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Microbial quality of equine frozen semen

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ABSTRACT

Bacteriological surveillance is little applied in management of equine frozen semen but it is quite important to verify the microbial contamination in order to find out the chance of transmission of pathology to the mare in AI. Authors describe a qualitative and quantitative analysis for bacterial contamination on long time (3–17 years) equine frozen semen stored in liquid nitrogen. The semen checked, produced in Italy and in another Europe country, was cryopreserved in liquid nitrogen inside sealed plastic straws. One hundred and ten straws were checked out for pathogenic and no pathogenic bacteria, aerobes and anaerobes and fungi (moulds and yeasts). The Total Microbial Charge was quite variable with an average of about 1.4×10^5 CFU/ml. Mostly the microbial agents identified were fungi (17.5%), *Enterobacter-coccus* spp. (15%), *Pseudomonas* spp. (6.25%), *Stenothophomonas maltophila* (6.25%) and anaerobic bacteria like *Propionibacterium granulosum* (7.5%) and *Clostridium* spp. (3.75%). 3.75% were unidentified Gram-negative rod and cocci. *Streptococcus* spp., *Staph. aureus*, *E. coli*, *Th. equigenitalis* and *Mycoplasma* spp. were not detected. The most represented species were *Enterobacter-coccus* spp. (1.1×10^5 CFU/ml), *St. maltophila* (8×10^4 CFU/ml) and *Pr. granulosum* (7×10^4 CFU/ml) while yeast and even more moulds were little abundant (4.7×10^4 and 3.4×10^4 CFU/ml respectively). The knowledge of equine frozen semen microbial quality is essential to check out transmission of venereal disease and improve the quality of cryopreserved germplasm.

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1. Introduction

Nowadays, in horse industry, the stallion semen cryopreservation technique has acquired great importance (Reger et al., 2003) and frozen semen is more and more employed (Loomis, 2001) in artificial insemination (AI). However the protocols proposed for its production are various (Graham, 1996; Devireddy et al., 2002) and not yet standardized (Alvarenga et al., 2005). Usually frozen semen is produced near equine reproduction centres where it is stored in small containers: 0.25 or mostly 0.5 ml straws filled with about 240×10^6 progressively motile spermatozoa showing not less than 30% progressive motility (Vidament et al., 1997; Barbacini et al., 1999; Loomis, 2001) and then it is plunged in liquid nitrogen (LN) for an indefinite time. When needed for AI the frozen semen dose is thawed and it is always assessed for kinetic parameters by a computer-assisted semen analyser (CASA) to value the hypothetic fertility (Varner et al., 1991). The bacteriological controls on frozen semen and also on fresh and cooled semen, are little mentioned in bibliography (Clément et al., 1993, 1995; Pickett et al., 1999; Corona et al., 2006) and overall are little applied in handling of equine frozen semen (Loomis et al., 2005). The stallion penis and prepuce are inhabited by a great variety of commensal bacteria or/and potentially pathogenic bacteria that contaminate sperm at ejaculation (Bristol, 1991; Tischner and Kosiniak, 1992; Varner et al., 1998; Pickett et al., 1999). They may be cultured from an ejaculated and from frozen–thawed semen. Opportunistic bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Streptococcus zooepidemicus*, *Taylorella equigenitalis*, if carried with AI, may cause infection and improve inflammation leading to infertility in susceptible mares (Parlevliet, 1997; Metcalf, 2001). Moreover, when semen for AI is referred for the international transportation of equine frozen, the control of microbial contamination acquire greater importance for the sanitary consequences (Metcalf, 2001). An extender with some added antibiotics is recommended to reduce the pollution or eliminate bacterial contamination during the preparation of the frozen semen. Different antimicrobial agents have been tested (Squires and McGlothlin, 1980; Arriola and Foote, 1982; Jasko et al., 1993; Varner et al., 1998) but few researches have demonstrated their effects on bacterial growth in extended semen and even more in the frozen one. Some authors put in evidence that none of the antibiotics tested were completely effective against bacterial growth in semen samples (Varner et al., 1998; Pickett et al., 1999). In frozen semen this problem is still underrated not only because of the difficulty of bacterial isolation but also because it is assessed that microbial and viral pathogens may survive in liquid nitrogen for a long time (Rall, 2003) For instance the agent of contagious equine metritis (CEM) is quite resistant in cryopreservation technique in LN (Guérin, 1992). For all these reasons it is very important to know which microflora is associated with germplasm cryopreservation that has been the least investigated one in equine medicine (Clément et al., 1993). However it is really important (Metcalf, 2001; Bielanski et al., 2003; Levy et al., 2004) because the frozen semen can be shipped and several exchanges are carried out in the world.

The purpose of this paper is to report the current state of knowledge on bacterial contaminations of equine frozen semen banked in liquid nitrogen for long time. Special attention will be given to the Total Microbial Charge (TMC) and to the microbial species contaminates (bacteria and fungi) in the frozen–thawed semen.

2. Materials and methods

2.1. Material

Semen was collected from 11 stallions (S1–S11) of age 6–16 years and then frozen in some equine reproduction stations in Italy (S1–S7) and in another European country (S8–S11). Afterwards it was automatically packaged in 0.5 ml straws (0.5 cm^3 semen straws, Minitüb), dipped in liquid nitrogen inside drums and stored from 3 to 17 years (1990–2004). Two straws of each stallion were tested 5 times (2×5) for a total of 110 straws (10×11). All samples were thawed in +37 °C water bath for 30 s.

2.2. Bacterial concentration determination

This calculus was carried out under sterile conditions: 1 ml of frozen–thawed semen was diluted 1:10 with buffered peptone sterile water, 100 μl from this suspension were surface plated on brain