Short communication: Effect of freezer storage time and thawing method on the recovery of *Mycoplasma bovis* from bovine colostrum

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ABSTRACT

Mycoplasma bovis is an important cause of mastitis in dairy cattle, and pneumonia, arthritis, and otitis in calves. Milk and colostrum are considered important sources of infection for calves. Knowledge on the effect of on-farm freezing (-18°C) and thawing methods on the recovery of *M. bovis* from colostrum samples is missing. In this study, 2 separate experiments were performed. The first experiment consisted of a longitudinal study examining the survival [as measured by $\log(10)$ reduction] of 2 *M. bovis* strains in frozen colostrum over 14 wk. The second experiment examined the effect of different thawing temperatures (45 and 20°C), thawing frequencies (once or twice), and initial colostrum titer (10^4 or 10^6 cfu/mL) on *M. bovis* survival. A single freeze-thaw cycle led to an approximate 1 log reduction of *M. bovis* titer, independent of the thawing temperature. Freezing for 14 wk did not significantly further reduce the titer of bacteria compared with freezing for 2 wk. A second freeze-thaw cycle further reduced the *M. bovis* count by approximately 0.5 log compared with a single freeze-thaw cycle. Thawing temperature and initial bacterial concentration did not significantly affect *M. bovis* reduction. In conclusion, storage of colostrum samples in the freezer at -18°C during epidemiological studies, herd monitoring, or test and cull programs will probably have little influence on qualitative bacteriological test results for *M. bovis*. The epidemiological or clinical relevance of an approximate 1 log reduction of *M. bovis* in colostrum is currently unclear.

Key words: *Mycoplasma bovis*, survival, freezing, colostrum

Short Communication

Mycoplasma bovis has been recognized as an important cause of untreatable mastitis in adult cattle and

chronic, unresponsive pneumonia in calves, frequently complicated by arthritis and otitis (Maunsell and Donovan, 2009; Maunsell et al., 2011). Annual losses due to *M. bovis* have been estimated to be above 4140 million in the United States (Rosengarten and Citti, 1999). It is generally accepted that the most important route of *M. bovis* introduction onto a farm consists of purchase of a carrier (Fox, 2012). Sperm can be infectious (Pfützner and Sachse, 1996), and recent work suggests the possibility of between herd spread through fomites or persons (Gille et al., 2016).

Known routes of transmission at individual animal level are the milking process, direct nasal contact, semen, and the consumption of infected milk (Pfützner and Sachse, 1996; Fox et al., 2005). For calves, in particular, consumption of infected milk is believed to be the primary route of infection (Walz et al., 1997; Butler et al., 2000; Maunsell and Donovan, 2009; Maunsell et al., 2012). Relatively few studies have documented the within-herd prevalence of *M. bovis* in milk samples. In a recent Swiss study, the within-herd prevalence of *M. bovis*-shedding cows was 2.4% (95% CI = 1.5–3.8) in milk by PCR assay (Aebi et al., 2015). In other studies, a within-herd prevalence between 2.6 and 43.0% has been reported (Fox, 2012).

Colostrum has been mentioned as a possible source of infection, but the prevalence of *M. bovis* in colostrum samples is currently unknown (Godden et al., 2006). Preliminary PCR testing of colostrum samples at the Belgian center for milk quality control (MCC-Vlaanderen, Lier, Belgium) did identify some positive samples (K. Supré, Milk Control Centre Flanders, Lier, Belgium, personal communication). The presence of *M. bovis* in colostrum is currently unaccounted for in *M. bovis*-preventive protocols. Indeed, the commonly recommended individual housing for 8 wk without nose-nose contact, together with replacing cow milk by milk replacer, might have limited efficacy to reduce mycoplasma infection if *M. bovis*-positive colostrum has been given previously (Maunsell and Donovan, 2009). Colostrum samples are often stored frozen before analysis (Godden, 2008); however, it is unknown how

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freeze-thaw cycles affect recovery of *M. bovis* from these colostrum samples. Freezing of milk samples has been shown to reduce the recovery of *M. bovis* by culture (Boonyayatra et al., 2010). Considering the different composition of colostrum compared with milk (Foley and Otterby, 1978), simple extrapolation of the survival data of *M. bovis* in milk to colostrum may be incorrect (Boonyayatra et al., 2010).

The objectives of the present study were to determine the effect of the duration of the freezing period, the number of freeze-thaw cycles, and the thawing temperature on the survival of *M. bovis* in colostrum samples inoculated with 2 concentrations of *M. bovis*. Two separate experiments were conducted to achieve these objectives.

The colostrum used in experiments 1 and 2 was gamma-irradiated frozen bovine colostrum (ECI, Marloie, Belgium), guaranteeing immunoglobulin levels over 70 g/L and a sterile product. Sterility was verified by plating the colostrum on standard blood culture, incubated at 37°C, 5% CO₂ for 24 h on Pleuropneumonia Like Organism (PPLO) agar (Difco, Becton Dickinson and Company, Franklin Lakes, NJ), and incubated for 1 wk to check for mycoplasmal growth specifically. Colostrum was thawed at room temperature before inoculation with *M. bovis*.

