Effects of Storage Time and Thawing Methods on the Recovery of *Mycoplasma* Species in Milk Samples from Cows with Intramammary Infections

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ABSTRACT

This study was executed to determine the effects of storage and thawing on the viability of *Mycoplasma* spp. in milk from cows with intramammary infections. The trial was designed using a control sample and seven handling regimens subjected to two methods of thawing. There was a significant treatment effect on the recovery of colony-forming units in milk samples when comparing the control sample with handling regimens 1 through 7. There was a continuous decline in log (10) mean number of cfu/mL recovered. Mean concentrations were 6.29, 4.64, 3.69, 3.01, 1.86, 4.41, 4.13, and 3.18 for control and handling regimens 1 to 7, respectively. To determine the best thawing method, handling regimen 1 through 7 samples were thawed using two methods. On average, more mycoplasma were recovered from milk samples thawed at ambient temperature (4.04 cfu/mL) than milk samples thawed in a 37°C water bath (3.76 cfu/mL). A final comparison was made between individual treatments. With the exception of the handling regimen 5 to 6 pair-wise comparison, all pair-wise comparisons between handling regimens were significantly different. The results of this study indicate that storage and thawing of milk samples is harmful to mycoplasma organisms. Fresh samples should be used to improve detection of *Mycoplasma* spp. from milk of infected cattle. If frozen samples are used, then length of storage time should be minimized, and thawing milk at ambient temperature will improve recovery of mycoplasma as opposed to thawing in a 37°C water bath.

(**Key words:** storage, thawing, mycoplasma, recovery)

INTRODUCTION

The objective of this study was to determine the effects of storage and freezing/thawing on the viability

of *Mycoplasma* spp. Freezing is lethal to many living systems. It preserves some cell structures while disrupting others; therefore, some organisms can withstand freezing whereas others cannot (Mazur, 1970). The ability to store milk samples collected from cases of subclinical and clinical mastitis, without marked loss of viability, in a frozen state for several weeks could be of great value to producers (Storper et al., 1982). Microbiological culturing is an important component of control programs to reduce prevalence of bovine mastitis in all stages of a cow's lactation.

Many studies have been performed to examine the survivability of pathogens that cause mastitis in cattle. Several studies have been executed to examine the effects of freezing and storage time on the recovery of bacteria in milk because freezing of milk samples has the potential to affect the survival of mastitis pathogens. Most studies have shown freezing to have no effect on the recovery of *Staphylococcus aureus* from milk samples (Luedecke et al., 1971; Bashandy et al., 1979; Pankey et al., 1987; Schukken et al., 1989; Murdough et al., 1996). Studies examining the effects of storage time and temperature on survival of *Streptococcus agalactiae* from milk samples have been mixed. Villanueva and colleagues (1991) found a 2.5 increase in the number of *S. agalactiae* colonies isolated when samples were stored at –20°C for 23 d. Whereas other studies found a significant decrease in the number of isolates of *S. agalactiae* recovered from samples (Luedecke et al., 1971; Storper et al., 1982; and Bashandy et al., 1979). Several studies have found there to be a significant decrease in the number of *Escherichia coli* isolates in samples after freezing (Storper et al., 1982; Pankey et al., 1987; Schukken et al., 1989). Studies have also consistently found a significant decrease in the number of *Streptococcus non-agalactiae* isolates recovered from milk samples that were stored at temperatures of –18 to –70°C for 14 to 28 d (Bashandy et al., 1979; Storper et al., 1982; Pankey et al., 1987).

Little work has been done in examining the viability of mycoplasma species. In observing avian, human, canine, and saprophytic types of mycoplasma, Kelton (1964) found considerable variation between strains in

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Table 1. Handling regimen designation for the assessment on recovery of mycoplasma as affected by different methods of storing and thawing of milk samples.¹

Portion	Handling	Method of storage sample
1	Control	No storage, sample plated freshly
2	1	Sample held frozen for 1 wk, thawed, plated, refrozen
2	2	Sample held frozen for 2 wk, rethawed, plated, refrozen
2	3	Sample held frozen for 3 wk, rethawed, plated, refrozen
2	4	Sample held frozen for 4 wk, rethawed, plated, refrozen
3	5	Sample held frozen for 2 wk, thawed, plated
4	6	Sample held frozen for 3 wk, thawed, plated
5	7	Sample held frozen for 4 wk, thawed, plated

¹Original milk sample collected from cows at weekly intervals. A sample was collected from each mammary quarter and a composite sample was collected. Original sample was apportioned into 5 different collection tubes and treated differently.

their resistance to alternate freezing and thawing. Most strains showed their greatest loss between the frozen sample and the first sample thawed after storage. Several strains displayed stunted colonies, and some strains required a longer incubation period to produce a colony.

