

## Short communication: The effect of centrifugation and resuspension on the recovery of *Mycoplasma* species from milk

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### ABSTRACT

Low sensitivity of a single bulk tank milk culture is a major limitation for detection of mycoplasma organisms. We hypothesized that sedimentation of *Mycoplasma* spp. in a milk sample by centrifugation followed by resuspension in a small volume of fluid before agar plating would increase the ability to detect *Mycoplasma* spp. compared with direct conventional culture. The experiment was conducted to determine recovery of *Mycoplasma* spp. from milk as affected by 1) treatment (centrifugation vs. conventional method); 2) 2 species (*Mycoplasma bovis* and *Mycoplasma californicum* and 4 strains for each species); and 3) 4 different concentrations of *Mycoplasma* spp. (1,000, 100, 10, and 1 cfu/mL). A 5-mL portion of mycoplasma suspension from each strain was inoculated into 45 mL of fresh bulk tank milk to achieve concentrations of 1,000, 100, 10, and 1 cfu/mL. Treatment samples were vigorously mixed and centrifuged at  $5,000 \times g$  for 30 min. Control samples were vigorously mixed. All samples were plated on modified Hayflick agar. Plates were incubated at 37°C and 5% CO<sub>2</sub> for 5 d. Mean ( $\pm$ SE) log<sub>10</sub> mycoplasma counts (cfu/mL) in the treatment groups ( $1.91 \pm 0.15$ ) were higher than those in the control groups ( $1.70 \pm 0.16$ ). Recovery of at least 1 mycoplasma colony on agar culture was 100% in both treatment and control groups at high, medium, and low concentrations. At the lowest concentration, recovery of at least 1 mycoplasma colony on agar culture in treatment and control groups was 75% ( $n = 12/16$ ) and 18.75% ( $n = 3/16$ ), respectively. Centrifugation of milk followed by suspension in a smaller volume of saline before conventional culture increased the ability to detect mycoplasma microorganisms in the milk sample compared with controls. Recovery by centrifugation appeared best at the lowest concentration where detection of a positive sample was 4 times more likely than when conventional methods were used.

**Key words:** centrifugation, *Mycoplasma* spp., mastitis, bulk tank milk

The conventional method of diagnosis of mycoplasma mastitis is microbiological culture of milk samples taken from individual cows or from herd bulk tank milk and the direct agar plating and culture of those samples (González and Wilson, 2003). It was suggested that culture of bulk tank milk be used for screening and surveillance of this disease because a positive result usually indicated that at least 1 cow in the herd has mycoplasma mastitis. Still, a negative result is not necessarily indicative of the absence of mycoplasma infection in a herd (Guterbock and Blackmer, 1984; Biddle et al., 2003) because the test is approximately 60% sensitive.

Low sensitivity of a single bulk tank milk culture is a major limitation for detection of mycoplasma organisms. Approximately 30 to 40% of bulk tank milk samples from herds with mycoplasma mastitis were mycoplasma negative, indicating that a herd would be erroneously considered free of mycoplasma mastitis (González and Wilson, 2003). The most common explanation for a false-negative bulk tank mycoplasma culture result is that the concentration of the pathogen in the milk sample is lower than the threshold of detection. Biddle and coworkers (2003) estimated that approximately one-third of the time a cow with mycoplasma mastitis would shed the pathogen in her milk at a low level such that when combined with milk from the rest of the herd, the concentration would be below the level of detection. One study showed that centrifugation of milk samples and resuspension of the bacterial pellet before culture improved the sensitivity of detection of *Staphylococcus aureus* (Zecconi et al., 1997).

We hypothesized that sedimentation of *Mycoplasma* spp. in a milk sample by centrifugation followed by resuspension in a small volume of fluid before agar plating would increase the ability to detect *Mycoplasma* spp. compared with conventional culture.

