Detection of *Staphylococcus aureus* in bulk tank milk using modified Baird-Parker culture media

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Abstract

The purpose of this project was to evaluate the use of 2 selective/differential culture media for detecting *Staphylococcus aureus* in bulk tank milk. One medium was Baird-Parker agar base supplemented with egg yolk tellurite emulsion and acriflavine. The other medium was Baird-Parker agar base supplemented with rabbit plasma/bovine fibrinogen and acriflavine. An increased inoculum of bulk tank milk (0.3 mL) was used to enhance the detection of *S. aureus* in samples containing low numbers of organisms. The sensitivity and specificity for detecting *S. aureus* in bulk tank milk were 94.8% and 100%, respectively, using Baird-Parker agar base supplemented with egg yolk tellurite emulsion and acriflavine, and 89.7% and 100%, respectively, using Baird-Parker agar base supplemented with rabbit plasma/bovine fibrinogen and acriflavine. Both media are practical for detecting *S. aureus* in bulk tank milk and monitoring its spread in lactating dairy herds in Alberta.

Résumé

Détection de *Staphylococcus aureus* dans le lait du réservoir en utilisant un milieu de culture modifié Baird-Parker

Le but de cette étude était d'évaluer deux milieux de culture sélectif/différentiel pour déceler la présence de *Staphylococcus aureus* dans le lait du réservoir. Le premier milieu de culture était composé d'agar Baird-Parker enrichie d'émulsion de tellurite de jaune d'œuf et d'acriflavine et le second, d'agar Baird-Parker enrichie de fibrinogène de bovin/de plasma de lapin et d'acriflavine. Un inoculum accru de 0,3 ml de lait a été utilisé pour améliorer la capacité à déceler *S. aureus* à partir d'échantillons renfermant une faible quantité d'organismes. La sensibilité et la spécificité de l'épreuve utilisant le milieu de culture Baird-Parker enrichi de jaune d'œuf et d'acriflavine étaient respectivement de 94,8 % et de 100 % et la sensibilité et la spécificité de l'épreuve utilisant le milieu de culture enrichi de fibrinogène et d'acriflavine étaient respectivement de 89,7 % et de 100 %. Les auteurs concluent que cette méthode de culture est valable pour déceler la présence de *S. aureus* dans le lait du réservoir et pour vérifier son étendu dans les troupeaux de vaches laitières en Alberta. *(Traduit par docteure Thérèse Lanthier)*

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Introduction

Staphylococcus aureus usually causes a chronic, subclinical mastitis in dairy cattle (1). It is a frequent isolate from mastitic milk samples in Alberta. Individual cow and bulk tank somatic cell counts (SCC) are a measure of the level of mastitis in a dairy herd, but do not indicate the cause of the mastitis. Therefore, the presence of *S. aureus* in a herd may go undetected by the dairyman until it has spread to a significant proportion of the lactating animals. As a result, implementation of the appropriate management procedures may be inadvertently delayed.

It is generally accepted that the primary source of *S. aureus* in a dairy herd is the udder of infected animals (2). Detection of this organism in bulk tank milk (BTM) indicates that infected lactating animals are present in the herd (3). However, culturing a single sample of BTM to detect the presence of *S. aureus* has a low sensitivity (4,5). For example, recent work done in Ontario indicated a sensitivity of 7% in using a single culture of BTM to detect the presence of *S. aureus* (6).

Bulk tank milk contains many environmental microorganisms originating from the surfaces of the teat skin and milking equipment (7). These organisms multiply when the temperature of the stored milk is temporarily raised by the addition of warm milk (8). When BTM is plated to blood agar medium, colonies of *S. aureus* are often overgrown by large numbers of these environmental microorganisms. This makes detection of *S. aureus* colonies very difficult, if not impossible.

Staphylococcus aureus is shed from infected udders intermittently and in relatively low numbers (9). Milk from infected cows is diluted out in the bulk tank with that from uninfected animals. As a result, the number of *S. aureus* organisms in BTM can be less than 100 colony forming units (CFU)/mL, the minimum detectable level when using an inoculum of 0.01 mL.

Use of a selective enrichment and/or differential culture medium has been valuable in detecting other pathogens in BTM (10,11). Several attempts have been made to develop a selective culture medium for detecting *S. aureus* in BTM or in other materials containing large numbers of mixed microbial flora (12–19). These studies have produced variable results.

The purpose of this project was to evaluate 2 modified selective culture media for detecting *S. aureus* in BTM.

