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ORIGINAL ARTICLE

Selective growth of *Staphylococcus aureus* from flushed dairy manure wastewater using acriflavine-supplemented mannitol salt agar

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Abstract

Aims: To investigate the use of mannitol salt agar (MSA) supplemented with acriflavine for selective growth and quantification of *Staphylococcus aureus* from flushed dairy manure wastewater (FDMW).

Methods and Results: Minimal inhibitory concentrations of acriflavine in MSA were determined by comparing the growth of *S. aureus* subsp. *aureus* (ATCC 33591) and *Staphylococcus epidermidis* (ATCC 155) in pure culture. Acriflavine concentrations of 1·3, 1·4 and 1·5 mg l^{-1} reduced CFU of *S. epidermidis* by 43%, 55% and 87%, respectively, while CFU of *S. aureus* subsp. *aureus* were only reduced by 15%, 20% and 26% at the respective concentrations of acriflavine. MSA supplemented with 1·5 mg l^{-1} acriflavine was tested for selective growth of indigenous *S. aureus* from three grab samples of FDMW. Acriflavine concentrations of 1·5 mg l^{-1} reduced background flora without significantly reducing ($P < 0·05$) indigenous *S. aureus* counts.

Conclusions: Acriflavine-supplemented MSA provides an effective media for selective growth and quantification of indigenous *S. aureus* from FDMW in the presence of high levels of background microflora.

Significance and Impact of the Study: *S. aureus* is implicated for mastitis infections in dairy cows. Therefore, a reliable means for monitoring and detecting the organism in FDMW provides a tool for measuring the effectiveness of treatment for reducing *S. aureus* levels and implementing flushwater recycling without affecting herd health.

Introduction

Mastitis causes an estimated $1·8 billion annual loss to US dairy operations (National Mastitis Council 1996) and controlling the transmission of mastitis is a major concern for dairy operations in the USA. *Staphylococcus aureus* is the leading cause of contagious bovine mastitis in the USA. Contagious bovine mastitis infections induced by *S. aureus* are usually chronic, subclinical and easily transmitted throughout the herd (Cullor 1993).

In warm climates of the USA, dairy operations utilize hydraulic flushing to remove manure from confinement barns and milking parlours. However, this practice creates large volumes of dilute manure wastewater. Typically, the wastewater undergoes primary treatment (i.e. mechanical screening, sedimentation or both) to remove coarse fibres and settleable solids. The resulting liquid fraction, flushed dairy manure wastewater (FDMW), can be recycled for barn flushing (Wilkie *et al.* 2004). Animals exposed to FDMW could potentially be infected by inherent concentrations of *S. aureus* (Janzen and Bishop 1983). Therefore, monitoring and detection of this organism in FDMW is necessary for developing intervention strategies to control transmission of *S. aureus* within the dairy operation.

Mannitol salt agar (MSA) is both a selective and differential medium for culturing staphylococci which was

originally described by Chapman (1945). The medium is selective because the presence of a high salt concentration (7·5%) suppresses the growth of most bacteria. However, other salt-tolerant bacteria are able to proliferate on this medium. This agar also contains mannitol, which serves as a differential agent. *Staphylococcus aureus* ferments the sugar alcohol to acidic by-products, lowering the pH of the agar. The reduction of pH is indicated by phenol red, resulting in a yellow halo around the colonies. Most coagulase-negative staphylococci are unable to ferment mannitol and colonies appear red on the agar, which are easily distinguished from yellow presumptive *S. aureus* colonies.

Staphylococcus aureus can be easily identified on MSA from samples with little to no contamination. However, samples with high concentrations of background flora can produce confluent growth on the media. Background flora in manure, such as *Lactobacillus* spp. and *Bacillus* spp. (Ouwerkerk and Klieve 2001), are salt tolerant and can proliferate on MSA. The use of evaporative cooling systems in dairy confinement areas (Fike *et al.* 2002) can potentially increase the concentration of *Staphylococcus epidermidis*, a common inhabitant on animal hides (Kloos 1980), in the wastestream and *S. epidermidis* can also proliferate on MSA. Additionally, samples containing high concentrations of dissolved organic matter, such as FDMW (Wilkie *et al.* 2004), provide a complex medium to support microbial proliferation, resulting in high concentrations of background flora. Confluent growth of background flora can mask or even limit the proliferation of *S. aureus* on MSA, making direct plate counting difficult and inaccurate. Enhancing the selectivity of MSA would provide a simple method for quantifying viable *S. aureus* in FDMW.

