RECOVERY AND ENUMERATION OF *STAPHYLOCOCCUS AUREUS* BY THE SELECTIVE AGAR OVERLAY METHOD

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MANUSCRIPT RECEIVED 2 JULY 2015; ACCEPTED 3 AUGUST 2015

ABSTRACT

Staphylococcus aureus is an example of a commensal bacterium responsible for emesis, acute diarrheal syndrome, and sepsis. S. *aureus* often must be isolated from patient samples in a clinical setting or from food samples during food processing in an industrial setting, although these bacterial cells may be injured by the human immune system or by food processing measures. Therefore, injured cells may not be fully recovered on media selective for S. *aureus* and enumeration (e.g., CFU/mL) may not reflect the true concentration of the original sample. The objective of this study was to determine whether the selective agar overlay method of recovery is more sensitive, selective, and time-effective for enumeration of artificially injured S. *aureus* cultures when compared to more traditional techniques. The selective agar overlay method involves pour plating S. *aureus* in non-selective medium, allowing the sample to incubate for a four hour recovery period, and then overlaying selective medium over the non-selective medium. Artificial injury of S. *aureus* cells was accomplished by treatment with carvacrol, an extract from oil of oregano. Our results indicated that carvacrol-injured S. *aureus* cells were recovered by the selective agar overlay at the same concentration as recovery on non-selective media, and at a significantly higher concentration than recovery on selective media. This method allows for more rapid and accurate diagnoses, and may be more cost-effective due to the reduction or elimination of false negative results.

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KEYWORDS

- *Staphylococcus aureus*
- Overlay
- Sublethally injured
- Carvacrol

INTRODUCTION

Staphylococcus aureus is a Gram-positive, ubiquitous, coccoid bacterium, which is a prevalent organism in the food and clinical microbiology arenas (1,10). This commensal organism is an opportunist and an emerging pathogen for both nosocomial and community-acquired infections (6).

Staphylococcus aureus enterotoxins, exotoxins, and hemolysin are responsible for emesis, acute diarrheal syndrome, and sepsis in humans (6,10). In addition, methicillin-resistant and vancomycin-resistant strains are troublesome because they are difficult to treat effectively (14).

A variety of media can be used to grow S. *aureus*, using both non-selective and selective techniques. However, the human immune response as well as food processing techniques may injure S. *aureus*, limiting growth and detectability in lab cultures, particularly if bacterial cells are not allowed to repair. Using a non-selective medium for isolating a pure bacterial culture is not warranted, as other species may grow (7). Employing a selective medium alone may be an improvement, but unfortunately, cells may not repair well enough to grow visible colonies on the medium (7), and may result in a false negative for presence of bacteria in the original sample. To solve this problem, enrichment of bacterial samples in broth is needed before plating onto selective media. However, enrichment in broth will not allow for accurate calculation of the original sample's bacterial cell concentration because only cells enriched in agar form countable colony forming units (CFU); each CFU represents one cell from the original sample. Therefore, the selective agar overlay method allows isolation of a pure strain and recovery of injured cells as well as calculation of the bacterial cell concentration from the original sample (13).

Unfortunately, methodology on the use of Mannitol Salt Agar (MSA; Hardy Diagnostics, Santa Maria, CA) as a differential and selective medium with clinical relevance (3) in the selective agar overlay method of enumerating S. *aureus* has not been documented. Others have found that Gram-positive Agar (GPA) was the most effective selective medium component of the overlay method for S. *aureus* (10). However, GPA is selective for all Gram-positive bacteria, not just S. *aureus*. Furthermore, the selective agar overlay method has been studied for S. *aureus* in the context of food microbiology but not for clinical microbiology (13).

Several stressors have the potential to sublethally injure S. *aureus* cells prior to recovery by the selective agar overlay method. Carvacrol, an extract from oil of oregano, increases the bacterial cell membrane permeability to potassium, which alters potassium and hydrogen concentration gradients. This disrupts ATP synthesis and leads to a decreased pool of ATP available within the cell (15). Carvacrol has the potential to be incorporated into the clinical arena in conjunction with antibiotics and, therefore, has been chosen as a clinically relevant stressor for this study.

Ultraviolet (UV) radiation injures bacterial cells by damaging DNA. UV light initiates a reaction between thymine bases in DNA to create a thymine dimer; two thymine bases are bonded together, which is not a normal base pairing. This mutation is stable and difficult for the cell to repair; therefore, the cell cannot transcribe its DNA normally and a loss of function occurs (11). UV light is used as a disinfecting measure in clinical settings and is, therefore, considered a clinically relevant stressor.

