Risk of *Salmonella* transmission via cryopreserved semen in turkey flocks

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ABSTRACT To investigate the possibility to carry pathogen bacteria in turkey flocks via cryopreserved semen, research was carried out 1) to investigate the microbial contamination of fresh and frozen thawed turkey semen and 2) to evaluate the effect of the freezing-thawing process on the survival of 3 serovars of *Salmonella* spp. experimentally inoculated in turkey semen. Five pools of semen diluted 4-fold were cooled, added with 8% of dimethylacetamide as a cryoprotectant, and aliquots of 80 μL were directly plunged into liquid nitrogen to form frozen pellets. Mesophilic viable counts, total and fecal coliforms, *Enterobacteriaceae*, enterococci, *Campylobacter* spp., and *Salmonella* spp. were investigated on fresh and thawed samples. Further, 5 pools of diluted semen were each divided into 3 subsamples, inoculated with 7.8 ± 0.2 log cfu·mL^{-1} of *Salmonella* Liverpool, *Salmonella* Montevideo, and *Salmonella* Braenderup, respectively, and cryopreserved before to assess the postthaw viability of *Salmonella* spp. strains. Fresh semen was highly contaminated by all of the saprophytic bacteria investigated and the cryopreservation process reduced the amount of mesophilic viable count and total coliforms ($P < 0.05$) and fecal coliforms, *Enterobacteriaceae*, and enterococci ($P < 0.01$) by about 1 log cfu·mL^{-1}. Conversely, neither *Campylobacter* spp. nor *Salmonella* spp. were found as endogenous bacteria in semen. In the inoculated semen, both *Salmonella* Liverpool, *Salmonella* Montevideo, and *Salmonella* Braenderup colonies were recovered postthaw, showing a significant reduction of 2.03 ± 0.28, 3.08 ± 0.22, and 2.72 ± 0.23 log cfu·mL^{-1}, respectively, compared with the fresh semen ($P < 0.001$). In conclusion, the cryopreservation process allowed us to obtain a low reduction of microbial count both in endogenous saprophytic bacteria and artificially inoculated *Salmonella* spp. strains; therefore, the possibility of *Samonella* spp. transmission to flocks through the use of infected cryopreserved semen does exist.

Key words: turkey, spermatozoa, cryopreservation, *Salmonella* species, microbial contamination

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INTRODUCTION

Turkeys are the only commercial livestock species completely dependent upon artificial insemination (**AI**) for fertile egg production because the difference in size between males and females caused by the advanced genetic selection makes it impossible for the turkeys to mate naturally. In the last few years, the dependence of the turkey industry on AI has prompted the search for technologies and methods to store the semen in frozen form. The use of frozen-thawed semen for AI could have practical advantages not only for the commercial breeding industry but also for cryobanking programs interested in maintaining and ensuring the long-term conservation of the genetic diversity of turkey breeds. However, irreversible damage seems to occur to the turkey spermatozoa during the freezing-thawing process; therefore, the on-field results with the use of cryopreserved turkey semen are unpredictable and generally do not allow satisfactory levels of fertility to be achieved (Long, 2006).

The most encouraging results in terms of fertility rates after AI with frozen-thawed avian semen have been obtained with rapid cooling of semen with dimethylacetamide (**DMA**) as a cryoprotectant and pellet packaging (Tselutin et al., 1995, 1999; Blesbois and Labbé, 2003). This method, coupled with straw packaging, was chosen as a reference for avian gene bank preservation programs in France (Blesbois and Labbé, 2003) and in the Netherlands (Woelders et al., 2006).

Nevertheless, little attention has been paid to investigate the level of security in sanitary requirements using cryopreserved turkey semen. Avian semen is not sterile but rather colonized at low levels by a variety of microorganisms. This occurs because bird anatomy is such that semen is exposed to the cloacal contents,

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providing ample opportunity for its contamination by saprophytic or pathogenous, or both, bacteria coming from the gastroenteric tract.

