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The effect of pre-enrichment on recovery of *Streptococcus agalactiae, Staphylococcus aureus* and mycoplasma from bovine milk

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SUMMARY

The study was conducted to determine whether pre-enrichment would increase sensitivity of detecting *Streptococcus* (*Str.*) *agalactiae*, *Staphylococcus* (*S.*) *aureus*, and mycoplasma in bovine milk. Two procedures were followed, one involving direct inoculation of milk on bovine blood agar, and the other involving pre-enrichment in broth followed by inoculation on agar. Logistic regression was used to predict the probability of isolation as a function of culture procedure and two additional covariates, the California Mastitis Test (CMT) score of the milk and the type of sample (indicating sample storage temperature and herd mastitis status). A total of 13778 milk samples was cultured for each of the three bacteria. By using results of both direct inoculation and pre-enrichment, the probability of isolation compared to use of direct inoculation only and adjusted for effects of other variables was increased 3·6-fold for *Str. agalactiae*, 1·6-fold for *S. aureus* and 1·7-fold for mycoplasma. The probability of isolation for all three bacteria increased as the CMT score increased. For *Str. agalactiae*, there was a statistical interaction predicting that enrichment improved the odds of isolation more from milk with high CMT scores than from milk with low scores. Results indicate that pre-enrichment can substantially increase the sensitivity of bacteriological screening of dairy cows for mastitis caused by *Str. agalactiae*, *S. aureus*, and mycoplasma.

INTRODUCTION

Programmes aimed at control and eradication of contagious mastitis typically attempt to minimize bacterial transmission from infected to susceptible cows by improving sanitation and identifying and segregating cows shedding contagious bacteria in their milk (1–4). Detectable shedding, however, may occur intermittently (5–7) and the spread of infection may still take place when the concentration of bacteria in milk fall below the sensitivity threshold of the microbiological test being used (8). In order to maximize benefits of control and/or eradication efforts, therefore, highly sensitive tests are desirable to screen cows for contagious bacteria. Improved sensitivity of tests could permit early detection,

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treatment, and/or removal of cows infected with *Streptococcus* (*Str.*) *agalactiae*, *Staphylococcus* (*S.*) *aureus*, or mycoplasma, thereby reducing the risk of mastitis to susceptible cows (1).

One way in which sensitivity may be improved is by increasing the numbers of micro-organisms through pre-enrichment. Although it is known that pre-enrichment can increase the likelihood of isolating *S. aureus*, *Str. agalactiae*, and mycoplasma present in low concentrations (6, 8, 9), it has not been established whether pre-enrichment offers any practical advantage (10). Because there are no estimates of increased sensitivity offered through pre-enrichment, it cannot be determined whether it would be worthwhile to incorporate pre-enrichment procedures in the screening of cows for contagious mastitis. Knowledge of the expected benefit of pre-enrichment would assist practioners and diagnosticians in efforts to control and eradicate mastitis from herds of dairy cows.

The purpose of this study was to quantify the effect of pre-enrichment on detection of *Streptococcus agalactiae*, *Staphylococcus aureus* and mycoplasma in bovine milk.

MATERIALS AND METHODS

Samples examined. The milk samples examined were routinely submitted to the Veterinary Medicine Teaching and Research Center (VMTRC) by dairymen and veterinarians in the San Joaquin Valley of California. Each sample was a composite of foremilk collected aseptically from all functional quarters. Samples were stored in one of two ways depending on the mastitis status of the herd. One type of sample (type 1) was collected from all cows through whole-herd tests of dairies involved in the initial phase of control and eradication of mastitis caused mainly by *Str. agalactiae* and *S. aureus*, with a few herds involved in eradication of mycoplasma. Milk samples from these herds were refrigerated at 5 °C for 1–2 h before being processed. The other type of sample (type 2) was collected from cows that had recently calved, were clinically mastitic, or were new entries into the herd. These cows were from herds engaged in ongoing mastitis surveillance and control programmes aimed at detecting and managing cows newly infected with *Str. agalactiae*, *S. aureus*, and/or mycoplasma. Most cows infected with these bacteria had been identified through previous whole-herd tests and had been either treated (*Str. agalactiae* infections) or removed from the herd (*S. aureus* and mycoplasma infections). Type 2 milk samples were frozen at −10 °C for 1–7 days before being collected for processing by the laboratory.

