Comparative Toxicity of Cryoprotectants on *Staphylococcus aureus* at Room Temperature

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Abstract

Staphylococcus aureus and Methicillin-Resistant *S. aureus* (MRSA) are important human and veterinary pathogens. Each year, thousands of researchers study these bacteria in detail; therefore, it is common practice for many *S. aureus* and MRSA isolates to be cryopreserved. Cryopreservation is a method by which bacterial samples are prepared and frozen for long periods of time, enabling future analysis and research. However, a comparison of different cryoprotectant methods for *S. aureus* preservation has not yet been done. The objective of this study was to determine which cryoprotectant is the most efficient in preserving *S. aureus*. Dimethyl sulfoxide (DMSO) is a commonly used cryoprotectant, but is also considered highly toxic to bacteria at room temperature. Alternative cryoprotectants such as 60% glycerol, a 50:50 solution of 7% milk and 60% glycerol, or a commercially available kit could be less toxic and thus better suited for cryoprotection. In our study, the four aforementioned cryoprotectants were compared by freezing eight strains of MRSA and two strains of Methicillin Susceptible *S. aureus* (MSSA) with each method. The samples were then thawed and allowed to sit at room temperature for up to 72 hours, with sampling occurring at predetermined intervals. The samples were serially diluted and plated on Mueller-Hinton agar, and colony-forming units were counted after 24 hours of incubation at 37°C to determine concentration and loss in bacterial population over time. Upon thawing, all samples showed a decrease in initial concentration of at least 80%. Within 72 hours at room temperature, the concentration of bacteria preserved with DMSO showed an additional decrease of 99.52% from the concentration determined at thawing. Samples preserved with glycerol, milk and glycerol solution, and the commercial kit decreased by 71.67%, 67.69%, and 35.10% respectively. No differences were observed between MRSA and MSSA. This suggests that using DMSO as a cryoprotectant is a potential risk because of its high bacterial toxicity. The use of glycerol, milk and glycerol solution, or a commercial kit is recommended based on their lower toxicities. Careful selection of a cryoprotectant is key to preserving valuable strains of *S. aureus* for future study.

Introduction

Staphylococcus aureus is a gram positive bacterium commonly found as part of the natural flora on the skin, skin glands, and mucous membranes of warm-blooded animals and humans. This bacterium is capable of becoming an opportunistic, blood-borne pathogen when it enters any open wound on the body (1). In hospitals and medical settings, multiple strains of Methicillin-Susceptible *S. aureus* (MSSA) and Methicillin-Resistant *S. aureus* (MRSA) have been the cause of nosocomial infections, representing a major public health issue (1, 3). In 2005, over 94,000 people were infected with MRSA, and over 18,000 people died due to MRSA infections (3). For the agricultural community, *S. aureus* not only represents a significant threat because it is one of the leading causes of mastitis in dairy cattle, but also because it has become an important emerging pathogen in animals (1). MRSA strains associated with these illnesses and deaths in both humans and animals are frequently preserved in bacterial banks to later study their phenotypic and genotypic characteristics. This allows for a better understanding of the molecular epidemiology of this pathogen. For example, based on the genotypic characteristics of these bacteria, one can determine whether an infection is from a hospital or community acquired strain.

Clones or related strains, outbreaks, and phenotypes such as antibiotic resistance may also be identified by using the strains that have been banked (2).