The endogenous intestinal microflora of turkeys, recovered also in semen, is composed principally by *Escherichia coli*, *Lactobacillus* spp., *Streptococcus* spp, *Enterococcus* spp., *Enterobacteriacae*, micrococci, Clostridia (Scupham et al., 2008), as well as by human pathogens such as *Salmonella* spp. or *Campylobacter* spp. (Donoghue et al., 2004). High amounts of saprophytic microorganisms such as coliforms, *Enterobacteriaceae*, and enterococci may compete with spermatozoa for the nutrients present in seminal plasma or in extenders, or both, especially during the semen handling before insemination, decreasing the semen quality and consequently also the fertility of hens (Boone and Hughes, 1970). In regard to pathogenic bacteria such as *Salmonella* spp., they may cause illness to humans through consumption of contaminated turkey meat (Hafez and Jodas, 2000). *Salmonella* spp. is the second most common cause of bacterial foodborne diseases in humans, and poultry and turkey products are implicated as a major source of human foodborne salmonellosis (Rostagno et al., 2006). For these reasons, the safe storage of avian germplasm and the consequent prevention of disease transmission between animals and from animals to humans via the food chain are of concern to both avian germplasm cryobanking programs and the avian breeding industry.

Recently, increased attention has been paid to investigate the possibility of avian infection through infected semen, particularly in turkeys. In this species, in fact, semen is routinely pooled and used to inseminate multiple hens and bacteria could be spread from semen throughout entire flocks via AI. Previous studies showed the possibility of spreading *Salmonella* spp. to hens, eggs, and chicks via contaminated turkey or rooster semen (Cole et al., 2004; Donoghue et al., 2004; Vizzier-Thaxton et al., 2006). However, to the best of our knowledge, no data on the effect of the freezing-thawing process on postthaw turkey semen contamination are available, as well as on the possibility of disease transmission through the use of cryopreserved semen.

This work had 2 main objectives: to investigate, quantitatively and qualitatively, the bacterial contamination of turkey semen before and after freezing-thawing and to evaluate the effect of cryopreservation on postthaw survival of *Salmonella* Liverpool, *Salmonella* Montevideo, and *Salmonella* Braenderup artificially inoculated in turkey semen.

MATERIALS AND METHODS

Chemicals

The chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). The Hektoen enteric agar was purchased from Biolife (Biolife S.r.l., Milan, Italy). All other media for culturing bacteria were purchased from Oxoyd (Oxoyd S.p.A., Rodano, Milan, Italy).

Birds and Semen Treatment

Mature turkey males from a hybrid large white strain reared under standard conditions in a private breeding (Azienda Agricola Santo Stefano, Castellalto-Canzano, Teramo, Italy) were used. The semen was collected via a dorso-abdominal massage technique from 42 toms and pooled (4 ejaculates/pool). A total of 10 pooled semen samples were used and they were each diluted 1:4 with the Tselutin extender (Na glutamate, 128 mmol·L^{-1}; K_2HPO_4, 20 mmol·L^{-1}; glucose, 44.4 mmol·L^{-1}; inositol, 11.1 mmol·L^{-1}; Mg acetate, 7 m*M*, mmol·L^{-1}; glycine, 13.3 mmol·L^{-1}; glutamic acid, 7.68 mmol·L^{-1}; pH 6.65).

Microbiological Analysis of Fresh and Frozen-Thawed Turkey Semen

Five of the 10 pools of diluted semen were assigned to study the microbial contamination of fresh turkey semen and following the freezing-thawing process. A part of each of these 5 pools of diluted semen was immediately used for the microbiological analysis on fresh semen, as described below, whereas the remaining part was cooled for 60 min at 5°C and then added with 8% (vol/vol) of DMA as cryoprotectant. After 5 min of equilibration with DMA, aliquots of 80 μL were directly plunged into a liquid nitrogen bath to form frozen pellets. The formed pellets were caught with sterilized pliers and immediately put in cooled sterile cryovials (from 5 to 7 pellets in a 1.5-mL cryovial) to avoid microbial cross-contamination between different pools of semen. The cryovials were put in a cryobox and stored in a liquid nitrogen tank for 2 mo before the microbiological analysis on thawed semen. At the moment of the analysis, thawing was quickly performed at 75°C, laying down the lower part of the cryovials in a water bath for 12 to 15 s.