Enhanced selectivity of MSA for *S. aureus* could be achieved by the addition of a selective agent, such as acriflavine. Acriflavine is an acridine dye and a chromophore that displays antibacterial properties, by binding to DNA (Wainwright 2001). Acriflavine is effective against staphylococci, with the exception of *S. aureus* (Roberson *et al.* 1992).

Supplementation with acriflavine has been investigated to improve the selectivity of different types of media for *S. aureus*. Devriese (1981) supplemented Baird-Parker agar with a combination of acriflavine, polymyxins and sulphonamide. Media supplemented with various concentrations of acriflavine were tested for selectivity of *S. aureus* using swabs from the nares of calves, swine, rabbits and chickens. Selective growth of *S. aureus* on Baird-Parker agar was achieved using 7 mg l^{-1} of acriflavine. Ollis *et al.* (1995) used Baird-Parker agar supplemented with 5 mg l^{-1} of acriflavine to detect *S. aureus* in bulk milk tank samples. Glassmoyer and Russell (2001) studied impedance microbiology using nutrient broth supplemented with 10 mg l^{-1} of acriflavine and selective detection of *S. aureus* from frozen and fresh poultry products was achieved using the modified media.

Although previous studies have shown that acriflavine can be used as a selective agent in media for *S. aureus*, initial screening for presumptive *S. aureus* can be laborious and costly due to the lack of effective differential properties of previously studied media. By contrast, mannitol fermentation provides easy visualization of presumptive *S. aureus*. Preliminary work by the authors on quantifying *S. aureus* in FDMW using MSA was unsuccessful when higher levels of *S. epidermidis* appeared in summertime conditions corresponding with the operation of fan and sprinkler cooling systems. Initial attempts to introduce acriflavine into the MSA media reduced but did not eliminate *S. epidermidis* CFU. Finding a concentration that reduces *S. epidermidis* without significantly inhibiting *S. aureus* would allow continued quantification of *S. aureus* in FDMW throughout the summertime conditions. The purpose of the current study was to investigate the use of acriflavine-supplemented MSA for selective growth and quantification of indigenous *S. aureus* from FDMW.

Materials and methods

Bacterial cultures

A methicillin-resistant strain of *S. aureus* subsp. *aureus* (ATCC 33591) was chosen as a model for antimicrobial-resistant *S. aureus* prevalent in livestock manure (Pitkälä *et al.* 2004). *Staphylococcus epidermidis* (ATCC 155) was also included in the laboratory studies because preliminary studies showed that in the summer months there was an increase in background flora, including *S. epidermidis*, in FDMW from the University of Florida Dairy Research Unit (DRU), Hague, FL, USA. Pure cultures of each organism were incubated overnight at 37°C in nutrient broth (Difco). A bacterial suspension in 1X phosphate-buffered saline (PBS), pH 7, was made by harvesting the cells with centrifugation (8000 ***g***, 10 min) and washing three times with PBS.

Media preparation and minimal inhibitory concentration testing

Mannitol salt agar (Difco) was prepared according to the manufacturer's instructions. Acriflavine neutral (Acros Organics, Morris Plains, NJ, USA) was prepared as a 10 g l^{-1} aqueous solution and filter sterilized using a 0·22-μm syringe filter (Millipore, Bedford, MA, USA). The dye was added to the media at the following concentrations (mg l^{-1}): 1, 1·1, 1·2, 1·3, 1·4, 1·5, 2 and 5. Media prepared without acriflavine supplementation served as a control.

Triplicate bacterial suspensions of *S. aureus* subsp. *aureus* and *S. epidermidis* each were serially diluted using PBS. An aliquot of each dilution was spread onto supplemented media and control media in duplicate. All cultures were incubated at 37°C for 48 h.