Long-term studies require these strains to be preserved, often with cryoprotectants, which lower the freezing point of cells and prevent formation of injurious intracellular ice crystals. A range of cryopreservation techniques are currently employed by laboratories, including use of dimethyl sulfoxide (DMSO), glycerol, milk/glycerol solutions, and commercially available kits. Prior research shows that cryoprotectants can be toxic to eukaryotic cells such as tobacco protoplasts and hamster fibroblasts, especially when the cells are allowed to thaw to room temperature and exposed to the cryoprotective chemical for a period of time (4, 5). However, we do not know how cryoprotectants will affect the survival of *S. aureus* in case of accidental or oversight situations in which storage samples are placed at room temperature for an extended period of time. Additionally, it was not known whether freezing would have a different impact on MSSA than on MRSA. This study addresses the survival of bacterial cells when exposed to different cryoprotectants at room temperature; the main purpose is to identify a cryoprotectant that provides better protection to the preserved strains in case of an extended exposure to room temperature. Since DMSO is considered highly toxic to bacteria at room temperature, then an alternative method such as the use of glycerol, a milk/glycerol solution, or a commercially available kit could be less toxic and thus better suited for cryoprotection. This information is valuable as many cryopreserved strains are irreplaceable and their destruction could mean the loss of many years of research and labor.

Materials and Methods

Two strains of MSSA and eight strains of MRSA were selected from the American Type Culture Collection (ATCC) and the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) for this research project. These strains were chosen because they are phenotypically and genotypically characterized worldwide control strains. First the selected strains were refreshed in blood agar plates to verify viability and to strengthen. Each individual strain was then frozen using four different methods of cryopreservation. These cryopreservation methods included use of the following cryoprotectants: dimethyl sulfoxide (DMSO), 60% glycerol, a 50:50 solution of 7% skim milk and 60% glycerol, and the commercially available CryoBank™ Bead System (Copan Diagnostics, Murietta, California) that contains both a proprietary cryoprotectant solution and beads to prevent large ice crystal formation.

Freezing Methods. For DMSO and glycerol, a 2 mL Eppendorf tube was filled with 550 µL of trypticase soy broth (TSB) and then inoculated with 1-3 colonies of a pure bacterial strain grown on blood agar plates. The tubes were incubated at 35°C for 24 hours. After the incubation, and prior to the addition of the cryoprotectant, 50 µL of each sample was collected to determine the initial concentration of colony forming units (CFU/mL) obtained in each tube. Then, 400 µL of DMSO or 500 µL of 60% glycerol solution was added to the broth. The samples were immediately frozen at -80°C. For the milk/glycerol solution, a 2mL Eppendorf tube was filled with 500 µL of TSB and inoculated with 1-3 colonies of a pure bacterial strain grown on blood agar plates. The tubes were incubated at 35°C for 24 hours. 50 µL of each sample was again taken to determine the initial concentration of CFUs/mL. Then, 500 µL of a solution containing a 50:50 mixture of 7% skim milk and 60% glycerol was added to each tube, and the samples were immediately frozen at -80°C. For the commercially available kit, a 2 mL Eppendorf tube was filled with 550 µL of TSB and inoculated with 1-3 colonies of a pure bacterial strain grown on blood agar plates. The tubes were incubated at 35°C for 24 hours. 50 µL of each sample was

again taken to determine the initial concentration of CFUs/mL. Then, 500 µL of the inoculated TSB was pipetted into the commercially available kit vials and immediately frozen at -80°C.

Experimental Design. After one week of freezing, each tube was removed from the freezer and allowed to thaw to room temperature (23°C). At 0 minutes, 30 minutes, 1 hour, 6 hours, 12 hours, 24 hours, and 72 hours after removal from the freezer, a sample was taken of each isolate and serially diluted to 10^{-8} of their original concentration. Each dilution was plated and evenly spread on the surface of Mueller-Hinton agar plates and incubated at 35°C for 24 hours. CFUs were then counted, and the concentration of bacteria was calculated for each sample over a period of 0 to 72 hours. CFUs were determined by multiplying the number of colonies present in the least diluted plate able to be counted by the dilution factor of the sample. Plates with more than three hundred colonies were considered uncountable.