More research needs to be done so that there is a good understanding of the consequences of freezing and storage on bovine mycoplasma so that misdiagnosis does not occur. The freezing of milk samples without compromising results would be extremely beneficial for producers monitoring mastitis within a herd.

MATERIALS AND METHODS

Milk Sample Collection

In an effort to determine the effects of storage and freezing/thawing on the viability of *Mycoplasma species*, the following trial was designed using a control sample and seven handling regimen samples. Eight lactating Holstein cows in various stages of lactation from four commercial dairies were enrolled for study. A commercial milk quality laboratory (Allan Britten, Udder Health Systems, Bellingham, WA) confirmed intramammary mycoplasma infections in each of the cows by culturing milk samples (Biddle et al., 2002). Five milk samples (four quarter and one composite) were collected from each cow on the same day, at 7-d intervals, for 4 consecutive weeks. Of the 160 samples collected from the eight infected cows, there were a total of 115 composite and mammary quarter milk samples positive for mycoplasma, and only these samples were used in this study.

Milk Sample Culturing

Portions of the original milk sample (n = 115), were split into four separate 5-mL tubes that were used for handling regimens 1 through 7, and stored at -20°C (Table 1). Each of the 115 samples (composite and mam-

mary) that were positive for mycoplasma received all seven handling regimens. The sample that was plated fresh served as the control sample. Following application of the treatments, the milk samples were cultured after serial dilutions of 1:100, 1:1,000, 1:10,000, and 1:100,000 in 0.01 M PBS (pH 7.4). One hundred microliters of each dilution was spread onto agar as described for the original sample. Each sample was plated in duplicate at 37°C, 10% CO₂, for 10 d before examination. Samples were examined under a 15× dissecting microscope and every colony with the distinctive "fried egg appearance" was counted and recorded from the dilution when colony-forming units ranged from 20 to 200 per agar plate (Hogan et al., 1999). The number of colony-forming units of *Mycoplasma* species per milliliter of milk sample was determined based on the counts and dilution factor used. In addition, a sample was recorded as positive for mycoplasma if at least one colony was detected from 100 µL of milk spread onto mycoplasma agar plates (National Mastitis Council, 1981). Mycoplasma were speciated when first isolated from a mammary quarter. Speciation was done by the California Animal Health and Food Safety Laboratory in Davis, CA, using the indirect immunoperoxidase test (Uchida et al., 1987).

The sample collected at each period was split into five different portions (Table 1). The first portion that was plated and cultured immediately after collection and was given the handling designation of control. The other portions were termed 2 to 5 and were subject to different handling regimens. Handling regimens 1, 2, 3, and 4 used the same milk sample. This portion (2) was repeatedly frozen and thawed for 4 wk starting on wk 1 postcollection, as indicated in Table 1. Thawing, plating, and refreezing of this sample were repeated weekly with a final plating 4 wk after original collection. Handling regimens 5, 6, and 7 were applied to three distinct portions; each was stored for varying lengths of time. Handling regimen 5 was stored frozen for 2 wk

Table 2. Evaluation of two different methods used to assess if recovery of mycoplasma colonies is affected by thawing methods.

Methods	Log ₁₀ cfu/mL ¹	SE (±)
Thawed at ambient temperature	4.04 ²	0.05
Thawed in 37°C water bath	3.76	0.05

¹Data are expressed as least square means of log₁₀ cfu/mL.

²Mean cfu/mL significantly greater ($P < 0.001$) for ambient temperature samples than water bath thawed samples.

and plated on wk 2 postcollection. Handling regimen 6 was stored frozen for 3 wk and plated on wk 3 postcollection. Handling regimen 7 was stored frozen for 4 wk and plated on wk 4 postcollection.

To assess the effects of temperature on mycoplasma viability, two thawing temperatures were applied in conjunction with the seven freezing regimens. Thawing method 1 involved thawing the samples at ambient temperature for one-half hour. Thawing method 2 involved thawing the samples in a water bath at 37°C for 10 min.

Statistical Analysis

The experimental design described in the above section is a randomized complete block design (cows acting as blocks) with a two-way treatment structure (length of freezing and temperature of thawing). The SAS Proc GLM (2001) was used to assess differences in the mean Log₁₀ (cfu/mL) for the control and the seven handling regimens, along with comparisons of the methods of thawing. Fisher's LSD multiple comparison procedure was used to assess differences in the length of freezing means and temperature of thawing means.