Direct inoculation procedures. Bovine blood (5% washed cells) and mycoplasma agar plates (11), were divided into quadrants so that each plate accommodated four samples. Three dots of *S. aureus* beta-toxin were deposited with a sterile cotton swab down the center of each blood agar quadrant and allowed to dry before milk was plated.

Frozen milk samples were thawed at room temperature. After mixing, milk was drawn in a single stroke of a sterile cotton swab down the center of a quadrant of a mycoplasma plate. The swab was rotated 180 degrees and the milk remaining on the swab was streaked throughout a quadrant of a blood agar plate. Milk initially deposited on the mycoplasma agar was then streaked with the same swab throughout the rest of the mycoplasma agar quadrant. This procedure reduced

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transfer of bacterial inhibitors (thallium acetate and penicillin) present in the mycoplasma media onto the blood agar, while maximizing the amount of inocula on mycoplasma media. Approximately 0·017 g of milk was deposited on mycoplasma agar and 0·013 g on blood agar.

Enrichment procedures. Following completion of the primary plating procedures, 1 ml milk was added to 5 ml brain heart infusion broth and to 3 ml mycoplasma broth (11). Brain heart infusion broth was incubated 6 h at 37 °C before inoculation on one-half of a bovine blood agar plate containing dried spots of *S. aureus* beta-toxin. An inoculum of about 0·015 g of milk and broth was swabbed onto the half plate and then streaked with a wire loop to produce isolated colonies for identification.

Mycoplasma broth was incubated 48 h at 37 °C in air before an inoculum of 0·016 g broth-milk mixture was swabbed onto a quadrant of a mycoplasma agar plate. Bovine blood agar plates were inoculated (with either milk or milk enriched with brain heart infusion broth) and incubated 18–24 h at 37 °C in air. Mycoplasma plates were incubated 7 days at 37 ° in an atmosphere of 4% CO_2

California Mastitis Test. The California Mastitis Test (CMT) was performed on milk after broths were inoculated; results were recorded as negative, trace, 1, 2 or 3 (12).

Identification of organisms. Presence of *Str. agalactiae* was determined by colony morphology, a positive CAMP reaction and a negative esculin hydrolysis reaction. Colonies of *S. aureus* were identified by alpha, alpha-beta, or beta zones of hemolysis on the agar, morphology, a cocci appearance, a positive Gram stain and a positive coagulase reaction.

At post-inoculation days 4 and 7, mycoplasma plates were examined using a dissecting microscope at 7–20× magnification. Suspect colonies were stained *in situ* with fluorescein-conjugated antisera to the various species, by methods previously described (11). Each suspect mycoplasma colony was stained initially with conjugate for the eight most common isolates (*M. bovis*, *M. californicum*, *M. bovigenitalium*, *M. canadense*, *M. arginini*, *M. bovarhinis*, *M. alkalescens* and *Acholeplasma laidlawii*) and, if all were negative, for three additional species (*M. bovocoli*, serotype L and group 7).

Analyses. Statistical analyses were performed to determine if pre-enrichment of milk altered the chance of isolating *Str. agalactiae*, *S. aureus*, or mycoplasma, compared to using only direct inoculation. In addition, data were analysed to determine if isolation of bacteria might be related to CMT and sample type. Multiple logistic regression was used to define appropriate models predicting culture results as a function of culture procedure, CMT, and sample type, and to estimate the magnitude of effects these variables may have had on predicted results. One model was created for each of the three organisms considered. Models were of the general form: culture result (0 = organism absent, 1 = organism present) = culture procedure (1 = direct inoculation only, 2 = pre-enrichment only, 3 = combined direct inoculation and pre-enrichment) + CMT result (0 = negative, 4 = trace, 1 = 1, 2 = 2, 3 = 3) and sample type (1 = type 1, 2 = type 2) + variable interactions + random error. For the combined procedure, a positive result was presence of bacteria following either direct inoculation or pre-enrichment; a negative result was the failure to detect bacteria using both direct

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inoculation and pre-enrichment. The models for each organism included only terms which contributed significantly ($P = 0{\cdot}15$ to remove, $P = 0{\cdot}10$ to enter) to the χ^2 goodness-of-fit. Computations were performed using the logistic regression (LR) program of BMDP (13).