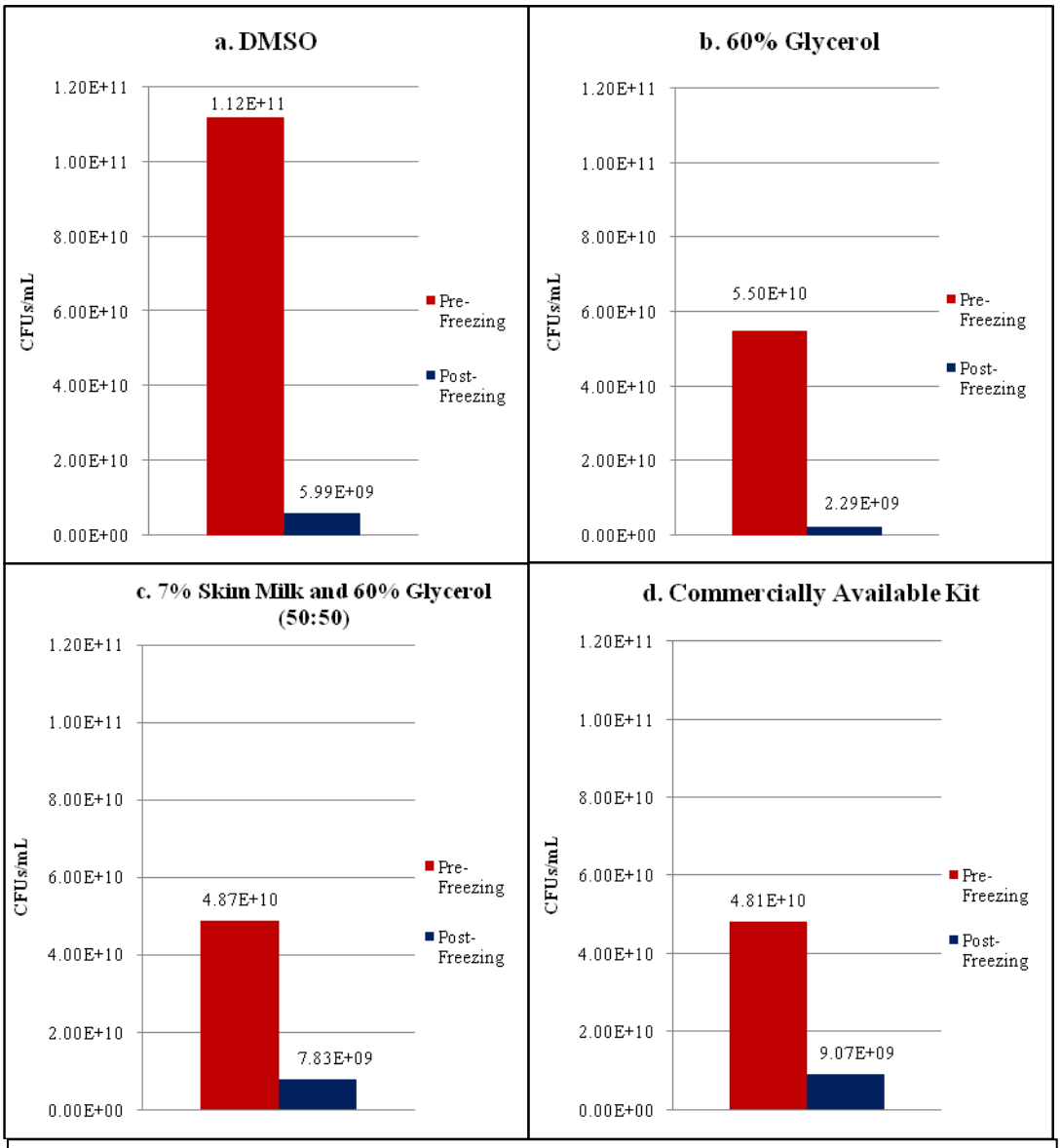

Figure 1. Comparison of average concentrations of bacteria before freezing and at 0 minutes after removal from the freezer. **a.** The population of bacteria treated with DMSO decreased by 94.65%. **b.** The population of bacteria treated with glycerol decreased by 95.83%. **c.** The population of bacteria treated with milk/glycerol solution decreased by 83.92%. **d.** The population of bacteria treated with the commercially available kit decreased by 81.13%.