Hydrogen peroxide acts at the bacterial cell membrane as an oxidizer, which would alter the activity of certain bacterial membrane proteins (9). Hydrogen peroxide produced by immune cells can be used to combat bacterial infection; therefore, hydrogen peroxide is relevant in the clinical arena.

By stressing S. *aureus* cultures with carvacrol, UV light, and hydrogen peroxide, a stressor which is most useful for testing the selective agar overlay method can be chosen. In order to be effective for this assay, the selected stressor needed to sublethally injure cells and therefore produce significantly lower cell counts on non-selective media than counts for non-stressed cells. This stressor also needed to allow for consistent

recovery of enough injured cells in order to conduct reliable statistical analyses.

The goal of this study was to develop a time-efficient selective agar overlay method of enumerating S. *aureus* which is sensitive and selective in the clinical arena. The objectives of this study were: 1) to show the selective agar overlay method is significantly more sensitive to enumerate S. *aureus* than plating directly onto selective media, such as MSA, and 2) to show the selective agar overlay method is equally as sensitive, but more selective than plating S. *aureus* onto non-selective Tryptic Soy Agar (TSA; Teknova, Hollister, CA). We hypothesize that if selective overlay plating (TSA-MSA) allows for recovery of injured S. *aureus* and selects for S. *aureus*, then selective overlay CFU/mL will be significantly higher than direct selective plating (MSA-MSA) CFU/mL alone and comparable to direct non-selective plating (TSA-TSA) CFU/mL.

MATERIALS AND METHODS

BACTERIA

Staphylococcus aureus strain ATCC BAA-977 was obtained from Microbiologics (St. Cloud, MN). Cultures were incubated in Tryptic Soy Broth (TSB; Alpha Biosciences, Baltimore, MD) for 24h at 37°C. Optical density (OD) was measured as 0.46 using a Beckman Coulter DU 530 spectrophotometer at 550 nm (Beckman Coulter, Brea, CA). All laboratory work was performed at Ball State University.

