pneimoniae*. Each sample of semen was diluted with buffered peptone sterile water with the rate of 10^{-1}. Then, 100µl of this suspension were inoculated on Petri dish of blood agar, blood agar colistin nalidixic acid, cetrimide agar, XLD agar and Chapman agar. Petri dish were incubated for 48 hours at 37°C in aerobic conditions for cetrimide, XLD and Chapman agar and in anaerobics conditions (7% CO_2) for blood agar and blood agar colistin nalidixc. Then, colonies were counted on Petri dishes and the number founded was multiplied by 1000 to indicate the number of bacteria per ml (CFU/ml) [6]. To identify the bacteria, colony was removed using the platinum loop and suspended in 0.5 ml of brain heart infusion after performing a Gram stain. Seeding is done on a proper gallery: Hajna-Kliger, Mannitol-mobility and urea-indole medium for Gram-negative, DNA agar and rabbit plasma for staphylococci. Final identification of the bacteria was performed by automated biochemical and enzymatic essays (mini API system, BioMérieux, France): API 20E for Enterobacteriaceae, API 20NE for Gram-negative non Enterobacteriaceae and API STREP for streptococci.

Statistical Analysis: Data were analysed using a software SAS (version 1997). Bacterial concentration of fresh, diluted and frozen-thawed semen was transformed into decimal logarithm and analysed. A general linear model was used to study the influence of stallions on bacteria flora concentration. The threshold of significance was fixed at 5 %.

RESULTS

Bacteria flora concentration was higher in fresh semen compared to diluted and frozen-thawed semen (1.10^6 CFU/ml *vs* 2,8. 10^5 CFU/ml and 3,2. 10^4 CFU/ml; $p < 0.05$) (Figure 1). Moreover, statistical analysis showed that bacteria flora concentration tends to decrease in frozen-thawed semen ($p < 0.1$).

Bacteria flora concentration varied also according to stallions in fresh, diluted and frozen-thawed semen ($p < 0.05$; Figure 2). In fresh and diluted semen, bacteria flora concentration varied respectively from $1.9\ 10^5$ and $2.5\ 10^4$ CFU/ml for stallion E to $2.1\ 10^6$ and $7.6\ 10^5$ CFU/ml for stallion A. However, in frozen-thawed semen bacteria flora concentration varied from $4.\ 10^5$ CFU/ml for stallions A and E to $5.8\ 10^3$ CFU/ml for stallion B. Statistical analysis showed also an ejaculate influence on bacteria flora concentration ($p < 0.05$).

Table 1: Percentage of ejaculates containing pathogenic bacteria in fresh, diluted and frozen-thawed semen

	Percentage of ejaculate contaminated with the pathogenic bacteria		
	Fresh semen	Diluted semen	Frozen-thawed semen
Pseudomonas aeruginosa	33 %	48 %	62 %
Staphylococcus aureus	10 %	0 %	0 %
Klebsiella pneumoniae	0 %	0 %	0 %
Streptococcus Zooepidemicus	0 %	0 %	0 %