Results

DMSO. The average pre-freezing concentration of bacteria frozen with DMSO was 1.12×10^{11} CFUs; immediately post-freezing, at 0 minutes, the average concentration was 5.99×10^{9} CFUs. The difference in the quantity of CFUs between pre and post-freezing indicates a loss of 94.65% of the original bacterial population just due to freezing (Fig. 1a). Within 72 hours of exposure to DMSO at room temperature, 99.52% of the bacterial population that survived freezing was also dead, for a final average bacterial population of 2.85×10^{7} CFUs (Fig. 2a).

60% Glycerol. The average pre-freezing concentration of bacteria frozen with 60% glycerol was 5.50×10^{10} CFUs; immediately post-freezing, at 0 minutes, the average concentration was 2.29×10^{9} CFUs. This indicates a loss of 95.83% of the original bacterial population just due to freezing (Fig, 1b). Within 72 hours of exposure to 60% glycerol at room temperature, 71.67% of the bacterial population that survived freezing was also dead, for a final bacterial population of 6.50×10^{8} CFUs (Fig. 2b).

50:50 Solution of 60% Glycerol and 7% Skim Milk. The average pre-freezing concentration of bacteria frozen with the milk/glycerol solution was 4.87×10^{10} CFUs; immediately post-freezing, at 0 minutes, the average concentration was 7.83×10^{9} CFUs. This indicates a loss of 83.92% of the original bacterial population due to freezing (Fig. 1c). Within 72 hours of exposure to the milk/glycerol solution at room temperature, 67.69% of the bacterial population that survived freezing was also dead, for a final bacterial population of 2.53×10^{9} CFUs (Fig. 2c).

Commercially Available Kit. The average pre-freezing concentration of bacteria frozen with the commercially available kit was 4.81×10^{10} CFUs; immediately post-freezing, at 0 minutes, the average concentration was 9.07×10^{9} CFUs. This indicates a loss of 81.13% of the original bacterial population due to freezing (Fig. 1d). Within 72 hours of exposure to the commercially available kit at room temperature, 35.10% of the bacterial population that survived freezing was also dead, for a final bacterial population of 5.89×10^{9} CFUs (Fig. 2d).

Concentrations of MSSA and MRSA frozen with the same cryoprotectants were very similar, indicating that there was no significant difference between MSSA and MRSA during the freezing process.

Discussion

A comparison of different cryopreservation methods for *S. aureus* has never been done before. Therefore, the main purpose of this study was to determine which commonly used cryoprotectant is the least toxic to *S, aureus* at room temperature, and therefore the most efficient for preservation. We compared four different cryopreservation methods, including DMSO, glycerol, milk/glycerol solution, and a commercially available kit that contained beads.

All cryoprotectants underperformed when we compared the pre-freezing and post-freezing bacterial concentrations. Samples treated with DMSO and glycerol only had about 5% of their original populations alive upon thawing. The milk/glycerol solution and the commercially available kit performed better, with almost up to 20% of the bacteria surviving the freezing and thawing process. Although enough bacteria survived in all cases to establish new samples, there is some concern that important plasmids, perhaps bearing genes for antibiotic resistance, could be lost due to the stresses of freezing and thawing. Thus, the population that we originally froze could potentially differ from the population remaining after freezing due to loss of plasmids. Therefore, it is important that bacteria preserved with cryoprotectants survive the freezing and thawing process with minimal decreases in population.