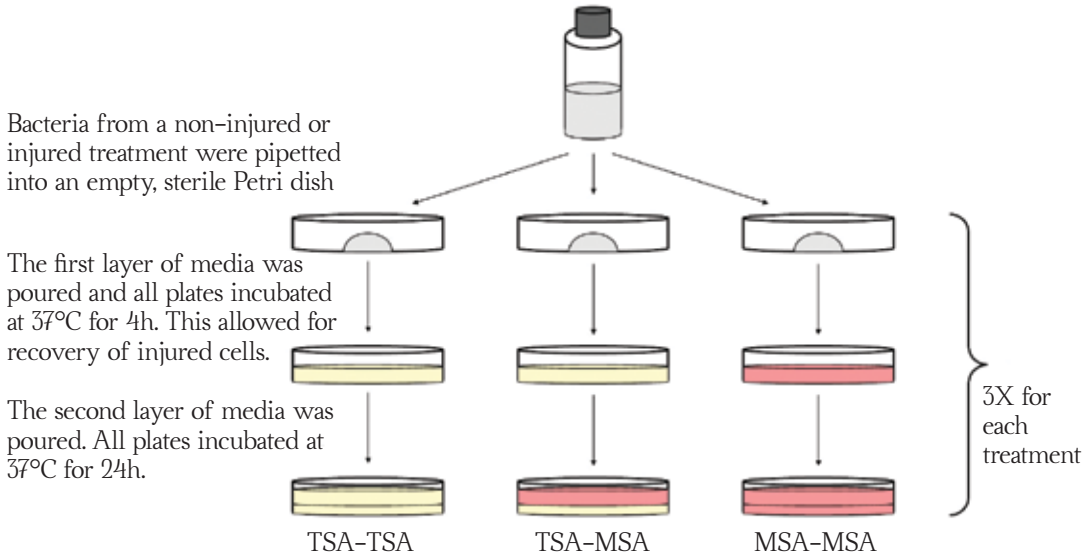
BACTERIAL INJURY

Staphylococcus aureus cells from TSB cultures were serially diluted 1:100 in sterile saline before undergoing stress treatments. Three stressors were tested: carvacrol (SAFC Supply Solutions, St. Louis, MO), UV light, and 3% hydrogen peroxide (VWR International, LLC, Radnor, PA). Our intent was to find a stressor that caused injury, but did not completely kill all cells. Carvacrol was added to the 10^{-2} diluted S. *aureus* cells at a volume of 100 uL, and these cells incubated for 5 min at room temperature. Serial dilutions up to 10^{-6} CFU/mL were then performed on the carvacrol-injured cells to determine efficacy. Injury by UV light was achieved by pouring cells from the 10^{-2} dilution to a layer of 5 mm thick into two separate, sterile Petri dishes. This allowed for injury by UV light for either 60 s or 90 s at room temperature, with agitation at every 30 s. Three percent hydrogen peroxide was added to the 10^{-2} diluted S. *aureus* cells at a volume of 5 mL, and these cells incubated for 5 min at room temperature. Serial dilutions up to 10^{-6} CFU/mL were then performed on the hydrogen peroxide-injured cells.

STRESSOR ANALYSIS

Carvacrol-injured, UV-injured (60s and 90s), hydrogen peroxide-injured, and 10^{-6} serially diluted non-injured (control) S. *aureus* cells from TSB cultures were pour plated with non-selective media (TSA) in accordance with methods used by Leboffe and Pierce (8). Plates were incubated 4 h at 37°C, and then overlaid with 20 mL of TSA. Plates were then incubated for 24 h at 37°C. Each of the five treatment groups was inoculated in triplicate. Colonies were counted and converted to CFU/mL.

Figure 1. Schematic of the methods used for recovery of *Staphylococcus aureus* cells by nonselective (TSA–TSA), selective (MSA–MSA), and selective overlay (TSA–MSA) plating techniques.



MEDIA ANALYSIS

Three base layer–top layer test media combinations were used in this study: non-selective (TSA–TSA), selective (MSA–MSA), and selective overlay (TSA–MSA). Non-selective plates and selective overlay plates used TSA as the base layer medium for pour plating, while selective plates used MSA as the base layer (Fig. 1). Non-injured *S. aureus* cells from TSB cultures were serially diluted to 10^{-6} CFU/mL and pour plated with the base layer of each test media (non-selective, selective, and overlay) and labeled. Carvacrol-injured *S. aureus* cells were pour plated at a 10^{-6} dilution with the base layer of each test media (non-selective, selective, and overlay). All plates were incubated 4 h at 37°C. Plates were then overlaid with a top layer of 20 mL of either TSA (non-selective plates) or MSA (selective overlay and selective plates). Plates were then incubated at 37°C for an additional 24 h after which colonies were counted and converted to CFU/mL. Each test media group for both non-injured and carvacrol-injured treatments was inoculated in triplicate.

STATISTICS

Two sets of statistical analysis were run. First, recovered *S. aureus* CFU/mL (response variable) were compared by a general linear model to determine how they were impacted by the four stressors and control (predictor variables). Once the most appropriate stressor was identified (i.e., carvacrol), *S. aureus* was subjected to the stressor and grown using non-selective, selective, and selective overlay media (predictor variables). Second, comparison of *S. aureus* CFU/mL (response variable) was done using a general linear model and a Tukey's honest significant difference test (Minitab 17, State College, PA). Significance was set at $p = 0.05$.

RESULTS

Concentration of recovered *Staphylococcus aureus* cells on non-selective media (TSA-TSA) varied depending upon the stressor type used (GLM, $F = 445.81$, $N = 15$, $P < 0.001$). Hydrogen peroxide-injured cell recovery was not statistically different from non-injured recovery, but was higher than carvacrol and UV-injured cell recovery. Carvacrol-injured were recovered at significantly lower concentrations than non-injured cells, but were recovered at significantly higher concentrations than UV-injured cells. UV-injured cells for both 60 and 90 seconds of exposure were recovered at significantly lower concentrations than all other treatment groups (Fig. 2).

Differences were shown in the recovery of carvacrol-injured *S. aureus* cells based on the media type. Cells were recovered from selective media at significantly lower concentrations than non-selective media and selective overlay (GLM; $F = 11.90$, $N = 9$, $P = 0.008$). Carvacrol-injured *S. aureus* cell recovery from selective overlay was not significantly different than recovery from non-selective media (Fig. 3).

Figure 2. *Staphylococcus aureus* density (CFU/mL) recovered on non-selective media (TSA-TSA) for five stressor treatments: non-injured, carvacrol-injured, hydrogen peroxide-injured, UV-injured for 60 sec, and UV-injured for 90 sec. Bars are 2 standard errors from the mean. Letters indicate significant difference as indicated by Tukey's honest significant difference test.