The relative effect variables had on culture outcome was estimated using odds ratios (OR) for each variable at each level of the other variables in the model. The overall odds ratio (OR) for the ith variable was estimated as

$$\exp \beta_i,$$

where β was the regression coefficient computed by the LR program. The confidence interval for the estimated odds ratio was computed as

$$\exp [\beta_i \pm Z\{\text{S.E.}(\beta_i)\}],$$

where Z was 1·96 or 2·58 for the 95 or 99% confidence interval and S.E. (β_i) was the standard error of the regression coefficient for the ith variable.

RESULTS

Isolation of Streptococcus agalactiae. Of 6250 type 1 milk samples, *Str. agalactiae* was isolated from 385 (6·2%) on direct inoculation, from 676 (10·8%) following pre-enrichment, and from 748 (12·0%) from either procedure. Of 7528 type 2 samples, *Str. agalactiae* was isolated from 224 (3·0%) on direct inoculation, from 320 (4·3%) following pre-enrichment, and from 358 (4·8%) from either procedure.

The model generated by stepwise logistic regression indicated statistically significant effects of sample type, CMT score and culture procedure ($P < 0{\cdot}001$). There also were interactions between these factors ($P = 0{\cdot}076$ for type × procedure, the last term fitted, and $P < 0{\cdot}001$ for the other two-factor interactions). The goodness of fit of the model with these six terms was very good ($\chi^2 = 2{\cdot}941$, D.F. $= 8$, $P = 0{\cdot}938$). The adjusted OR estimated from the model, which indicated the relative advantage of a procedure compared to using only direct inoculation, was 3·3 for the pre-enrichment procedure and 3·6 for the procedure combining pre-enrichment and direct inoculation, or a 230 and 260% increase, respectively. The odds of isolating *Str. agalactiae* increased as CMT score increased. The OR increased from 0·9 for milk with a CMT score of 'trace' to 9·5 for milk with a CMT score of 3, compared to milk with a zero CMT score (Table 1). An interaction between sample type and CMT score appeared as a decrease in the odds of isolation for type 2 samples with a CMT score of zero, 'trace' or 1, and an increase in the odds for a CMT score of 3 (Table 2). An interaction also was found between procedure and CMT score. The odds of isolation following pre-enrichment were 2–3 times as high for milk with a zero CMT score, compared to milk with a CMT score of 3. The odds of isolation from type 2 samples was three-fold less than from type 1 samples (OR = 0·3).

Isolation of Staphylococcus aureus. For type 1 samples, *S. aureus* was isolated from 422 (6·8%) on direct inoculation, from 572 (9·1%) following pre-enrichment, and from 639 (10·2%) from either direct inoculation or pre-enrichment. The number of isolations for type 2 samples, were 277 (3·7%) on direct inoculation, 355

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(4·7%) following pre-enrichment, and 411 (5·5%) from either direct inoculation or pre-enrichment.

The model generated by stepwise logistic regression indicated statistically significant effects of sample type, CMT score and culture procedure ($P < 0·001$), although there was no indication for a significant strength to the association with sample type (OR = 0·9, 95% confidence interval = 0·7 − 1·2). There also was an interaction between type and CMT ($P < 0·001$). The goodness of fit of the model with these four terms was very good ($\chi^2 = 9·979$, D.F. = 18, $P = 0·933$). The adjusted OR for the pre-enrichment procedure (OR = 1·4) and the combination of direct inoculation and pre-enrichment procedures (OR = 1·6) were both significant at or above the 99% level (Table 1). These estimates of OR represented increases in isolations of 40% and 60%, respectively. As with *Str. agalactiae*, increasing CMT scores were associated with increased OR. An interaction between sample type and CMT score appeared as a decrease in the chance of isolation for type 2 samples with a high CMT score (Table 2).