From both fresh and frozen-thawed samples, 1 mL of semen was taken with sterile tips and put into a sterile test tube together with 9 mL of sterile saline solution (0.9% NaCl). This suspension was used to obtain a 10-fold serial dilution used for microbial counts.

Mesophilic bacteria were counted on plate count agar after 48 h at 28°C. *Enterobacteriaceae* were enumerated on violet red bile glucose agar after an incubation of 36 h at 37°C. Purple colonies, 1 to 2 mm in diameter, surrounded by a purple halo, were assumed to be *Enterobacteriaceae*. Total and fecal coliforms were counted on violet red bile agar after 36 h at 37 and 44°C, respectively. Enterococci were counted on Slanetz and Bartley medium after an incubation of 36 h at

37°C. *Campylobacter* spp. were determined on Campy medium, after 48 h at 35°C in a jar containing an AnaeroPack Campylo sachet (Mitsubishi Gas Chemical America Inc., New York, NY), necessary to provide the microaerophilic conditions for *Campylobacter* spp. growth. Viable counts of *Salmonella* spp. cells were determined by superficial inoculation on Hektoen enteric agar as described below.

Salmonella spp. Recovery in the In Vitro Inoculated and Frozen-Thawed Turkey Semen

The remaining 5 pools of diluted semen were assigned to be challenged in vitro with exogenous *Salmonella* spp. to evaluate the effect of cryopreservation on *Salmonella* spp. recovery after thawing. Each pool of diluted semen sample was divided in 3 subsamples. Suspensions of known concentrations of *Salmonella* Liverpool, *Salmonella* Montevideo, and *Salmonella* Braenderup, 3 emerging serovar of *Salmonella enterica* ssp. *enterica*, were individually used to respectively contaminate the 3 subsamples of semen in a concentration of about 7.8 $\pm$ 0.2 log cfu/mL of diluted semen (log cfu·mL^{-1}). Successively, the subsamples were cooled, added with the cryoprotectant, equilibrated, frozen, stored, and thawed as described before.

Viable counts of *Salmonella* spp. were determined by superficial inoculation on Hektoen enteric agar (Oxoid, Basingstoke, Hampshire, UK). Exactly 0.1 mL of each serial dilution was placed and spread onto an agar plate using a glass spreader. The plates remained for some minutes in the same position before they were incubated at 37°C for 24 h. Colonies developed on Hektoen enteric agar were recognized as described by Feldsine et al. (2003) and counted.

Microbial Count

All analyses were carried out in duplicate. Colonies grown on the specific agar were recognized and counted. Colonies were enumerated at the dilution producing at least 30 to a maximum of 300 colonies. The total colony-forming units of each microbiological group per milliliter of semen were obtained by multiplying the number of colonies by the dilution factor.

Statistical Analysis

Statistics were done with SPSS (SPSS 14.0 for Windows, SPSS, Chicago, IL). The mean prefreeze and postthaw bacterial counts for the microbiological groups investigated in semen were compared by a *t*-test. Data were $\log_{10}$-transformed for statistical analysis. A 2-tailed *P*-value less than 0.05 ($P < 0.05$) was regarded as significant. The results are presented as means of $\log_{10}$ cfu·mL^{-1} $\pm$ SEM.

RESULTS

Microbiological Analysis in Fresh and Frozen-Thawed Turkey Semen

Fresh semen was highly contaminated by commensal bacteria and the cryopreservation process caused a reduction in microbial counts (Figure 1), passing from 5.22 $\pm$ 0.66 and 4.35 $\pm$ 0.79 to 4.32 $\pm$ 0.58 and 3.32 $\pm$ 0.4 for mesophilic viable counts and total coliforms ($P < 0.05$), respectively, and from 4.07 $\pm$ 0.22, 4.21 $\pm$ 0.53, and 4.26 $\pm$ 0.08 to 3.18 $\pm$ 0.52, 3.19 $\pm$ 0.37, and 3.67 $\pm$ 0.08 in fecal coliforms, *Enterobacteriaceae*, and enterococci ($P < 0.01$), respectively. Conversely, neither *Campylobacter* spp. nor *Salmonella* spp. were found in both fresh and cryopreserved samples.