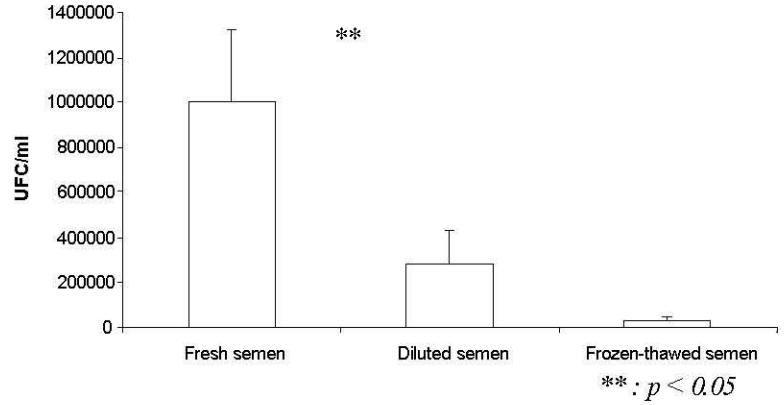

Fig. 1: Bacteria flora concentration in fresh, diluted and frozen-thawed semen

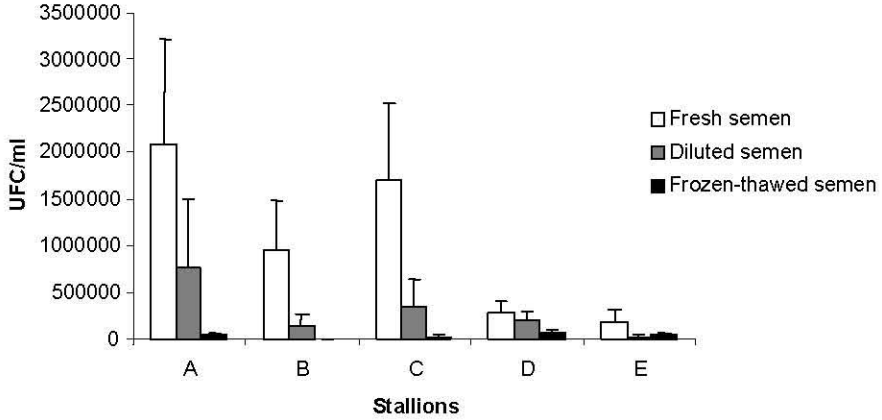

Fig. 2: Bacteria flora concentration in fresh, diluted and frozen-thawed semen according to stallions

Only two potentially pathogenic bacteria (Table 1), *Pseudomonas aeruginosa* and *Staphylococcus aureus*, was found in fresh semen respectively at 33 % and 10 % of ejaculates. Our results showed that *Staphylococcus aureus* disappears from ejaculates after dilution and freezing-thawing of the semen. However, *Pseudomonas aeruginosa* remains present and the percentage of ejaculate contaminated with this bacteria increases to 48 % after semen dilution and 62 % after freezing-thawing semen.

DISCUSSION

The decrease in bacteria flora concentration found after semen dilution is due to the effect of antibiotics Penicillin and Gentamicin. This result is in agreement with Clément *et al.* [5]. These authors reported that the milk extender added with only Gentamicin or with an association of Gentamicin and Penicillin, lead to minimize bacteria flora concentration. However, they [5] indicated that the two concentration antibiotics must not exceed 50 IU/ml for Penicillin and 50 µg/ml for Gentamicin because they become toxic for spermatozoa. Moreover, Varner *et al.* [4] found that bacteria growth was eliminated when semen was diluted in milk - glucose added only with 1000 µg/ml of Gentamicin compared to the one when semen was diluted in milk - glucose added only with 1000 IU/ml of Penicillin G.

Our results showed a decrease in bacteria flora concentration after freezing - thawing semen and after one month of semen storage in nitrogen liquid, which is in agreement with Clément *et al.* [5] and Corona et Cherchi [6]. Clément *et al.* [5] found on the one hand, that if semen was stored in nitrogen liquid at - 196°C for 3 days, the bacteria flora concentration was about 2,6. 10^4 CFU/ml and on the other hand, if semen was stored for 3 months, the bacteria flora concentration was 1,5. 10^4 CFU/ml. Corona et Cherchi [4] reported also that bacteria flora concentration decreased after freezing - thawing semen and established a mean bacteria flora concentration of 1,4 10^5 CFU/ml when semen stored in liquid nitrogen between 3 and 17 years.

Our results revealed that *Staphylococcus aureus* which was presnt in 10 % of ejaculates disappeared after semen dilution. However, percentage of ejaculates contaminated with *Pseudomonas aeruginosa* increased after dilution and freezing - thawing semen. This indicated that *Pseudomonas aeruginosa* was resistant to the effect of the dilution containing antibiotics and the effect of freezing - thawing semen. Guérin [9] reported that *Pseudomonas aeruginosa*, which is responsible of endometritis in mares, resists to the effect of freeze cooling. In addition, Clément *et al*. [5] and Corona et Cherchi [4] reported that *Pseudomonas aeruginosa* was frequently isolated from frozen - thaw semen. Consequently, it was revealed resistant to the freeze. Bielanski *et al.* [10] showed that this bacterium can survive in liquid nitrogen at -196°C for a long period of semen storage. Therefore, it is harmful for spermatozoa and decreases sperm fertility. Corona et herchi [4] added that *Pseudomonas aeruginosa* is present in environment. For this reason, antibiotics extender's cannot protect semen from this bacterium, which explains in our study the increase of percentage of contaminated ejaculates with *Pseudomonas aeruginosa*.

In conclusion, this study showed that we could decrease bacteria flora concentration after semen dilution extender containing antibiotics Penicillin and Gentamicin and decreased furthermore this concentration after freezing - thawing semen. Two potentially pathogen bacteria founded in fresh semen. Unfortunately, one of them, *Pseudomonas aeruginosa*, revealed resistant to the effect of antibiotics Penicillin and Gentamicin and to the effect of freezing semen. In conclusion, it seems that a bacteriological control is necessary to know bacteria flora associated with fresh, diluted and frozen - thawed semen used in artificial insemination program, in order to explain some problem of fertility in mares.

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