Appropriate precautions were taken to prevent skin exposure and inhalation during preparation of acriflavine solutions and media. All used and unused media were autoclaved and disposed of as biohazardous material.

Flushed dairy manure wastewater sample collection

Three discrete FDMW samples were collected daily from the DRU, over a 3-day period. For each sampling event, three 1-l grab samples were collected from a pump sump leading to the dairy's wastewater treatment system. The samples were transported on ice and analysed in triplicate on the same day as collected.

Enumeration of *S. aureus* from FDMW samples

Each sample was serially diluted (1 : 100) using PBS and 0·1 ml was spread onto MSA supplemented with 1·5 mg l^{-1} acriflavine. Inoculated MSA without acriflavine supplementation served as a control. The plates were incubated as previously stated. To confirm the presence of *S. aureus*, where possible, 10% of all presumptive colonies were subjected to coagulase (Remel) and DNase (Remel, Lenexa, KS, USA) testing. Colonies that fermented mannitol and exhibited coagulase and DNase activity were considered to be *S. aureus*.

Statistical analysis

Average CFU values recorded from each media inoculated with FDMW were subjected to analysis of variance and Student's *t*-test with a significance level α of 0·05.

Results

Determination of minimal inhibitory concentration of acriflavine

Initial experiments were performed to determine the minimal inhibitory concentration (MIC) of acriflavine required to achieve selective growth of *S. aureus* on MSA. The percentage CFU reductions of *S. aureus* subsp. *aureus* and *S. epidermidis* were determined at each acriflavine concentration tested (Fig. 1). Acriflavine concentrations of 1·3, 1·4 and 1·5 mg l^{-1} reduced *S. epidermidis* by 43%, 55% and 87%, respectively. Using the same concentrations of acriflavine, *S. aureus* subsp. *aureus* was reduced by 15%, 20% and 26%. At 2 mg l^{-1} of acriflavine, *S. aureus* subsp. *aureus* and *S. epidermidis* were reduced by 96% and 99%, respectively. Both organisms were completely inhibited at 5 mg l^{-1} acriflavine.

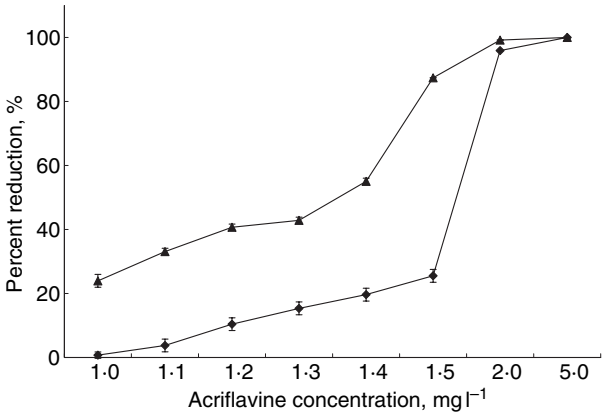

Figure 1 Percentage CFU reductions of *Staphylococcus aureus* subsp. *aureus* ATCC 33591 (◆) and *Staphylococcus epidermidis* ATCC 155 (▲) on mannitol salt agar supplemented with various concentrations of acriflavine. Each data point represents an average percentage reduction and error bars represent standard deviations of triplicate plates.

Selective growth of *S. aureus* from FDMW samples

From the MIC experiments, an acriflavine concentration of 1·5 mg l^{-1} was found to yield the highest reduction of *S. epidermidis* without greatly sacrificing *S. aureus* subsp. *aureus*. MSA supplemented with 1·5 mg l^{-1} acriflavine was tested for quantifying indigenous *S. aureus* from three discrete FDMW samples (FDMW samples 1, 2 and 3). Total colonies, presumptive *S. aureus* colonies and confirmed *S. aureus* colonies were determined (Table 1). The control media yielded confluent growth at the tested dilution of FDMW for all three samples. However, using the same dilution, supplementation with 1·5 mg l^{-1} acriflavine reduced the total number of colonies from all three FDMW samples. Figure 2 shows an example of the reduction of confluent growth on acriflavine-supplemented MSA. Additionally, the number of presumptive *S. aureus* colonies was similar to the number of confirmed *S. aureus* colonies for all three samples using 1·5 mg l^{-1} acriflavine (Table 1). These results suggest that, due to increased selectivity, the use of acriflavine minimizes the occurrence of false-positive colonies on MSA.