Salmonella spp. Recovery in the In Vitro Inoculated and Frozen-Thawed Turkey Semen

As shown in Figure 2, in the in vitro challenged semen, *Salmonella* Liverpool, *Salmonella* Montevideo, and *Salmonella* Braenderup colonies were recovered in thawed samples.They showed a significant reduction of 2.03 $\pm$ 0.28, 3.08 $\pm$ 0.22, and 2.72 $\pm$ 0.23 log cfu·mL^{-1}, respectively, with respect to the fresh semen ($P < 0.001$).

DISCUSSION

Our research showed that the cryopreservation process was able to reduce but not eliminate either the amount of endogenous saprophytic bacteria or *Salmonella* spp. strains artificially inoculated in turkey semen. The gastroenteric tract of birds is normally inhabited by a variety of commensal and saprophytic bacteria, most of which are regarded as nonpathogenic. The particular bird anatomy is such that the cloaca is the common opening for the urinary, reproductive, and gastrointestinal tract of avian, so the external openings of the vas deferens are close to ureter and coprodeum outlets. In this way, the semen, coming from vas deferens, is predisposed to fecal contamination by bacteria coming from the gastroenteric tract, as shown by the results of the microbiological analysis on fresh semen, in which high amounts of total and fecal coliforms, *Enterobacteriaceae*, and enterococci were found. Even if such microorganisms are considered as nonpathogenic for animals, a high amount of these bacteria may have a negative effect on semen quality and potential fertility of hens (Boone and Hughes, 1970). In investigations on human and stallion semen (Villegas et al., 2005; Ortega-Ferrusola et al., 2009), the bacterial contamination of the ejaculates was also considered responsible for the lowered life span of spermatozoa postthaw. It is well known that the microbial amount of semen may