In addition to recording the number of cfu/mL, samples were also recorded for presence or absence of mycoplasma, for the control and the seven handling regimens when thawed using methods 1 and 2. Because the response is binary (presence/absence) and the experimental design was a randomized complete block design, Cochran's Q (Zar, 1996) was therefore used to assess differences among the treatment proportions.

RESULTS

On average, more mycoplasma were recovered from milk samples thawed slowly at ambient temperature than milk samples thawed quickly in a 37°C water bath ($P < 0.001$) (Table 2). The results show that thawing method played a significant role ($P < 0.001$) in affecting the recovery of mycoplasma (Table 3). As samples were repeatedly frozen and thawed or stored for longer lengths of time, less mycoplasma was recovered. The

Table 3. Evaluation of mycoplasma survival in samples¹ that were treated by being repeatedly frozen and thawed or stored frozen for varying lengths of time.

Handling regimens	Log ₁₀ cfu/mL ²	Samples negative (%) ³
Control sample	6.29 ^a	0
Treatment 1	4.64 ^b	22
Treatment 2	3.69 ^c	34
Treatment 3	3.01 ^d	40
Treatment 4	1.86 ^e	59
Treatment 5	4.41 ^f	24
Treatment 6	4.13 ^f	27
Treatment 7	3.18 ^g	45

^{a-g}Means within the column not sharing a common superscript were significantly different from control by ANOVA ($P < 0.001$).

¹Samples were subjected to seven different handling regimens (Table 1).

²Data are expressed as least square means for log₁₀ cfu/mL.

³Percentage of samples where mycoplasma numbers were below the level of detection and assumed to be zero.

frequency distribution of mycoplasma isolated from the control sample and handling regimens 1 through 4 (Table 3) indicates that with repeated freezing and thawing there is a loss of up to 59% of the positive mycoplasma samples. Likewise, the frequency distribution of mycoplasma isolated from the control sample and handling regimens 5 through 7 (Table 3) indicates storage time alone had a large effect on recovery of mycoplasma from samples, with as great as 45% loss in the number of positive mycoplasma samples recovered. A final comparison was made between individual treatments. Pair-wise comparison of handling regimen means indicated that they were significantly different from each other ($P < 0.001$), with the exception of the handling regimens 5 to 6 pair-wise comparison ($P = 0.05$), indicating a significant loss in mycoplasma positive samples over storage time. Thirty-one percent ($n = 36$) of the samples were positive for *Mycoplasma californicum*, the remaining were positive for *Mycoplasma bovis*.

DISCUSSION

One method of controlling mastitis is by the identification of organisms in milk samples. The ability to be able to freeze and store milk samples without loss is essential so that misdiagnosis can be prevented. Several studies have been done to examine the survivability of pathogens that cause IMI. Because most studies have shown no adverse effect on the recovery of *S. aureus*, it is generally accepted that freezing of milk samples will not result in the misdiagnosis of cows (Luedicke et al., 1971; Bashandy et al., 1979; Pankey et al., 1987; Schukken et al., 1989; Murdough et al., 1996). For organisms such as *E. coli* (Storper et al., 1982; Pankey et al., 1987; Schukken et al., 1989) and

S. non-agalactiae (Bashandy et al., 1979; Storper et al., 1982; Pankey et al., 1987) routine freezing and thawing is not accommodating to these organisms; therefore, the possibilities of misdiagnosis may be high. Storper and co-workers (1982) found a 29% loss in *E. coli* and a 36% loss in *S. non-agalactiae* after samples were stored at -18°C for 4 wk. This study found as high as a 59% reduction on *Mycoplasma* species positive samples after being stored for 4 wk. Thus, results would suggest that freezing and thawing pose an even greater risk to the detection of mycoplasma species than has been found for other milk pathogens.

These trial results indicated that storage and thawing of frozen intramammary milk samples is harmful to mycoplasma organisms. Recovery of mycoplasma was significantly greater in samples thawed slowly at ambient temperature. Therefore, this would suggest that a mycoplasma cell is susceptible to injury when thawed too rapidly. There was a significant reduction in the number of mycoplasma colonies as samples were repeatedly frozen and thawed or stored for various lengths of time. A loss in numbers of mycoplasma colonies was found, such that nearly half the samples would not yield mycoplasma after storage. These results indicate that storage and thawing of milk samples is harmful to mycoplasma organisms, which could lead to misdiagnosis. Thus, it is best to culture fresh milk samples suspected of mycoplasma to keep loss of mycoplasma colonies to a minimum and avoid misdiagnosis.

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