A completely randomized design with a  $2 \times 2 \times 4$  factorial arrangement was used to test the factors: 1) 2 treatments (centrifugation vs. direct agar plating);

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2) 2 test species (*Mycoplasma bovis* and *Mycoplasma californicum*; 4 strains for each species were used); and 3) 4 concentrations of *Mycoplasma* spp. (1,000, 100, 10, and 1 cfu/mL). Sixty-four milk samples were randomly assigned to receive the combination of these treatment factors.

Fresh bulk tank raw milk from the Washington State University dairy farm was used as the test medium. The herd had no history of mycoplasma mastitis as determined from periodic cultures of cows with clinical and subclinical mastitis, and from bulk tank milk cultures over a 20-yr period. Broth cultures of mycoplasma strains in the logarithmic phase of growth were centrifuged at  $5,000 \times g$  for 30 min. Four strains of *M. bovis* and 4 of *M. californicum* were used. Strains were from cows with mastitis and had different genotypes as determined by pulsed-field gel electrophoresis (Biddle et al., 2005). The cell pellet was suspended in PBS. The mycoplasma suspension was diluted to achieve an optical density of 2.0 at 540 nm corresponding to a theoretical concentration of  $10^8$  cfu/mL. This mycoplasma suspension was serially diluted with PBS to achieve theoretical concentrations of 10,000, 1,000, 100, and 10 cfu/mL. At each concentration, one of the duplicates was randomly allocated as the treatment tube, the other as the control tube.

A 5-mL portion of mycoplasma suspension from each control and treated tube was added to 45 mL of fresh bulk tank milk to achieve a theoretical concentration of 1,000, 100, 10, or 1 cfu/mL. These concentrations correspond to what was termed high, medium, low, and very low, respectively. Treatment tube milk samples were vigorously mixed and centrifuged at  $5,000 \times g$  for 30 min. After centrifugation, the fat layer was removed and the skim decanted. Then, 400  $\mu$ L of PBS was added to resuspend the centrifuged pellet. The resuspension was vigorously mixed and 200  $\mu$ L removed and spread on a modified Hayflick agar plate (National Mastitis Council, 1999). Control tube milk samples, which were not centrifuged, were vigorously mixed and 200  $\mu$ L of mycoplasma-inoculated milk was removed and plated on a modified Hayflick agar plate in duplicate. The actual concentration of *Mycoplasma* spp. used to inoculate the milk was determined by plating and culturing 200  $\mu$ L of the original PBS suspension of 1,000 cfu/mL and enumerating the colonies as described. All plates were incubated at 37°C with 5% CO<sub>2</sub> for 5 d. Plates were examined using 15 $\times$  microscope for evidence of growth of mycoplasma microorganisms with the distinctive fried-egg appearance and colonies counts made. Plates were classed as a positive culture if at least 1 colony of mycoplasma organism was found. Bulk tank milk samples were plated and cultured by using these conventional methods (National Mastitis Council, 1999)