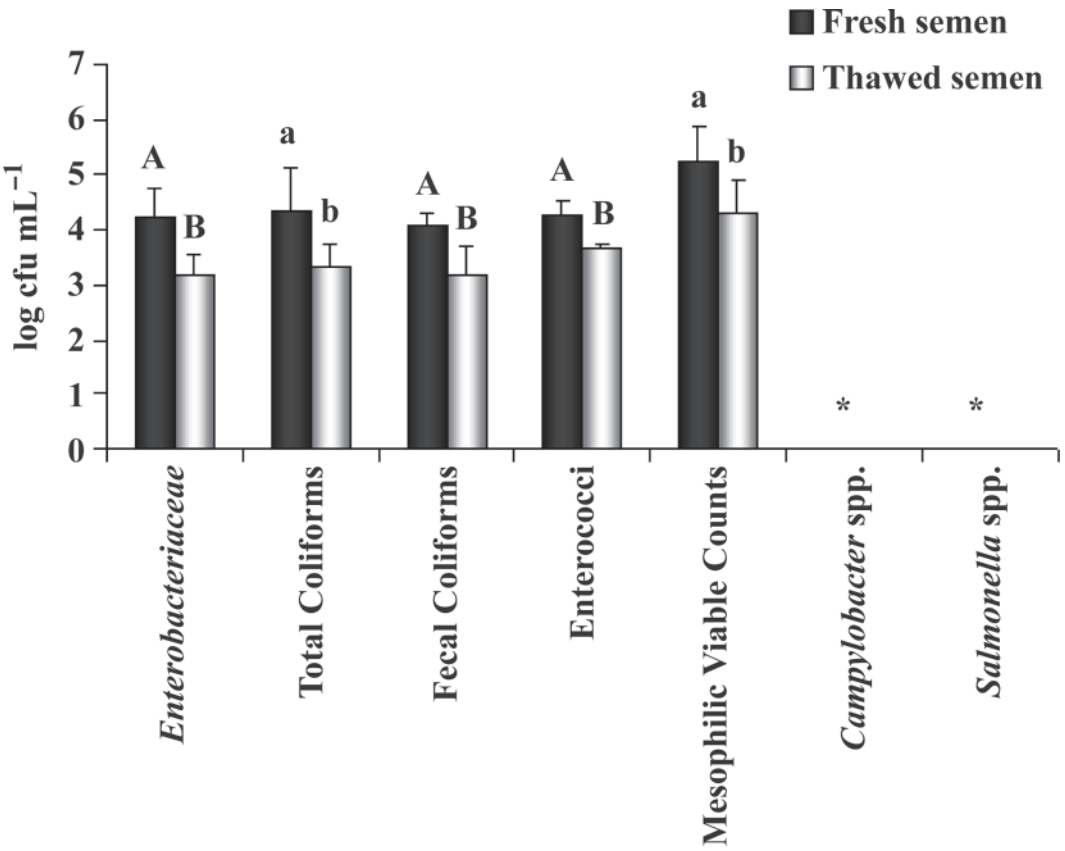

Figure 1. Comparison between the microbial content of fresh and frozen-thawed turkey semen. Different letters within columns indicate significant differences. Capital letters differ for $P \leq 0.01$; lowercase letters differ for $P \leq 0.05$. An asterisk indicates that microbial count was undetectable in 1 mL of turkey semen.

be controlled, after semen collection, by adding antibiotic to semen extenders; however, in turkey semen, it has been shown that the common antibiotic in semen extenders either did not reduce the *Campylobacter* spp. and *Salmonella* spp. amount or reduced but did not eliminate these bacteria from semen (Donoghue et al., 2004). Moreover, in avian and other livestock species, it has been shown that the addition of antibiotics to

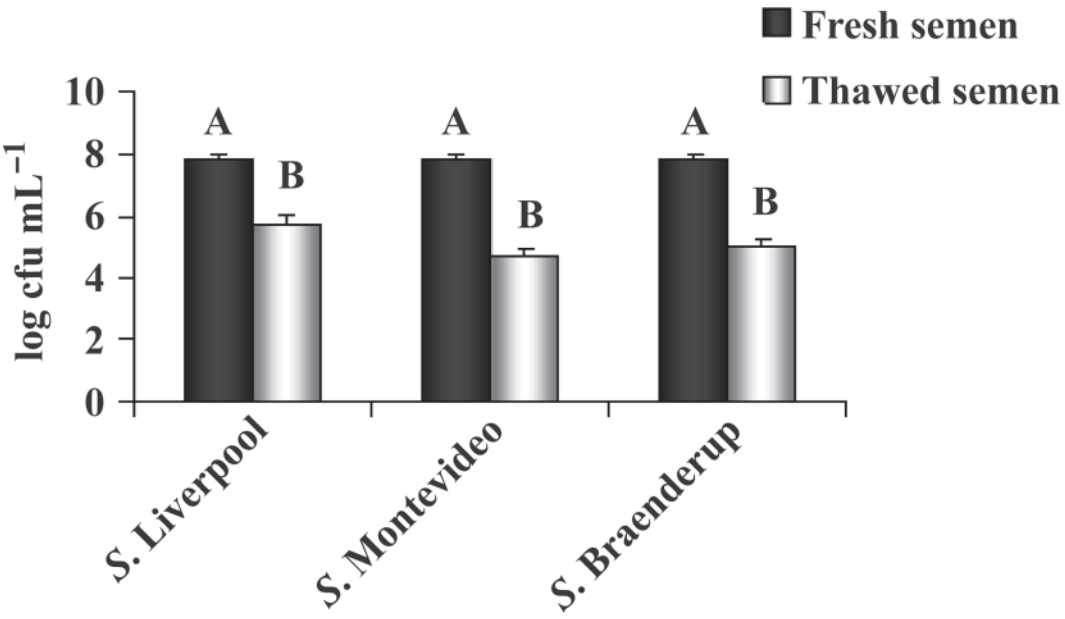

Figure 2. Comparison between the content of artificially inoculated *Salmonella* Liverpool, *Salmonella* Montevideo, and *Salmonella* Braenderup in fresh and frozen-thawed turkey semen. Different letters within columns indicate significant differences ($P \leq 0.001$). S = *Salmonella*.

semen extender may also cause detrimental effects on spermatozoa (Sexton et al., 1980; Aurich and Spergser, 2006; Akhter et al., 2008).