Discussion

Previously, increased background flora levels during summer confounded detection and quantification of *S. aureus* in the FDMW using MSA and *S. epidermidis* appeared as

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Table 1 Enumeration of *Staphylococcus aureus* colonies from flushed dairy manure wastewater (FDMW) samples using mannitol salt agar (MSA) with and without 1·5 mg l^{-1} acriflavine supplementation

Acriflavine concentration (mg l^{-1})	FDMW sample 1*			FDMW sample 2*			FDMW sample 3*		
	Total colonies	Presumptive *S. aureus*	Confirmed *S. aureus*	Total colonies	Presumptive *S. aureus*	Confirmed *S. aureus*	Total colonies	Presumptive *S. aureus*	Confirmed *S. aureus*
0·0	TNTC	TNTC	180 ± 23[a]	TNTC	4 ± 4[a]	2 ± 2[a]	TNTC	267 ± 52[a]	143 ± 21[a]
1·5	239 ± 29	169 ± 18	168 ± 17[a]	222 ± 57	164 ± 44[b]	165 ± 43[b]	165 ± 26	124 ± 15[b]	117 ± 32[a]

TNTC, Too numerous to count.

*Values are mean CFU ± SD ($n = 9$). Values with the same letter (a, b) designation within the same column are not significantly different ($P < 0·05$).

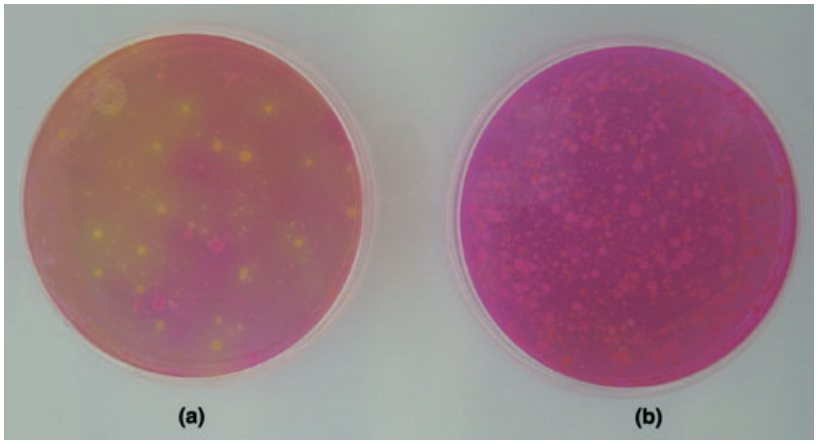

Figure 2 Reduction of confluent growth on acriflavine-supplemented mannitol salt agar (MSA): MSA supplemented with 1·5 mg l^{-1} acriflavine (a) and without acriflavine (b).

the principal interference. In this study, MSA supplemented with 1·5 mg l^{-1} of acriflavine was found to be an effective medium for selective growth of *S. aureus* from FDMW. The determination of MIC of acriflavine in MSA found that 1·5 mg l^{-1} acriflavine resulted in 26% and 87% CFU reductions for *S. aureus* subsp. *aureus* and *S. epidermidis*, respectively. The 26% CFU reduction was not reflected in field samples with indigenous FDMW organisms. As shown in Table 1, the number of confirmed *S. aureus* from supplemented media and control media from FDMW samples 1 and 3 did not differ significantly ($P < 0·05$), suggesting that indigenous *S. aureus* were not inhibited by acriflavine. In sample 2, detection of indigenous *S. aureus* was significantly higher on supplemented media compared with the control media. The advantage of using acriflavine in the current study was the reduction of confluent background growth (as shown by FDMW sample 2) and the reduction in the number of false presumptive colonies (as shown by FDMW samples 1 and 3).