Upon exposure to cryoprotectants for 72 hours, decreases in bacterial population were observed in all protocols. As hypothesized, samples treated with DMSO experienced a drastic reduction in concentration over the 72 hour exposure period due to its high toxicity to bacteria. By 72 hours at room temperature, less than 0.48% of the bacteria that survived freezing and thawing is still viable. This sample could be at high risk to be lost since the amount of bacteria is dangerously low. Glycerol had slightly better results than DMSO at room temperature. Sharp decreases in population are not observed in glycerol as in DMSO, indicating that glycerol is less toxic to bacteria at room temperature. By the end of the 72 hour exposure period, 28.33% of the bacteria that survived freezing and thawing are still viable. The milk/glycerol solution fared even better than the glycerol. Although there is an initial decrease in population within 30 minutes of exposure to the solution at room temperature, the population appears to recover within 60 minutes (Fig. 2c). This is followed by a steady decline in population until the 72 hour exposure period ended, resulting in up to 32.31% of bacteria surviving after freezing and thawing. The increase in population after 30 minutes could be due to the bacteria utilizing the nutrients found in milk, resulting in a surge in population. The decline could be a result of acidic or otherwise toxic metabolic products produced by the bacteria during consumption of milk nutrients. These additional toxins produced by the bacteria helped to contribute to the steady decline in population. The commercially available kit fared the best out of all the cryoprotectants, with over 64.90% of the bacteria surviving freezing and thawing dying alive after exposure to the kit for 72 hours at room temperature. It is interesting to note that at 6 hours and 24 hours of exposure, the bacterial populations decrease and then appear to recover again (Fig. 2d). This may be due to the presence of both nutrients and buffers in the commercially available kit. Nutrients could help boost bacterial population as it declines, as in the case of the milk/glycerol solution, and buffers could help combat the toxic effects of acids produced by the bacteria while consuming the nutrients. Further study of the effects of the commercially available kit and the milk/glycerol solution is needed to verify.

Recommendations

Based on the results of this study, 60% glycerol, a 50:50 mixture of 60% glycerol and 7% skim milk, or a commercially available kit are recommended for use as cryoprotectants, especially in scenarios where accidental thawing of samples is a concern. The commercially available kit performed better than the other methods used in this experiment, and the milk/glycerol solution is suggested as an alternative because it also performed well. Use of DMSO as a cryoprotectant represents a higher risk due to its high toxicity to bacteria at room temperature. When choosing a cryoprotectant, it is important to consider its toxicity to bacteria, in addition to other concerns such as cost and practicality within the laboratory. Glycerol is less expensive and easy to use, whereas the milk/glycerol solution and the commercially available kit are more labor intensive and/or more expensive, but also more effective. Careful selection of a cryoprotectant can effectively preserve laboratory samples for years.