However, the most interesting point stressed in this study is the possibility to vehiculate *Salmonella* spp. via cryopreserved semen. If turkey semen is contaminated, the freeze-thawing process is inefficient to contrast the presence of *Salmonella* spp. In this way, the use of infectious cryopreserved semen may serve as a vehicle for the spread of *Salmonella* spp. from males to the hens via AI. In avian, *Salmonella* spp. usually cause asymptomatic infection and infected turkey breeders became reservoirs and excreters of these bacteria. In fact, the possibility exists that hens vertically transmit *Salmonella* spp. to progeny by the production of contaminated eggs that will hatch infected chicks (Reiber and Conner, 1995; Reiber et al., 1995a,b; Cole et al., 2004; Donoghue et al., 2004). Once chicks grow, they will produce contaminated meat (Arsenault et al., 2007); in fact, during slaughter and processing, *Salmonella* spp. from the gastrointestinal tract of carrier birds can contaminate turkey carcasses and the slaughter and processing line (Rostagno et al., 2006) with a consequent cross-contamination of meat (Nde et al., 2007).

In our study, the turkey semen was negative for *Salmonella* spp. presence, and we were able to perform the second part of the research recurring to an experimental challenging of semen. However, the presence of pathogens such as *Salmonella* spp. and *Campylobacter* spp. as endogenous microbiota in turkey and poultry semen from commercial breeder farms has been found (Donoghue et al., 2004).

Even if in our study, after the freezing and thawing process, there was a significant reduction both in all of the microbiological groups detected in fresh semen and in vitro inoculated *Salmonella* spp., the majority of microorganisms survived in thawed turkey semen samples. Investigations on human semen related to the risk of semen samples cross-contamination during cryostorage (Bielanski et al., 2003; Mazzilli et al., 2006) or sexually transmitted diseases (Garcia et al., 1981; Glander et al., 1986) have also shown a high survival of microorganisms during cryostorage procedures.

It is known that the cryopreservation of biological cells and tissue (i.e., gametic cells) is a complex process in which damage of cells occurs. Spermatic cells, particularly those from avian, are particularly sensitive to the stresses imposed by the freezing and thawing (Long, 2006; Iaffaldano et al., 2009). The sperm cells cryoinjuries are a consequence of intracellular or extracellular ice crystal formation, osmotic imbalance during the passage to isotonic to hypertonic condition induced by the addition of the cryoprotectant, osmotic swelling after thawing, changes in lipid phase transition and increased lipid peroxidation, alterations in membrane carbohydrate content, and adenosine triphosphate generation (Massip et al., 2004; Long, 2006).

Even bacteria cells are sensitive to this stress (Mazur, 1966). This was demonstrated by the lower microbial content found in thawed semen samples in respect to the microbial content found in the fresh semen samples; however, all of the bacterial groups detected in fresh semen were also found after thawing.

The probable explanation of these results is that the steps of the cryopreservation process that intended to successfully preserve the sperm cells by cold shock were also able to protect the bacteria cells (Hubálek, 2003). Bacteria cells are usually maintained as frozen cultures after fast-freezing protocols. It is well known that cryopreservation of bacteria at temperatures of −196°C in liquid nitrogen is a reliable method for their long-term storage (Hubálek, 2003). In this way, the cooling of semen, the cryoprotectant used, and the rapid freezing technique chosen in this study for cryopreserving turkey spermatozoa were also effective for the long-term culture maintenance of the microorganisms present in semen, keeping the microbial cells in a non-growth-promoting stable condition. Even if cryostorage reduces the concentration of detectable bacteria in turkey semen immediately after thawing, a few viable microorganisms are able to increase quickly if bacteria, originating from the turkey intestinal environment, can pass, via AI of thawed semen, to a favorable environmental condition of growth, such that of a hen's reproductive tract.

In conclusion, cryopreservation by the pellet method allows a reduction but not an elimination of endogenous saprophytic bacteria or artificially inoculated *Salmonella* spp. strains in turkey semen. Therefore, the use of cryopreserved semen may represent a serious risk of disease transmission in vivo by AI. For this reason, we recommend the microbiological analysis of fresh turkey semen to control the absence of pathogen bacteria in semen before sperm cryopreservation.

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