In highly contaminated environmental samples, presumptive *S. aureus* colonies may be identified easily by reducing background flora. The current study showed the effectiveness of applying supplemented MSA to FDMW, where fluctuations in daily water volume and manure production resulted in varying sampling conditions (Wilkie *et al.* 2004). Our data has shown that confluent growth of background flora from FDMW can be dramatically reduced by the addition of 1·5 mg l^{-1} acriflavine.

The acriflavine resistance and mannitol fermentation in MSA offers advantages over another widely used media for culturing *S. aureus*, Baird-Parker agar (Baird-Parker 1962). Baird-Parker agar contains two selective agents, lithium and potassium tellurite. Tellurite and egg yolk constituents serve as differential agents to distinguish *S. aureus* from coagulase-negative staphylococci. The activity of lecithinase causes the production of halos to identify *S. aureus* colonies. However, the formation of black colonies and the halos formed by lecithinase activity of *S. aureus* are not distinctive in the presence of high concentrations of contaminating flora (De Buyser *et al.* 1998). Using Baird-Parker media, *S. aureus* cannot be distinguished from coagulase-negative staphylococci or background flora without further analysis (Schoeller and Ingham 2001). The inability to easily detect presumptive *S. aureus* colonies on the medium makes direct quantification of *S. aureus* from samples containing high concentrations of background flora difficult. Ollis *et al.* (1995) also reported

similar difficulties using Baird-Parker agar. By contrast, MSA supplemented with acriflavine allows for easy visualization and identification of presumptive colonies by individuals with minimal training in identifying *S. aureus*.

Another advantage of using acriflavine in MSA as opposed to Baird-Parker agar is nonreactivity with any of the constituents of MSA. In addition to nucleic acids, acriflavine also binds to proteins (Wainwright 2001). The dye binds to proteins found in the egg yolk supplement required for the preparation of Baird-Parker agar. Therefore, Baird-Parker agar requires higher concentrations of acriflavine to be effective. Previous studies (Devriese 1981; Ollis *et al.* 1995) have reported using higher concentrations of acriflavine in Baird-Parker agar when compared with the current study using MSA to achieve selective growth of *S. aureus*. A lower concentration of acriflavine was found to be effective in MSA, probably due to the low protein content of the media. Furthermore, the lower concentration required with MSA reduces the amount of the toxic dye to be handled by laboratory personnel.

Acriflavine-supplemented MSA can potentially reduce the time and labour for identifying *S. aureus*. Numerous auxiliary tests, including acriflavine-supplemented P agar (Roberson *et al.* 1992), are required for confirmation of *S. aureus* from clinical, food and environmental samples. The use of acriflavine in MSA could obviate the need for numerous confirmation tests due to the selective properties of the dye for *S. aureus* and the inherent selective and differential properties of MSA.

The ability to produce coagulase does not influence acriflavine resistance in *S. aureus*. Acriflavine is known to inhibit the growth of coagulase-negative staphylococci. However, coagulase-positive species of staphylococci, *Staphylococcus intermedius* and *Staphylococcus hyicus*, have been shown to be sensitive to acriflavine (Roberson *et al.* 1996). Acriflavine resistance in *S. aureus* is attributed to the presence of drug efflux genes, which are also involved in multiple drug resistance. These genes code for drug efflux pumps, which remove antiseptics and dyes from the bacterial cell (Paulsen *et al.* 1998). One such gene, *sepA* (staphylococcal efflux pump gene), codes for a protein that has been shown to effectively remove acriflavine from *S. aureus*, resulting in acriflavine resistance (Narui *et al.* 2002).

The current study has shown that MSA supplemented with 1·5 mg l^{-1} acriflavine is effective in selecting for *S. aureus* in complex wastewater samples containing flushed dairy manure. In the FDMW samples, the amount of background flora was reduced, increasing selectivity for *S. aureus* and allowing for this mastitic pathogen to be more easily detected and quantified in the wastestream. The enhanced selectivity of MSA by acriflavine supplementation may have potential application for other environmental samples where isolation or quantification of *S. aureus* is difficult due to confluent growth of background microflora.

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