Materials and methods

Two modifications of Baird-Parker medium were developed. One was Baird-Parker agar base, supplemented with rabbit plasma/bovine fibrinogen and acriflavine (BPRA). The other was Baird-Parker agar base, supplemented with egg yolk tellurite and acriflavine (BPA). Their formulations are summarized in Table 1.

Seventy-seven milk producers were selected on the basis of their proximity to the Edmonton Animal Health

Table 1. Formulae for Baird-Parker agar base, supplemented with acriflavine and egg yolk tellurite (BPA), and for Baird-Parker agar base, supplemented with acriflavine and rabbit plasma/bovine fibrinogen (BPRA)

Ingredients	BPA	BPRA
Distilled Water	1000 mL	900 mL
Baird-Parker agar base[a]	63 g	63 g
RPF Supplement[b]	—	100 mL
Egg yolk tellurite emulsion[c]	50 mL	—
Acriflavine hydrochloride[d]	1.05 mL	1 mL
Antifoam B emulsion[e]	1 mL	1 mL

[a]Catalogue # CM275, Oxoid Canada Inc, Nepean, Ontario K2E 7K3
[b]Catalogue # SR122, Oxoid Canada Inc
[c]Catalogue # SR54, Oxoid Canada Inc
[d]Catalogue # B27043-26, BDH Inc, Edmonton, Alberta T6B 2L8. The working stock of acriflavine hydrochloride (5 mg/mL) is sterilized and filtered. The final concentration of acriflavine hydrochloride in the medium is 5 μg/mL
[e]Catalogue # A5757, Sigma Diagnostics Canada, Mississauga, Ontario L4N 9Z9

Laboratory and their willingness to participate in the project. A composite foremilk sample was obtained by our technician from every lactating cow in each herd. The udders were washed and dried and the teat ends swabbed with 70% isopropyl alcohol. The first strippings were discarded, then approximately 10 mL of milk were collected from each quarter into separate sterile vials. In 61 of the 77 herds, this sampling was done at the 1st or 2nd milking into the bulk tank, after the emptying and sanitization of the tank.

After all the animals had been milked and the bulk tank had been agitated for at least 5 min, a 40 mL sample of BTM was obtained from the top of the bulk tank, using a disinfected stainless steel dipper, and deposited into a sterile vial. All milk samples were immediately chilled and then transported to the laboratory on ice.

The individual cow milk samples were kept refrigerated after reaching the laboratory and processed within 24 h. Samples were brought to room temperature and thoroughly mixed manually using a mechanical platform shaker for 5 min. An inoculum of 0.03 mL was spread on the surface of sheep blood agar (Columbia agar base, catalogue # CM331, Oxoid Canada Inc, Nepean, Ontario), supplemented with 5% v/v defibrinated sheep blood.

The BTM samples were frozen within 24 h of reaching the laboratory and then stored for a variable time at −80°C until processing. (Preliminary studies have shown that storage of BTM samples at −80°C for up to 6 mo do not have a major effect on the recovery of *S. aureus*.)

To culture the BTM samples, they were brought to room temperature (at least 1 h) in batches of 50 to 100. They were thoroughly mixed both manually and on a mechanical platform shaker for 5 min. An inoculum of 0.3 mL was spread confluently over both BPA and BPRA plates (15 × 100 mm) with a bent glass rod. Care was taken to keep the inoculum away from the edges of the plate. The lids were left ajar for about 30 min prior to eversion to allow for absorption of the inoculum.

All culture plates were incubated at 35°C for 48 h. Suspect colonies were confirmed as being *S. aureus* by both a positive tube coagulase test (coagulase plasma EDTA, Bacto/Difco, Detroit, Michigan, USA) and a positive hyaluronidase test. Staphylococcal colonies showing negative reactions for both tube coagulase and hyaluronidase were considered not to be *S. aureus*. If only 1 of these 2 tests was positive, or if atypical colony morphology was observed, ancillary tests were performed to identify the colony. These ancillary tests included a catalase reaction, slide coagulase test, Gram's stain, latex agglutination (Staphaurex, Wellcome Diagnostics, Dartford, England), and/or commercial identification panels for *Staphylococcus* spp. (API Staph or Vitek GPI, Biomerieux Vitek, Hazelwood, Missouri, USA). A milk sample, either bulk milk or individual cow milk, was considered positive if 1 or more colonies of *S. aureus* were identified.