Isolation of mycoplasma. For type 1 milk samples, mycoplasma were isolated from 31 (1·1%) on direct inoculation, from 32 (1·1%) following pre-enrichment, and from 44 (1·6%) from either direct inoculation or pre-enrichment. For type 2 samples, there were 119 (3·2%) isolations on direct inoculation, 159 (4·3%) following pre-enrichment, and 203 (5·5%) from either direct inoculation or pre-enrichment.

The model generated by stepwise logistic regression indicated the same significant effects of sample type, CMT score and culture procedure ($P < 0·001$) as for *S. aureus*. There also was an interaction between type and CMT ($P < 0·001$). The goodness of fit of the model with these four terms was very good ($\chi^2 = 9·071$, D.F. = 18, $P = 0·958$). The adjusted OR was 1·3 for the pre-enrichment procedure, and 1·7 for the combined procedures, which represented a 30 and 70% increase in isolations, respectively. An interaction between sample type and CMT score appeared as an increase in the chance of isolation for type 2 milk with a low CMT score (Table 2). In contrast to models for *Str. agalactiae* and *S. aureus*, type 2 milk was associated with a significant increase in odds of isolation (OR = 4·3, Table 1).

DISCUSSION

This study found that pre-enrichment substantially improved the sensitivity of screening for *Str. agalactiae*, *S. aureus*, and mycoplasma in bovine milk. The greatest benefit from pre-enrichment was seen in detection of *Str. agalactiae*, for which a 3·6-fold increase in isolations, or an increase of 260%, was predicted with the use of both direct inoculation and pre-enrichment procedures. For the diagnostician attempting to decide if pre-enrichment is worthwhile, an expected increased number of infections detected using both procedures could be calculated using the OR and an estimate of the percent of cows detected by the usual method of direct inoculation only. For example, if screening only by direct inoculation were expected to identify infection in 10% of cows in a herd, use of both procedures would be predicted generally to isolate the bacterium from 36% of the cows.

It is anticipated that the actual OR for isolation of *Str. agalactiae* or *S. aureus*

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Table 1. *Odds ratios and confidence limits of isolating* Streptococcus agalactiae, Staphylococcus aureus *and mycoplasma associated with culture procedure, CMT score, and sample type, compared to direct inoculation from type 1 milk with a negative CMT score*

Variable	Odds ratio	Confidence (OR) limits
Streptococcus agalactiae		
Type 2	0·3	0·2, 0·5*
CMT trace	0·9	0·7, 1·8
CMT 1	1·5	1·1, 2·2†
CMT 2	2·9	1·9, 4·6*
CMT 3	9·5	6·3, 14·2*
Type 2 × CMT trace	1·6	1·1, 2·5†
Type 2 × CMT 1	2·0	1·4, 3·0*
Type 2 × CMT 2	1·7	1·1, 2·5*
Type 2 × CMT 3	0·8	0·9, 1·5
Enrichment procedure	3·3	2·1, 5·0*
Both procedures	3·6	2·4, 5·5*
Type 2 × enrichment procedure	0·8	0·6, 1·0†
Type 2 × both procedures	0·8	0·6, 1·0†
CMT trace × enrichment procedure	0·7	0·4, 1·2
CMT 1 × enrichment procedure	0·7	0·5, 1·1
CMT 2 × enrichment procedure	0·5	0·3, 0·9*
CMT 3 × enrichment procedure	0·5	0·3, 0·7*
CMT T × both procedures	0·7	0·5, 1·2
CMT 1 × both procedures	0·7	0·5, 1·0
CMT 2 × both procedures	0·5	0·3, 0·9*
CMT 3 × both procedures	0·5	0·3, 0·7*
Staphylococcus aureus		
Type 2	0·9	0·7, 1·1
CMT trace	1·6	1·2, 2·2*
CMT 1	2·1	1·7, 2·8*
CMT 2	3·9	3·1, 5·1*
CMT 3	7·4	5·9, 9·4*
Type 2 × CMT trace	0·9	0·7, 1·3
Type 2 × CMT 1	0·9	0·7, 1·2
Type 2 × CMT 2	0·5	0·4, 0·8*
Type 2 × CMT 3	0·2	0·2, 0·3*
Enrichment procedure	1·4	1·2, 1·6*
Both procedures	1·6	1·4, 1·8*
Mycoplasma spp.		
Type 2	4·3	1·8, 9·9*
CMT trace	1·6	0·6, 3·9
CMT 1	0·9	0·4, 2·1
CMT 2	6·4	2·7, 15·2*
CMT 3	5·0	2·0, 12·9*
Type 2 × CMT trace	0·5	0·2, 1·4
Type 2 × CMT 1	3·7	1·0, 12·5*
Type 2 × CMT 2	0·6	0·3, 1·2
Type 2 × CMT 3	0·6	0·3, 1·2
Enrichment only	1·3	1·0, 1·6†
Both procedures	1·7	1·3, 2·2*