In experiment 1, the effect of the duration of the freezing period on survival of *M. bovis* in colostrum was evaluated. Two strains of *M. bovis* were inoculated in colostrum. At inoculation and after 1, 4, 10, and 14 wk of storage at -18°C, 3 colostrum samples for each strain were thawed at 20°C (room temperature) and serial culture was performed to determine the *M. bovis* titer.

In experiment 1, 2 strains of *M. bovis* (LG1 and LG2) were used. Strain LG1 originated from a bronchoalveolar lavage sample from a calf with pneumonia; LG2 originated from a milk sample from a cow with mastitis, arthritis, and an infected seroma (Gille et al., 2016). *Mycoplasma bovis* species identification was confirmed by use of real-time PCR targeting the *uvrC* gene of filter-cloned isolates (Rossetti et al., 2010). In preparation for this study, 200 µL of a *M. bovis* strain suspended in a storage medium consisting of 75 mL of horse serum (Thermo Fisher Scientific, Carlsbad CA) and 25 mL of brain heart infusion broth (Bio-Rad, Hercules CA) supplemented with 10% (wt/vol) glucose (Merck, Darmstadt, Germany) was inoculated into 10 mL of modified PPLO broth (Difco). After inoculation, the broth was incubated for 5 d at 35°C and 5% CO₂, after which the PPLO broth was centrifuged at 4500 × *g* for 30 min at 21°C to sediment the bacteria. The supernatant was discarded and the

pellet was resuspended in sterile PBS. Dilution of the bacterial suspension to approximately 10¹⁰ cfu/mL was done by use of an optical density meter (Ultrospec III, Pharmacia Biotech, Cambridge, UK). The sample was diluted until an optical density of 0.2 absorbance units at 540 nm was achieved, based on specifications by Boonyayatra et al. (2010). Further dilution was done to achieve starting titers of 10⁶ cfu of *M. bovis*/mL of colostrum. The inoculated colostrum samples were stored in sterile 15-mL Falcon centrifugation tubes (Thermo Fisher Scientific, Waltham, MA) and frozen at -18°C. *Mycoplasma bovis* titer was determined immediately after complete thawing (no ice visible). One hundred microliters of colostrum was plated on PPLO agar using serial dilutions. Samples were incubated for 1 wk at 35°C and 5% CO₂, and colonies (with the typical fried-egg appearance) were counted with help of a microscope (10× magnification). Each sample was only plated once, but for each time point multiple samples were thawed.

To determine the effect of freezing over a 14-wk period and the effect of *M. bovis* strain on the survival of *M. bovis* in colostrum samples, a linear mixed model with repeated measures was used (PROC MIXED). Five time points (at inoculation, after 1, 4, 10, and 14 wk) were included as the within-subject factors; strain type (LG1 vs. LG2) was added as a between-subject factor. The sample size (3 observations per group per time point) was based on the detection of a 1 log difference between both strains, with a standard deviation of 0.4 log, 80% power, and 95% certainty. Mauchly's test of sphericity was used to determine equality of variances. Model validity was checked through inspection of the residuals. Bonferroni corrections were used for pairwise comparisons between the different time points. A compound symmetry repeated variance structure was used. In all models significance was set at $P < 0.05$, and $0.05 < P < 0.10$ was considered a trend. All analyses were performed in SAS version 9.4 (SAS Institute Inc., Cary, NC).

In the second experiment, the effect of repeated thawing (once or twice) and temperature of the first thawing process [either 20°C (room temperature) or 45°C] on *M. bovis* recovery was determined using 2 inoculum titers (10⁴ and 10⁶ cfu of *M. bovis*/mL). The sample size required to detect a 1 log reduction in *M. bovis* count between 2 storage and thawing methods, with 95% certainty and 80% power, was 18 observations per group (Win Episcopo 2.0, Zaragoza, Spain).

A full factorial design was used with 4 test groups: (1) freezing for 2 wk and a single thawing at 20°C; (2) freezing for 2 wk and a single thawing at 45°C; (3) freezing for 1 wk and thawing at 45°C, refrozen for

Figure 1. Mean concentration of colony-forming units per milliliter of colostrum after freezing and thawing a single time over a set time period. Time = 0 = inoculation; time = weeks of freezing. Values with different letters (a,b) are significantly different ($P < 0.05$) within subjects (time effect). Error bars represent SD. Color version available online.

1 wk and thawing at 20°C; and (4) freezing for 1 wk and thawing at 20°C, refrozen for 1 wk and thawing at 20°C. Sample preparation was identical to experiment 1, except that only the LG1 strain was used, in titers of 10^4 and 10^6 cfu of *M. bovis*/mL of colostrum. The inoculated colostrum samples were split in 16 portions of 10 mL of colostrum, which were distributed over the 4 treatment groups. The experiment was repeated 3 times. One week after inoculation, repeated thawing groups at 20 and 45°C and refrozen at −18°C. All groups were thawed at their respective temperatures 2 wk after inoculation. *Mycoplasma bovis* titer was determined using the same method as in experiment 1.

