Original paper

THE POSSIBILITY OF CONTAMINATION OF DEEP FROZEN BULL SEMEN DURING LONG PERIODS OF STORAGE IN CONTAINERS WITH LIQUID NITROGEN

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Abstract

Preservation of bull sperm by deep freeze is a technological process that allows you to store semen in theory for an unlimited period of time, national and international transport, without significant loss of quality and safety of semen. According to the literature, some microorganisms successfully survive the low temperatures during storage of semen in liquid nitrogen (-196 ° C), in deep-frozen semen, seeds, and in liquid nitrogen and ice sediment in the storage container for deep-frozen bull semen. The aim of this study was to do microbiological analysis of samples semen frozen bull and liquid nitrogen in containers for bull semen storage. 414 samples of frozen bull semen, and 53 samples of liquid nitrogen ice sediment were examined. From the deeply frozen semen were isolated *Candida albicans, Citrobacter freundii and Pseudomonas stutzeri*. Microorganisms isolated from the storage container of frozen semen are mostly members of the family *Enterobacteriacae*, and *Citrobacter freundii* was isolated from the largest number of samples. Considering the findings of microorganisms in the semen, and liquid nitrogen, there is the possibility of connection of contamination of the semen with microorganisms of the liquid nitrogen, and reversely, as would be the goal of our future investigations.

Key words: *Citrobacter freundi, Candida albicans, deep frozen bull semen, liquid nitrogen*

Introduction

For the production and distribution of quality bull semen in artificial insemination the bulls that have passed the relevant tests are used. The bulls must be disease free, and their semen is collected and processed according to standard protocols. However, the microorganisms are present in most of the semen samples, in a smaller or larger number. These organisms are opportunistic pathogens of low importance for causing infection. Nevertheless, even such microflora can lead to infection (OIE Appendix 3.2.1.), under the conditions where the immune system of the body is compromised. According to literature data, the individual microorganisms can successfully survive the low-temperature storage of semen in liquid nitrogen (-196 ° C), both in semen straws, as well as in liquid nitrogen and ice sediment in the containers for storage of bull frozen semen. According to the "Regulations on the manner of sperm marking, keeping records of the production of sperm, and the conditions that must be

met in terms of semen quality of the Republic of Serbia" Article 5, the number of microorganisms after thawing of frozen semen should not exceed 500 cfu / ml. Vakanjac et al. (2013) isolated samples of *Candida albicans* in 9 and *Citrobacter Freundi* in 5 of 351 samples of deep frozen bull semen after thawing. Abro et al (2009) examined 100 samples of frozen bull semen and isolated seven different bacterial species (*Acinetobacter, Actinobacillus lignieisis, Citrobacter, Micrоccus luteus, Pseudomonas auruginosa, Staphylococcus epidermidis* and *Staphylococcus intermedius*). Bielanski et al. (2003) in their work indicated that they isolated 13 different bacterial species from deep-frozen bull semen which were stored in liquid nitrogen from 6 to 35 years. The same author also states that in 69% of samples of the liquid nitrogen, 62% of samples of the deep frozen semen and 32% of the samples of embryos the microorganisms were isolated, and in 14 (35%) of the samples is isolated from any microorganism of the sample. Bielanski (2012) states that the viruses are isolated from the liquid nitrogen, but a larger number of ubiquitous microorganisms were isolated from the liquid nitrogen and the ice sediment. Morris (1999) in his paper states that he isolated 100 cfu anaerobic microorganisms and aerobic microorganisms 10 cfu per 10 kg of liquid nitrogen. Radnoti and Farkas (1966) isolated 1 cfu of microorganisms on 5-10 ml of liquid nitrogen. Piasecka-Seraline (1972) describes in his work that there were 94% samples of frozen semen showing bacterial contamination after freezing and storage in liquid nitrogen, which was experimentally contaminated with *Escherichia coli* and *Staphylococcus aureus*. Nedić et al. (2013) in total of 35 samples of liquid nitrogen confirmed the presence of microorganisms in 21 samples, and identified *Citrobacter freundi, Klebsiella oxitoca, Acinetobacter braumannii, Acinetobacter Iwoffi, Pseudomonas stutzeri, Citrobacter diversus, Citrobacter koseri, Proteus mirabillis* and *Aspergillus sp*.