Sensitivity of BTM culture in this study was defined as the proportion of positive herds that yielded *S. aureus* from a single BTM culture. Specificity of BTM culture was defined as the proportion of negative herds from which *S. aureus* was not isolated from a single BTM culture.

Kappa, the ratio of the difference between the observed and expected levels of agreement and the maximum possible agreement beyond chance, was used to evaluate how well the 2 culture media agreed in their ability to predict the *S. aureus* infection status of BTM. The McNemar chi-square (X^2) was used to determine whether the proportion of BTM samples testing positive for *S. aureus* on BPA was statistically the same as the proportion testing positive on BPRA.

The sensitivity and specificity for each of the 2 culture media were calculated for each of 8 cutoff values, which represented the percentage of cows known to be infected with *S. aureus* in each of the 58 infected herds. Each cutoff value was used arbitrarily to designate whether the herd was to be labelled positive or negative for *S. aureus*. The predictive values, positive and negative, for detecting *S. aureus* were calculated for both media using the sensitivity and specificity values generated above, and a prevalence of infected herds of 66.8%.

A single BTM sample from every dairy herd in Alberta was cultured on either BPRA or BPA culture medium. These BTM samples were part of the provincial regulatory Grade and Price Program and were obtained from the Central Milk Testing Laboratory during January to March 1993. They were stored at −80°C then processed in groups of 50 to 100 samples (as described above for BTM samples).

Results

Several herds had to be resampled due to microbial overgrowth on the blood agar plates. Following incubation on BPRA medium, *S. aureus* colonies were greater than 1 mm in diameter, muddy in appearance and varied in color from grey/black to grey/green to grey/brown. There was an opaque or cloudy zone of variable intensity and size surrounding each colony. On BPA medium, *S. aureus* colonies were similar in appearance, but were usually shiny or glistening, occasionally appeared dull or 'matt-like'. Colonies on BPA varied in color from black or charcoal to grey or grey/brown and sometimes had a clear, opaque or dark zone (halo) surrounding them.

Table 2. Sensitivity and specificity of BPA and BPRA for culturing *Staphylococcus aureus* in bulk tank milk (BTM)

Prevalence of infection in herd (%)	Number of infected farms	BPA sensitivity	BPA specificity	BPRA sensitivity	BPRA specificity
1 or more	58	94.8	100.0	89.7	100.0
2 or more	54	94.4	82.6	88.9	82.6
3 or more	49	98.0	75.0	93.9	78.6
4 or more	42	97.6	60.0	95.2	65.7
5 or more	40	100.0	59.5	97.5	64.9
6 or more	38	100.0	56.4	97.4	61.5
8 or more	33	100.0	50.0	100.0	56.8
10 or more	26	100.0	43.1	100.0	49.0

Table 3. Predictive values for culturing BTM from herds of varying infection levels of *S. aureus* on BPA, based on a prevalence of infected herds of 66.8% and using sensitivity and specificity values generated in Table 2

Sensitivity	Specificity	PVP[a]	PVN[b]
94.8	100.0	100.0	90.5
94.4	82.6	94.3	87.8
98.0	75.0	88.8	92.6
97.6	60.0	83.1	93.7
100.0	59.5	83.3	100.0
100.0	56.4	82.2	100.0
100.0	50.0	80.1	100.0
100.0	43.1	77.9	100.0

[a]PVP — Predictive value positive
[b]PVN — Predictive value negative

Table 4. Predictive values for culturing BTM from herds of varying infection levels of *S. aureus* on BPRA, based on a prevalence of infected herds of 66.8% and using sensitivity and specificity values generated in Table 2

Sensitivity	Specificity	PVP[a]	PVN[b]
89.7	100.0	100.0	82.8
88.9	82.6	91.1	78.7
93.9	78.6	89.8	86.4
95.2	65.7	84.8	87.2
97.5	64.9	84.8	92.7
97.4	61.5	83.6	92.3
100.0	56.8	82.4	100.0
100.0	49.0	79.8	100.0

[a]PVP — Predictive value positive
[b]PVN — Predictive value negative

Thirty-five hundred and seventy individual cow milk samples from 77 herds were cultured. Based on these results, 58 of the 77 herds were designated positive for *S. aureus* (75.3%) and 19 were designated negative. The mean number of lactating cows was 47 (s = 18) and 45 (s = 19) in the positive and negative herds, respectively. Five hundred and fifteen cows from the 58 positive herds were positive on culture for *S. aureus*. The number of animals infected on each farm ranged from 1 to 73 ($\bar{x}$ = 9.4, s = 13). The prevalence of infected cows ranged from 1.6% to 93.2% of the lactating animals ($\bar{x}$ = 20.7%).