* Significant at the 1% level. The 99% confidence limits are presented.
† Significant at the 5% level. The 95% confidence levels are presented.

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Table 2. *Number of isolates and odds ratios for combinations of procedure, sample type and CMT in the isolation of* Streptococcus agalactiae, Staphylococcus aureus *and mycoplasma from bovine milk*

	No. isolates			Odds ratio		
CMT	Direct	Enrichment	Both	Direct	Enrichment	Both
			Streptococcus agalactiae			
			Type 1 samples			
0	37	118	130	1·0	3·3	3·6
T	16	39	43	0·9	2·1*	2·3
1	49	120	130	1·5	3·5	3·8
2	75	124	141	2·9	4·8	5·2
3	208	275	296	9·5	15·7	17·1
			Type 2 samples			
0	17	41	45	0·3	0·8	0·9
T	12	18	21	0·3	0·8	0·9
1	39	58	66	0·5	1·7	1·8
2	45	61	77	0·9	2·0	2·1
3	111	144	159	2·9	3·0	3·3
			Staphylococcus aureus			
			Type 1 samples			
0	33	58	66	1·0	1·4	1·6
T	26	46	51	1·6	2·2	2·6
1	68	109	126	2·1	2·9	3·4
2	111	134	160	3·9	5·5	6·2
3	184	225	236	7·4	10·4	11·8
			Type 2 samples			
0	23	60	73	0·9	1·3	1·4
T	28	40	48	1·4	1·8	2·1
1	62	78	90	1·9	2·4	2·7
2	63	70	83	3·5	2·5	2·8
3	81	107	117	6·7	1·9	2·1
			Mycoplasma spp			
			Type 1 samples			
0	3	3	5	1·0	1·3	1·7
T	1	3	4	1·6	2·1	2·7
1	4	1	4	0·9	1·2	1·5
2	13	14	19	6·4	8·3	10·9
3	10	11	4	5·0	6·5	8·5
			Type 2 samples			
0	11	24	29	4·3	5·6	7·3
T	7	10	13	3·4	4·5	5·8
1	35	50	67	14·3	18·6	24·3
2	34	42	50	16·5	21·5	28·1
3	36	38	44	12·9	16·8	21·9

* Odds ratio was computed as the product of odds ratios for significant main and interaction effects as indicated in Table 1. For example, odds of isolating *Streptococcus agalactiae* from an enriched type 1 sample with a CMT trace was 2·1 [3·3 (enrichment) × 0·9 (CMT = T) × 0·7 (CMT = T × enrichment)] times that using the direct procedure on a type 1 sample with a negative CMT score.

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would vary from herd to herd. For herds in the early phases of the disease, with cows not yet showing a high CMT score or evidence of clinical mastitis (14), the OR would probably be lower than for herds with many cows and quarters chronically infected. This was suggested by the interaction found here with CMT score, where pre-enrichment had a less pronounced effect on improving detection for milk with a zero or 'trace' CMT score, than for milk with a score of 1, 2 or 3 (Table 2).

The combined use of both procedures could be employed at the onset of an eradication program as a highly sensitive screening test. At that time it may be desirable to reduce the prevalence of disease as rapidly as possible, even at the expense of a high false positive rate (15). Pre-enrichment would likely increase the rate of false positive tests because any *S. aureus* or *Str. agalactiae* contaminating the sample would be enhanced in numbers and could not be distinguished from those causing inflammation. Nevertheless, a highly sensitive test may be preferred to screen cows for further examination. Quarter samples of cows giving positive screening test results could be examined by a procedure of higher specificity to confirm or deny the presence of bacteria in the gland.