Very extensive testing possibilities of cross-contamination of samples and liquid nitrogen are described by Bielanski (2005). In his work he states three experiments, the first experiment, 15 ml of bacterial culture and 10 ml of viral culture poured into liquid nitrogen in which bovine embryos are stored, in the second experiment, samples of bovine semen and embryos exposed to viral and bacterial culture prior to freezing, and in the third experiment 1 ml of the bacterial and viral culture is frozen and stored, and wherein the same container and the sample of bovine embryos and semen. The results of this study show that there is no cross-contamination of samples in one of these three experiments if they were frozen 7 days in the tested container.

Materials and methods

A total of 414 samples of frozen bull semen and 53 samples of ice sediment of liquid nitrogen were checked. After dissolution in the water bath at 37 ° C, with a deletion of straws 70% alcohol, and careful trimming of the top, straws were seeded in medium (blood agar supplemented with 10% sheep blood, Mac Conkey agar, and Sabouraud dextrose agar). Samples of liquid nitrogen were taken with sterile swabs, dip in bowl filled with liquid nitrogen, in which frozen bull semen straws are stored. After enrichment in nutrient broth (24 hours at 37 ° C) samples were streaked onto solid medium (blood agar supplemented with 10% sheep blood, Mac Conkey agar, and Sabouraud dextrose agar). All plates were incubated for 24-48h at 37°C. Bacterial strains obtained in pure cultures were examined on the basis of morphological, cultural and biochemical characteristics. As a test to confirm, in the process of identification Microgen ID (Camberley, UK) was applied.

Results and discussion

Results of 414 samples of frozen bull semen show that *Candida albicans* was isolated in pure culture in nine samples, and *Citrobacter freundi* in 5 samples. We isolated two types of microorganisms in samples of deep frozen bull semen, in contrast to Bielanski et al. (2003) who in his work isolated as many as 13 different bacterial species. Abro et al. (2009) who examined 100 samples of frozen bull semen isolated seven different bacterial species. Although the temperature of liquid nitrogen is -196 °C it is considered that contamination of the samples stored in liquid nitrogen is possible when the container is not properly sealed.

Piasecka-Serati in 1972 was first to describe contamination of deep frozen bull semen in liquid nitrogen, which is experimentally contaminated with *Escherichia coli* and *Staphylococcus aureus.* From a total of 53 samples of ice sediment of liquid nitrogen that we examined in our study, organisms were isolated in 28 samples. *Citrobacter freundi* were isolated in 9 samples, *Pseudomonas aeruginosa* and *Klebsiella oxitoca* in 2 samples, *Acinetobacter iwoffi* in 3 samples ,*Pseudomonas stutzeri* in 4 samples, *Staphylococcus saprophyticus, Citrobacter diversus, Klepsiella ozaenae, Proteus mirabillis* and *Aspergillus sp*. in one sample, Candida albicans in 3 samples. High levels of liquid nitrogen sediment contamination, of the container for storage of human embryos are described by Morris (2005). The author states that the bacteria are isolated from all of the samples, the mold in 9 of the 10 samples, wherein *Acinetobacter baumannii, Micrococcus sp. Chrysenomonas luteola, Klepsiella oxytoca, Sphinogobacterium spiritivorum, Weaksella virosa* and nonhemolytic streptococci were identified. A large number of different microorganisms isolated from liquid nitrogen (*Staphylococcus auricularis, Bacillus sp, Alcaligensis faecalis, Stenotrophomonas maltophilia,* etc.). Bielanski got in his work (2003). Fountain et al. (1997) reported the isolation of *Aspergillus sp, Penicillium sp, Paecilomyces*, α hemolytic *Staphylococcus, Bacillus sp, Corynobacterium* and coagulase-negative *staphylococci* in liquid nitrogen for storage of hematopoietic stem cells.

Conclusion

Although there is no conclusive evidence about the possibility of transmission of microorganisms from a container of liquid nitrogen in the samples of stored deep frozen bull semen, it is still necessary to implement measures to ensure that contamination of liquid nitrogen and samples is reduced to a minimum. Containers in which deep-frozen bull semen are kept, should be new or previously thoroughly cleaned and disinfected, filled with new liquid nitrogen and sealed under veterinary supervision. Containers must be annually emptied, cleaned and disinfected to reduce possible contamination of liquid nitrogen and stored samples.

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