Fifty-two of the 58 BTM samples from the positive herds were positive for *S. aureus* after culture on BPRA. All 6 BTM samples that were false negatives were from herds with a low proportion of infected animals (1/35, 1/44, 2/48, 1/33, 2/41, 2/74). The sensitivity and specificity of BPRA for detecting *S. aureus* in bulk milk were 89.7% and 100%, respectively.

Fifty-five of the 58 BTM samples from the positive herds were positive for *S. aureus* after culture on BPA. The 3 BTM samples that were false negatives were from herds containing low numbers of infected cows (1/35, 1/44, 2/48). These 3 samples were also negative for *S. aureus* on BPRA. The sensitivity and specificity of BPA for detecting *S. aureus* in bulk milk were 94.8% and 100%, respectively.

The results of the 2 culture media were in agreement on 74 of the 77 cultures. Twenty-two BTM samples did not show growth of *S. aureus* on either media, and 52 BTM samples were positive for *S. aureus* growth on both media. Kappa was 0.63, indicating that the 2 media were in agreement beyond a level expected by chance alone. The X^2 was insignificant; [(3-0)2]/3 = 1.33, indicating a high degree of agreement between the 2 media.

Table 2 summarizes the sensitivity and specificity calculations. The sensitivities were high at all cut offs used, but the specificity of both media falls below 50% when at least 10% of the herd must be infected in order to designate that herd as positive.

Tables 3 and 4 summarize the predictive values, positive and negative, for BPA and BPRA, respectively. Sensitivity increased and specificity decreased as the cutoff value was increased. These predictive values were very good, especially when the cutoff values for the percentage of infected cows in a herd was set at 1%. The rate of false positives increased greatly as more farms with higher infection levels were categorized as negative farms.

The results of culturing a single BTM sample from every dairy herd in Alberta are summarized in Table 5. Of the 1273 BTM samples cultured, 851 (66.8%) yielded at least 1 colony of *S. aureus* and were considered positive.

Discussion

Both BPA and BPRA are effective media for suppressing or inhibiting most environmental organisms present in BTM without jeopardizing the recovery of

Table 5. Results of culturing a single BTM sample from every dairy farm in Alberta for *S. aureus* on either of 2 modified Baird-Parker media

	BTM positive for *S. aureus*	BTM negative for *S. aureus*
Number of herds	851	422
Average SCC[a] (cells/mL)	237,800	117,400
s of SCC (cells/mL)	211,600	90,150
Minimum SCC (cells/mL)	27,000	28,000
Maximum SCC (cells/mL)	2,667,000	989,000

[a]Somatic cell count

S. aureus. However, they do allow the growth of some staphylococci other than *S. aureus* and occasionally other genera, such as, *Streptococcus* spp. and enteric bacteria.

The opaque or cloudy zone surrounding colonies of *S. aureus* on BPRA 'flags' the activity of coagulase, an enzyme produced by *S. aureus* but lacking in most other staphylococci. We found this opaque zone to be a reliable diagnostic marker for *S. aureus* colonies on BPRA.

The clear, opaque or dark halo, which may surround *S. aureus* colonies on BPA, flags a positive "egg yolk reaction" and is not specific for *S. aureus*. Other staphylococcal colonies can be "egg yolk-positive". As well, many strains of *S. aureus* found in BTM produce egg yolk-negative colonies. A trained and discriminating eye is required to achieve high recovery of *S. aureus* on BPA medium. This is because of the poor discrimination of *S. aureus* colonies from those of other *Staphylococcal* spp. Verification of all suspect *S. aureus* colonies is required.

The larger inoculum size of 0.3 mL permitted the detection of *S. aureus* in BTM containing as little as 4 CFU/mL, compared to 100 CFU/mL when using an inoculum of 0.01 mL, and provided excellent sensitivity for detecting *S. aureus* in BTM using either culture medium. Both culture media provide excellent sensitivity and specificity in herds with 1% of cows infected. This compares to reports where the sensitivity of a single bulk milk culture for detecting *S. aureus* ranged from 7.0% to 42.2% (4,5).