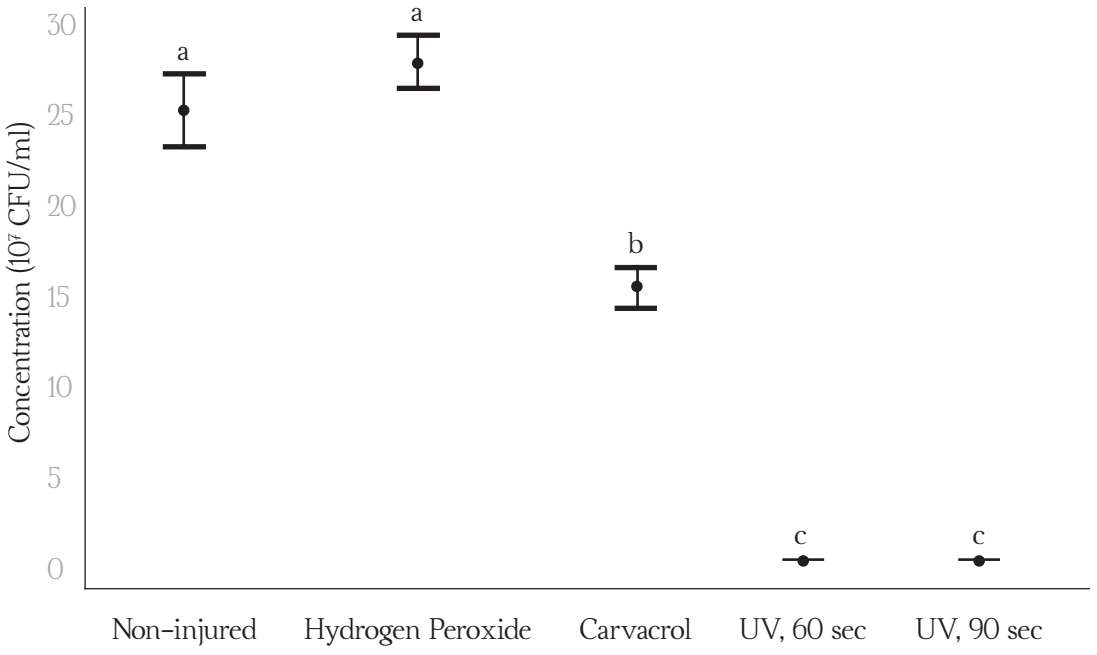
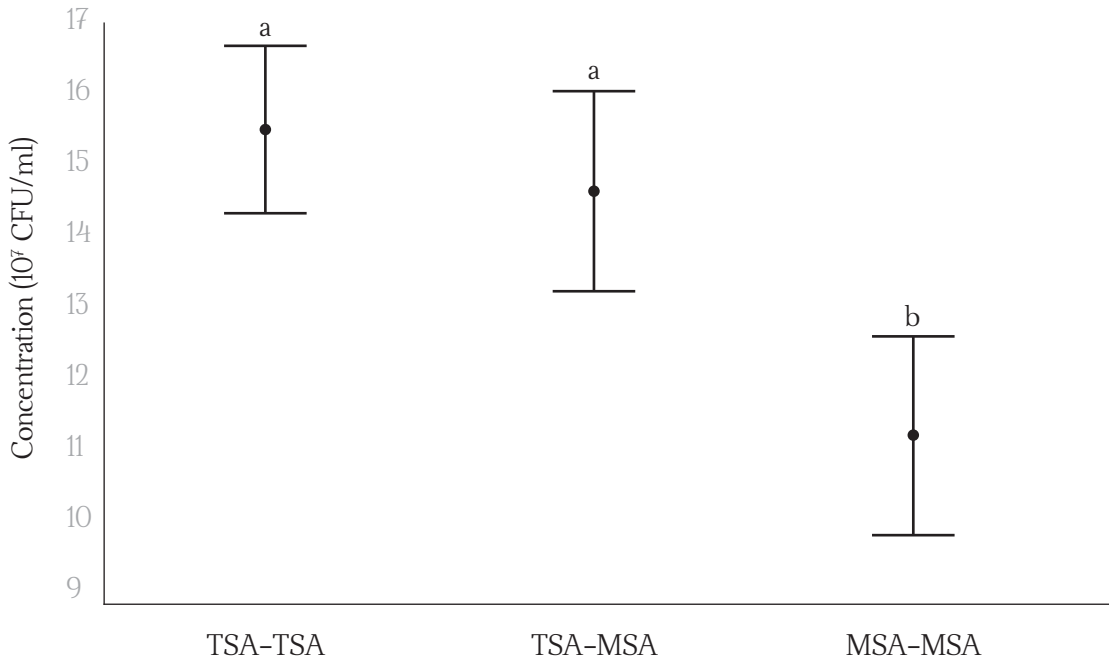