A linear mixed-model (PROC MIXED) was used to determine the effects of the initial *M. bovis* concentration in colostrum (10^4 vs. 10^6), number of freeze-thaw cycles (1 vs. 2), and thawing temperature (20 vs. 45°C). The test run was added as a random effect to account for clustering of measurements within a run. A maximum likelihood estimation with Satterthwaite approximation was used. All factors were forced into the model and interactions between significant main effects were tested. Post-hoc comparisons were made using Bonferroni corrections. Model validity checking and significance definitions were as described for experiment 1.

In experiment 1, the effect of freezing duration on the survival of *M. bovis* in colostrum was determined. Freezing significantly reduced *M. bovis* concentration by a mean of 0.81 to 1.02 log (SD = 0.13–0.15) for LG1 and LG2, respectively, between inoculation and 1 wk later (Figure 1). Longer freezing times did not result in further decrease in the number of *M. bovis* recovered from colostrum. No significant effect of strain was observed (Figure 1).

The effects of repeated thawing and thawing temperature tested in experiment 2 are shown in Table 1. Independent of the starting titer (10^4 or 10^6 cfu/mL), freezing and thawing of *M. bovis*-infected colostrum reduced the *M. bovis* concentration by approximately 1 log compared with the initial concentration. Thawing temperature (20 vs. 45°C) did not have a significant effect on the survival of *M. bovis* in colostrum ($P = 0.43$). Repeated thawing of colostrum further decreased the number of *M. bovis* by approximately 0.5 log compared with single thawing, regardless of the starting concentration of *M. bovis* ($P < 0.05$).

As expected, freezing and subsequent thawing of colostrum did not result in a complete elimination of *M. bovis*, similar to previous observations in milk samples (Boonyayatra et al., 2010). After a single freeze-thaw

Table 1. Effect of different thawing temperatures and repeated thawing on recovery of *Mycoplasma bovis* from bovine colostrum samples, with a titer of 10⁴ or 10⁶ cfu/mL of *M. bovis*¹

Item	Low starting titer (10 ⁴ cfu/mL)				High starting titer (10 ⁶ cfu/mL)				P-value ²			
	Single thaw		Double thaw		Single thaw		Double thaw		Freq.	Temp.	Conc.	Freq. × Conc.
	20°C	45°C	20°C	45°C	20°C	45°C	20°C	45°C				
<i>M. bovis</i> (cfu/mL)	3.55 ± 0.33	3.73 ± 0.28	3.18 ± 0.35	3.14 ± 0.36	5.80 ± 0.23	5.65 ± 0.40	5.18 ± 0.35	5.0 ± 0.68	<0.001	0.43	<0.001	0.17

¹Results presented as log-transformed concentration means of 3 independently repeated experiments ± SD.

²Freq. = thawing frequency; Temp. = temperature; Conc. = inoculation concentration.

cycle, the *M. bovis* concentration was reduced by approximately 1 log, independent of the starting titer, which is comparable to the findings reported for milk samples (Boonyayatra et al., 2010). Unlike Boonyayatra et al. (2010), longer freezing intervals were tested in our study as well. This did not result in a significant further decline of *M. bovis* colony-forming units. *Mycoplasma* concentration in milk ranges between 10² and 10⁸ cfu/mL, with the vast majority above 10⁶ cfu/mL (Biddle et al., 2003; Cai et al., 2005). If the *M. bovis* concentrations in colostrum and naturally infected milk are similar, an approximate 1 log reduction will result in *M. bovis* concentrations ranging between 10 and 10⁷ cfu/mL of colostrum. Such *M. bovis* concentrations can be detected with most of the commonly used screening methods, such as PCR, ELISA, and culture (detection limit = 10 cfu/mL; Biddle et al., 2003). Intermittent shedding has been described for several *Mycoplasma* species, so negative results should be interpreted carefully (Biddle et al., 2003).

Extension of storage time at −18°C for up to 14 wk did not further decrease the survival rate of *M. bovis* in colostrum as compared with storage for 1 wk. These results further illustrate that long-term freezing of colostrum is not a valid control strategy to prevent *M. bovis* infection of neonatal calves. Double freeze-thaw cycles resulted in a significantly larger reduction of *M. bovis* in colostrum. However, multiple freeze-thaw cycles can cause an undesirable decline in maternal antibody levels (Argüello et al., 2003) and are thus not recommended.

In conclusion, single and double freeze-thaw cycles reduced *M. bovis* concentration in colostrum by 1 to 1.5 log, respectively. Thawing temperature and initial bacterial concentration did not significantly affect the survival of *M. bovis* in colostrum. The storage of colostrum samples in the freezer at −18°C during epidemiological studies, herd monitoring, or test and cull programs likely has little influence on qualitative bacteriological test results for *M. bovis*. As there are currently no indications of the minimal infective dose of *M. bovis* in colostrum, the epidemiological or clinical relevance of an approximate 1 log reduction of *M. bovis* in colostrum is unclear and deserves further attention in future research. In case a lower *M. bovis* titer reduction is desired for epidemiological studies, addition of glycerol might be a valid aid, as described for milk samples by Boonyayatra et al. (2010).

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