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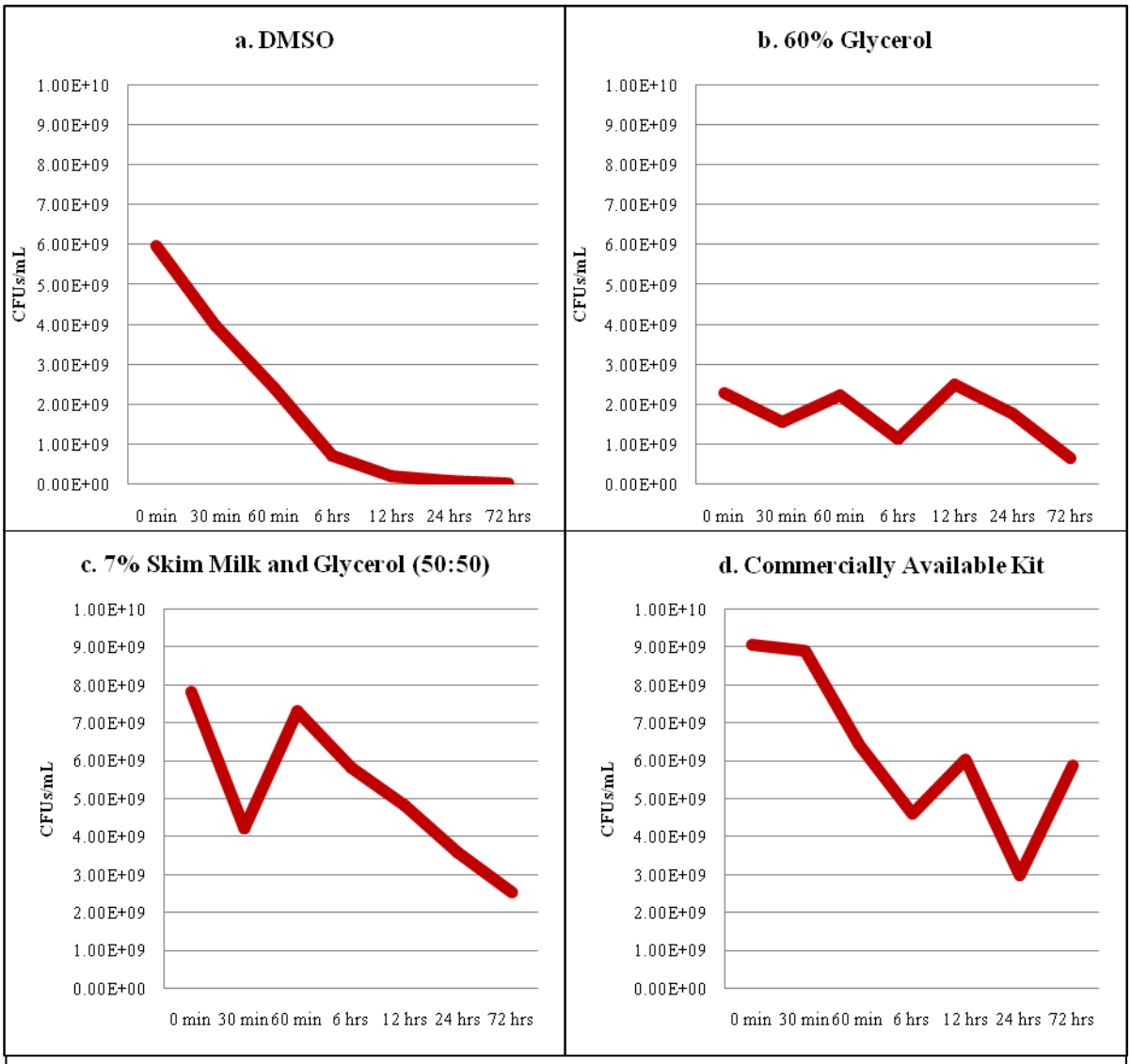

Figure 2. Comparison of decrease in CFU concentration over a period of 72 hours of exposure to cryoprotectants at room temperature: **a.** DMSO, **b.** 60 % glycerol, **c.** a 50:50 solution of 60% glycerol and 7% skim milk, and **d.** the commercially available kit.

References

1. Crossley, K. B., and Archer, G. L. 1997. The Staphylococci in Human Disease. Churchill Livingston, Inc. New York, NY, pgs. 1-38..
2. Keane, C. T. 1992. Methicillin-Resistant *Staphylococcus aureus* (MRSA): An Overview, in Cafferkey, M. T., ed., Methicillin-Resistant *Staphylococcus aureus*: Clinical Management and Laboratory Aspects. Marcel Dekker, Inc. New York, NY, pgs. 1-20.
3. Klevens, M. K., Morrison, M. A., Nadle, J., et al., Invasive Methicillin-Resistant *Staphylococcus aureus* Infections in the United States, (http://www.cdc.gov/mrsa/pdf/InvasiveMRSA_JAMA 2007.pdf), 26 November, 2010.

4. Muldrew, K., and McGann, L. E., Cryoprotective Compounds, (http://people.ucalgary.ca/~kmuldrew/cryo_course/cryo_chap8_1.html), 9 November, 2010.
5. Steponkus, P. L., Langis, R., and Seizo, F. 1992. Cryopreservation of Plant Tissues by Vitrification. Advances in Low Temperature Biology vol 1: pgs 1-61.