However, in our study, the lowest prevalence of lactating cows infected with *S. aureus* in positive herds was 1.6%. Bulk tank milk from large dairy herds with a very small number of cows infected with *S. aureus* can be expected to give some false negative culture results, because the number of *S. aureus* organisms in the BTM could be less than 4 CFU/mL, the approximate minimum detection limit for this culture technique. Culturing additional samples of BTM from these herds would increase the chance of detecting low levels of *S. aureus*. Strategic sampling and culturing of BTM after specific groups of animals have been milked into the tank may overcome the effect of diluting the number of *S. aureus* organisms. Strategic sampling could also be a valuable management tool for monitoring specific groups in the milking herd.

Although, in this study, the specificity of both BPA and BPRA was 100%, this level would not be expected with routine use of this BTM culture technique. Subsequent work in our laboratory with these media has indicated that false positive culture results can occur. Care must be exercised to eliminate inadequate sampling procedures. Using a contaminated dipper or milk vials and obtaining BTM samples from the drain valve on the bottom of the bulk tank have resulted in false positive culture results. Inadequate tank sanitation could also adversely affect the culture results.

The predictive value of BTM culture represents the probability that the culture result actually reflects the infection status of the herd. Even when only 1% of the herd is infected with *S. aureus*, the predictive values for both of these culture media are very good. This is because both the sensitivity and specificity criteria are high. The high predictive values indicate that a high degree of confidence can be placed on the *S. aureus* culture results using either medium.

Using a 0.3 mL inoculum on either BPA or BPRA is a highly reliable and effective method for detecting *S. aureus* in BTM. We prefer using BPRA, because it has a reliable presumptive *S. aureus* colony marker, which is lacking on BPA. Improved efficiency for *S. aureus* colony discrimination on BPRA, compared with BPA, reduces the time required for colony selection and verification. The disadvantages of BPRA include the cost and sporadic commercial availablility of the rabbit plasma/bovine fibrinogen supplement.

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CROSS-CANADA DISEASE REPORT

RAPPORT DES MALADIES DIAGNOSTIQUEES AU CANADA

Ontario

Neospora abortions in eastern Ontario dairy herds

Fifteen of approximately 80 cows in a dairy herd in eastern Ontario aborted during an 18-day period in January and February of 1994. Most aborting cows were 3 to 7-years old and aborted at 4 to 8-months gestation. Four fetuses were submitted to the Kemptville regional veterinary laboratory of the Ontario Ministry of Agriculture, Food and Rural Affairs for examination. All fetuses had lesions that were consistent with abortion due to *Neospora* spp., including multifocal nonsuppurative encephalitis, nonsuppurative skeletal and cardiac myositis, and necrotizing placentitis; hepatitis, nephritis, and pneumonia were inconsistently present. Formalin-fixed brain from 1 fetus tested positive in an avidin-biotin complex immunoperoxidase test, using *Neospora caninum* antiserum (Dr. D. Haines, Western College of Veterinary Medicine, Saskatoon, Saskatchewan).

Neosporosis was first diagnosed at the Kemptville regional veterinary laboratory as a cause of bovine abortion in eastern Ontario in 1993. From February 1993 to July 1995, neosporosis was diagnosed by histology and/or immunoperoxidase testing in 24 herds; it was suspected in 7 additional herds from which brains of aborted fetuses had not been submitted for histologic examination, but from which other fetal tissues had lesions compatible with neosporosis. Almost all cases occurred in dairy herds, and most affected herds had a history of multiple abortions; at least 2 herds had more than 10 abortions.

Neosporosis is considered a significant cause of bovine abortion. However, the life cycle and definitive host(s) of the parasite are unknown. Histopathology of the fetal brain is similar to that associated with protozoal infections in other species, such as *Toxoplasma gondii* in sheep (1). In one study of abortion in dairy cattle in California, 88 of 95 fetal brains with focal encephalitis reacted with antiserum to *Neospora caninum* to the immunoperoxidase procedure (1). Fecal contamination of feed by a carnivorous host was the suspected source of these infections.

Calves exposed in utero to *Neospora* spp. may be born with neurological signs, or develop them within a few days of birth. Some cows with a history of neospora fetal infection may abort again, or deliver neospora-infected calves in their next pregnancy (2).

Formalin-fixed brain (especially medulla), even if autolysed, heart, skeletal muscle, placenta and other tissues routinely submitted for diagnosis of abortions are essential for confirmation of neosporosis. At present, fetal and maternal serology are not considered as determinate as histopathology and immunohistochemistry in the diagnosis of this disease (3).

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