Figure 3. Carvacrol-injured *Staphylococcus aureus* concentration (CFU/mL) recovered on non-selective media (TSA-TSA), selective overlay (TSA-MSA), and selective media (MSA-MSA). Bars are 2 standard errors from the mean. Letters indicate significant difference as indicated by Tukey's honest significant difference test.



DISCUSSION

We hypothesized that using a selective agar overlay method would result in more accurate enumeration of viable cell counts of S. *aureus* compared to direct plating onto a selective agar. As hypothesized, the recovery of injured cells *via* the selective agar overlay method was comparable to recovery on non-selective media, but greater than recovery on selective media.

We intended to choose a stressor which would result in significantly less recovery of S. *aureus* cells on non-selective media than non-injured cells, and would allow for high enough recovery to ensure reliable colony counts. Hydrogen peroxide and UV light were not chosen as stressors for assessing the selective agar overlay because they did not meet this requirement. Hydrogen peroxide treatment allowed for recovery of enough cells to perform statistical analyses, but did not stress cells significantly from control, which is a necessity for ensuring the presence of sublethally injured S. *aureus*. UV-injured cells were recovered at a significantly lower concentration than non-injured cells, but viable cell counts from cells treated with this stressor were too low (fewer than 100 colonies per plate) to ensure consistency upon replication of the experiment. Carvacrol significantly stressed S. *aureus* cells compared to control, but not

to the extreme degree as seen in the UV treatments. In recent studies, carvacrol has been used to cease the synthesis of proteins that are important in bacteria such as *Escherichia coli* (2). Therefore, the selection of carvacrol to sublethally injure S. *aureus* was ideal for determining the effectiveness of the selective agar overlay method.

The selective agar overlay assay was effective because recovered carvacrol-injured viable cell counts on the selective agar overlay were not statistically different from non-selective counts. In addition, the selective agar overlay counts were statistically higher than selective media cell counts. This indicates that the four hour recovery period in the selective overlay method promotes sensitivity. Our findings and others (12) support the conclusion that the selective agar overlay method does allow for sensitive recovery of injured cells in the same manner that TSA would, but the overlay method does not compromise the selectivity of MSA for S. *aureus*. This was concluded because the recovered CFU/mL for the overlay method are comparable to non-selective CFU/mL but higher than selective CFU/mL.

The selective agar overlay method is a relevant detection method for the clinical microbiology arena. This assay is more sensitive than direct selective plating, but maintains the selective and differential nature of selective media. These traits together minimize the potential for false negative diagnoses of S. *aureus* infection and allow for effective enumeration of S. *aureus* in samples with multiple bacterial species. The selective agar overlay assay is also time-efficient, which is necessary to expedite detection of S. *aureus* cells in patients and to prevent long-term damage resulting from illness. This may reduce financial burdens that long-term hospital stays can place on patients and families. The selective agar overlay method has advantages compared to current clinical methods of S. *aureus* isolation, which are direct plating on selective media containing antibiotics and multiplex polymerase chain reaction (PCR; 4,5). As shown in our results, recovery of S. *aureus* on selective media results in lower enumeration compared to the original sample. Multiplex PCR is both rapid and sensitive, but it is extremely expensive and is a difficult technique to execute. The selective agar overlay method is inexpensive and can be performed correctly with minimal training.

Future studies may use alternative methods of stressing cells, including higher concentrations of hydrogen peroxide or less exposure time to UV. Additional *in vitro* and *in vivo* studies, using clinical samples and/or animal models, are required before application of this assay to a clinical setting.

ACKNOWLEDGMENTS

We thank Dr. Thomas Lauer for guidance and support in manuscript writing and editing. We also thank Dr. John McKillip for assisting with experimental design and for supplying materials. In addition, we acknowledge the Ball State University Department of Biology for funding the project.

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