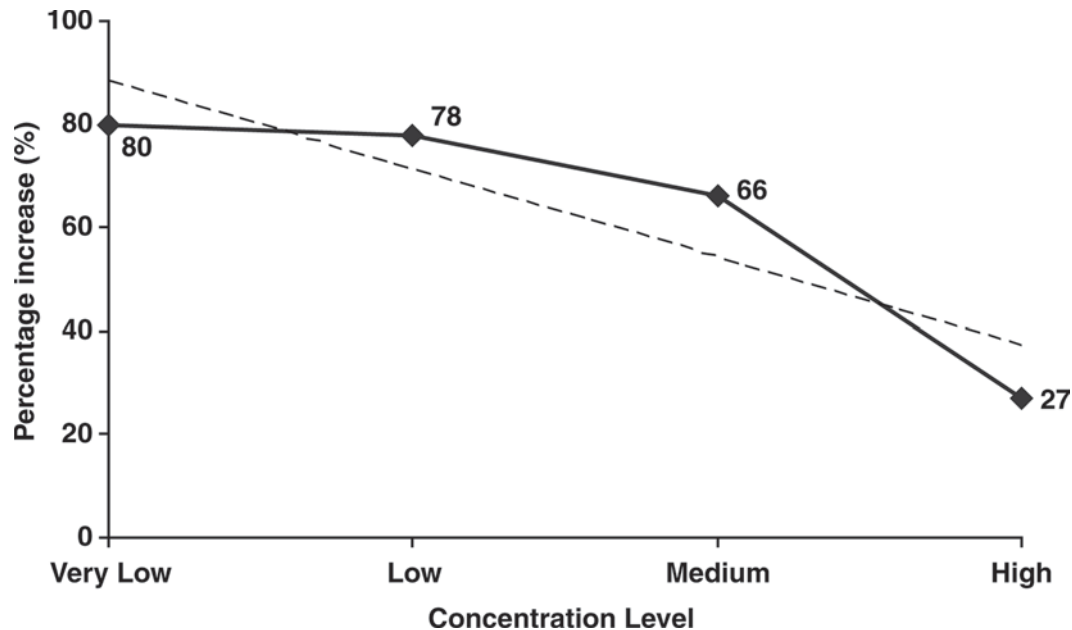
to determine that samples were free from *Mycoplasma* spp.

The differences in mean number of mycoplasma counts (cfu/mL) between treatment tube milk and control tube milk were analyzed using ANOVA, mixed linear model (PROC MIXED; SAS Institute, 2002). Treatment, concentration, and species were defined as fixed effects. Strain nested within species was defined as a random effect. Mycoplasma counts were transformed to log<sub>10</sub> values to satisfy normal distributional requirements of ANOVA. Multiple comparisons were done using least significant difference method. A trend line was created by Excel (Microsoft Corp., Redmond, WA) to note the recovery of *Mycoplasma* spp. by concentration level (Figure 1).

Mean ( $\pm$ SE) mycoplasma counts in the treatment groups ( $1.91 \pm 0.15$ ) were higher than in the control groups ( $1.70 \pm 0.16$ ;  $P < 0.05$ ). At each theoretical concentration level (1,000, 100, 10, and 1 cfu/mL), the mean treatment recoveries were significantly higher than those of the controls (Table 1). The counts of all mycoplasma species and strains were pooled to create the means. Moreover, the trend in the increase in recovery of *Mycoplasma* spp. was linear but negatively associated. The relative increase in recovery was lowest at the highest concentration and highest at the lowest concentration (Figure 1). Recovery of at least 1 mycoplasma colony on agar was 100% in both treatment and control groups at the high, medium, and low concentrations. At the very low concentration, recovery of at least 1 mycoplasma colony on agar culture in treatment and control groups was 75% ( $n = 12/16$ ) and 18.75% ( $n = 3/16$ ), respectively. Species and strain had no effect on mycoplasma counts. No significant interactions were found among factors.

Evidence from the current study supports the hypothesis that centrifugation of milk with *Mycoplasma* spp. followed by resuspension in a small volume of PBS before culture increases the ability to detect the organisms. Centrifugation improved the recovery of mycoplasma for both species tested, all strains within species, and at all levels of initial concentration. Moreover, the procedure was able to detect a mycoplasma-positive milk sample 75% of the time at a very low concentration (1 cfu/mL). This concentration is below the normal threshold of detection, as the minimum level of detection would range from 10 to 20 cfu/mL corresponding to plating volumes of 100 to 200  $\mu$ L of milk (Farnsworth, 1993).