Although fewer isolates can be anticipated using direct inoculation only, most positive cows would be identified sooner if direct inoculation were included with pre-enrichment. Retention of a direct inoculation in screening, therefore, is recommended so that preliminary information can be disseminated as soon after sampling as possible.

The interactions identified by the statistical analysis indicated that, in assessing benefits of pre-enrichment for *Str. agalactiae*, consideration should be given to the type of sample and the CMT score. Results predicted that the chance of isolation was better for pre-enriched type 1 samples than for pre-enriched type 2 samples, which likely reflects infection status of herds making up type 1 samples. Type 1 samples were collected in the whole-herd screening of cows and probably consisted of a higher proportion of cows in early stages of infection, when low concentrations of *Str. agalactiae* may not be detected by direct inoculation (16). It also is possible that sample storage could explain the interaction because the freezing of type 2 samples may have decreased the number of isolations of *Str. agalactiae* (17). A practical application of this interaction between procedure and sample type is that pre-enrichment may be more appropriate (cost-effective) in an initial whole-herd screening of cows for *Str. agalactiae*, than during later phases of surveillance.

Another interesting interaction was found between pre-enrichment for *Str. agalactiae* and CMT. It appeared that pre-enrichment procedures were more beneficial in detecting *Str. agalactiae* from high CMT milk than from low CMT milk. The reason for this interaction is not known, but it may reflect a reduced ability for pre-enrichment to detect the low concentrations of the bacteria present during early stages of infection (16, 18).

It was apparent that several other factors may influence the likelihood of isolating either of the three bacteria from bovine milk studied. It cannot be determined from these results whether the effects associated with sample type were related to sample storage (freezing or refrigeration) and/or to the infection and inflammation status of the herds. Reduced numbers of mycoplasma would be expected if samples were frozen at temperatures higher than −70 °C and if the rate of cooling were low (19), as was the case in the present study. If freezing had

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reduced the concentration of mycoplasma below the level detectable by direct inoculation, but not below the level detectable using pre-enrichment, an interaction between sample type and procedure probably would have been apparent in the model. Because there was no interaction, the effect on mycoplasma isolations attributable to sample type probably was due to the fact that type 2 samples were from dairies with a known history of mycoplasma mastitis, whereas type 1 samples tended to be from herds mainly with mastitis caused by *Str. agalactiae* and *S. aureus*.

The low odds of isolating *S. aureus* from type 2 samples was not likely due to freezing because, in contrast to mycoplasma and *Str. agalactiae*, freezing may improve the chance of isolating *S. aureus* from milk (17). Instead, the low OR probably reflects the low level of *S. aureus* infections in type 2 herds, compared to type 1 herds that were only in the early stages of a program for controlling this bacterium.

It is not surprising that the sensitivity of detecting *Str. agalactiae* and *S. aureus* would be increased by pre-enrichment (8). Jasper (10), however, concluded that there is little advantage to pre-enrichment in detecting mycoplasma, even though early work on diagnosis of mycoplasma mastitis found pre-enrichment to increase the isolation rate (6, 9). In one study, a 9% increase in isolations, found using 0·01 ml of milk in broth, was considered too small to justify the additional costs of pre-enrichment (20). The comparatively high benefit of 30% (OR = 1·3) for pre-enrichment only and of 70% (OR = 1·7) for both procedures found here may be due to the large volume of milk (1 ml) added to broth, which would be expected to increase the probability of detecting bacteria in low concentrations (8). A potential problem encountered with enrichment, however, can be overgrowth by other bacteria (8).

Although overgrowth was not a major constraint in this study, it is conceivable that it could occur in samples not collected in a sanitary manner or in composite samples representing milk from several mastitic quarters. Presence of *S. aureus* or *Str. agalactiae* in the latter situation may be obscured by growth of other types of bacteria infecting the udder.

In summary, results presented here suggest that the combined use of direct inoculation and pre-enrichment may yield 260, 60 and 70% more isolates of *Str. agalactiae*, *S. aureus*, and mycoplasma, respectively, from bovine milk. Before this procedure is incorporated into mastitis control programs, consideration should be given to cost, increased sensitivity, decreased specificity, types of samples collected, the extent of herd udder infection and inflammation, and contaminant overgrowth.

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