This ability to detect a positive sample at the lowest concentration occurred 4 times more frequently than if the sample were cultured using the conventional (control) technique. Thus, the technique, through a theoretical 250-fold increase in cell concentration, im-



**Figure 1.** The relative difference in mycoplasma counts from all treatment versus control group samples by high, medium, low, and very low concentrations. The number above the point corresponding to each concentration level is the percentage increase in counts of the treatment mean compared with control mean. The dashed line shows the trend. Treatment was centrifugation followed by resuspension of small volume of fluid before plating on agar.

proved recovery at all levels (Figure 1). Still, recovery was not nearly as high as the theoretical expectation. It is possible that not all mycoplasma organisms in milk samples were recovered in the pellet after centrifugation. The organisms may be partitioned into the skim and fat portions. For example, *Mycobacterium avium* ssp. *paratuberculosis* was partitioned into the cream and whey portions as well as the pellet, leading to a lower bacterial count than expected (Gao et al., 2005). Alternatively, centrifugation could have resulted in aggregation of mycoplasma colonies so that when plated, individual colony-forming units could not be distinguished after growth of the colony. The trend in the recovery of mycoplasma colonies increased with a decreasing concentration of inoculum (Figure 1). This finding supports the contention that aggregation of colonies decreased as concentration decreased and thus, the percentage recovery of colonies improved. The advantage of centrifugation was consistent with the previous report that showed that the centrifugation procedure increased the recovery of *Staphylococcus aureus* from milk samples (Zecconi et al., 1997). Recovery of mycoplasma organisms was increased by inoculation of enrichment medium with milk and incubation for 4 d (Biddle et al., 2003). Although the enrichment method increased the recovery of the organisms from 24 of 104 samples (24%), it failed to recover mycoplasma organisms from 9 of 104 samples (9%) that were positive using the conventional method. The centrifugation method

described herein has the advantage over preincubation with enrichment medium, because it does not require additional time for incubation.

Single sampling is commonly used during herd-monitoring programs (Jayarao et al., 2004), although multiple sampling and culture of bulk tank milk increases the sensitivity of detection of *Mycoplasma* spp. (González and Wilson, 2003). The concentration of mycoplasma organisms may often be lower than the threshold of detection using the conventional culture method. At times, a cow or cows will shed mycoplasma intermittently and there can be variation in the amount

**Table 1.** Contrast of means<sup>1</sup> ( $\pm$ SE) of log<sub>10</sub> mycoplasma counts (cfu/mL) in treatment and control groups<sup>2</sup>

Concentration <sup>3</sup>	Treatment	Control
High	3.07 $\pm$ 0.28 <sup>a</sup>	2.97 $\pm$ 0.45 <sup>b</sup>
Medium	2.18 $\pm$ 0.07 <sup>a</sup>	1.93 $\pm$ 0.10 <sup>b</sup>
Low	1.36 $\pm$ 0.10 <sup>a</sup>	1.14 $\pm$ 0.13 <sup>b</sup>
Very low	1.02 $\pm$ 0.06 <sup>a</sup>	0.77 $\pm$ 0.32 <sup>b</sup>

<sup>a,b</sup>Values in a row with different superscripts were significantly different ( $P < 0.01$ ).

<sup>1</sup>Least squares means of all mycoplasma counts from all replicates of all strains and species, within group (treatment or control).

<sup>2</sup>Treatment was centrifugation followed by resuspension of small volume of fluid before agar plating; control was conventional direct plating.

<sup>3</sup>Concentration of high, medium, low, and very low represent theoretical concentrations of 1,000, 100, 10, and 1 cfu/mL of mycoplasma.

of organism shed when daily comparisons are made (Biddle et al., 2003). The results from the current study indicate that recovery of mycoplasma organisms can be improved so that single rather than multiple consecutive samples can be used to monitor bulk tank milk. Moreover, the technique was not affected by species or strain of mycoplasma organism tested and the technique appeared to be most efficient when the concentration of mycoplasma was lowest.

In conclusion, centrifugation of milk followed by suspension in a smaller volume of saline before conventional culture increased the ability to detect mycoplasma microorganisms in the milk sample compared with controls. Recovery appeared best at the very low concentration (1 cfu/mL), where detection of a positive sample was 4 times more likely compared with when conventional methods were used.

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