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Viability of *Methicillin-Resistant Staphylococcus aureus* (MRSA) Bacteria Preserved at Different Temperature and Time

Sekar Karima Rachma[1], Kurniawan[1*], Ikhsan Mujahid[1] and Kurnia Ritma Dhanti[1]
[1]Program Studi Teknologi Laboratorium Medik D4, Fakultas Ilmu Kesehatan, Universitas Muhammadiyah Purwokerto Banyumas, Indonesia, 53182.
*Corresponding author: kurniawan@ump.ac.id

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Abstract: *The bacteria Staphylococcus aureus, a normal component of human flora, is easily mutated and antibiotic-resistant, leading to the creation of Methicillin-Resistant Staphylococcus aureus (MRSA). This bacterium is a difficult-to-control nosocomial infection in healthcare settings, so it must be stored using specialized preservation agents, like DMSO, to investigate the nature and characteristics of these bacteria for prevention, control, and therapy. The purpose of this study was to evaluate the survival of MRSA bacteria that had been kept in a DMSO solution at various temperatures and periods. This study was carried out between July and October2022 using a one-shot study case research design, data analysis, and quantitative descriptive analysis. According to the findings, MRSA bacteria held in 15% DMSO solution lost viability over time and at various storage temperatures. Among various bacterial species held at various storage temperatures, MRSA bacteria had the lowest colony loss rate (-20 °C). Based on the findings of this study, it can be said that the preservation of MRSA bacteria at freezing temperature (-20 °C) and storage length of one month resulted in the greatest survivability of the bacteria when stored in 15% DMSO solution.*

Keywords: *Methicillin-Resistant Staphylococcus aureus (MRSA), preservation, viability, and DMSO*

1. INTRODUCTION

Staphylococcus aureus is a member of a group of Gram-positive bacteria that make up the normal flora of the skin, glands, and mucous membranes in warm-blooded animals and humans (Dibah *et al*., 2014). As the name implies, this bacterium has a coccal (round) cell shape with a grape-like arrangement. At the same time, the bacterial colonies are large and round with a diameter of 1-3 mm, convex, opaque, and yellow. *S. aureus* bacteria grow aerobically and anaerobically (facultatively) with a temperature range of 18-40 ℃ and can grow in media with a high salt content (10%) (Taylor & Unakal, 2022).

S. aureus bacterium is one of several bacterial species that have the property of changing quickly (mutating) so that it is straightforward to develop resistance to antibiotics, for example, the strain of *Methicillin-Resistant Staphylococcus aureus* (MRSA) bacteria. These bacterial strains are known to act as opportunistic pathogenic bacteria that cause nosocomial infections in hospitals and other health facilities (Dibah *et al*., 2014; Soltys *et al*., 2012). The MRSA strain of bacteria shows different microbiological, therapeutic, and clinical features when compared to other groups of bacteria that are sensitive to the antibiotic methicillin because it has a gene that can encode resistance to all types of antibiotics from the β-lactam group, making it difficult to control (Kurniawan *et al*., 2021).

As a strain of pathogenic bacteria in humans that is difficult to control, this MRSA bacterium needs to be preserved as a collection of cultures in bacterial storage banks so that later it can be explored and studied concerning its phenotypic and genotypic characteristics for prevention,

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control and treatment due to bacterial infection. This preservation effort will provide a better understanding of various aspects, including the morphological, biochemical, physiological and molecular characters of this MRSA bacterium (Soltys *et al*., 2012).

Bacterial preservation is maintaining and storing bacterial cultures to remain alive and not change their nature and character. The mechanism for preserving these bacteria is to provide particular treatment to the bacterial culture so that the metabolic activity in the bacterial cells will decrease without a decrease in viability so that the recovery process and its survival remain high (Fitriana, 2019). Preserving a bacterium is essential for every laboratory, especially research laboratories. This effort requires commitment and hard work from all parties because bacterial cultures' metabolic capabilities and authenticity must be appropriately maintained (Susilawati & Susiawati Purnomo, 2016).

When viewed based on the period, bacterial preservation can be divided into long-term and short-term preservation. Generally, each laboratory carries out short-term preservation of bacteria for routine purposes such as practice. In contrast, long-term preservation is carried out to store bacterial cultures of research results that have potential and can be developed or utilized. More than 20 types of bacterial preservation methods have been identified, of which they can be grouped into four categories, namely preservation with periodic subcultures, drying, freeze-drying, and cryopreservation.

Out of these four categories, the best methods suggested by the researchers are freeze-drying and cryopreservation. According to Missiakas and Schneewind (2018), *S. aureus* bacteria are best preserved using the cryopreservation method using a temperature of -80 °C. This is related to the advantages or advantages possessed by this method, such as its long storage time (> ten years) without experiencing significant changes in phenotypic and genotypic characters. However, the facts on the ground show that most laboratories cannot preserve bacteria using this method, considering that this cryopreservation method requires complete equipment and infrastructure, high costs, complex and complicated operations, large and stable energy requirements, and the support of educated and trained human resources (Guo *et al*., 2020).

Several bacterial preservation methods require a preservative agent that can lower the freezing point of cells and prevent the formation of ice crystals inside the cells so that the bacterial cells do not suffer damage. Some of these agents include Dimethyl Sulfoxide (DMSO) solution, glycerol, a mixture of milk and glycerol, and preservation kits that are available and sold commercially (Soltys *et al*., 2012).

DMSO has been widely used as a preservation agent for mammalian and bacterial cells due to its low cost and low cytotoxicity. DMSO could reduce the electrolyte concentration in the residual solution and, at a specific temperature, does not freeze in and around cells (Jang, 2017). In addition, DMSO can also prevent the formation of water crystals in cells by increasing the concentration of intracellular solutes, thereby helping the vitrification of water at low temperatures (Whaley *et al*., 2021).

The concentration of DMSO solutions widely used for bacterial preservation is 10%; however, there has yet to be an absolute consensus regarding the optimal concentration. Therefore, determining the optimal concentration of DMSO in bacterial preservation is important because, according to Siddiqui *et al.* (2016), DMSO solution can cause severe adverse reaction to bacterial cells due to its cytotoxic properties.

According to Cabello-Olmo *et al.* (2020), time and temperature can affect the survival of bacterial cells during the preservation process, so variations and manipulation of ambient temperature need to be considered so that bacterial viability can be maintained and not lost. High temperatures can significantly reduce bacterial viability. Conversely, low temperatures have been reported to be used for bacterial preservation. Therefore, choosing the optimal temperature during the bacterial preservation process is very important to maintain the viability of MRSA bacteria. This is also related to the laboratory's sustainability of the MRSA bacterial stock.

Based on the selection of the temperature and time of preservation of MRSA bacteria, as well as the results of previous studies, an assessment should be made regarding the most optimal temperature and storage time for the preservation

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of MRSA bacteria. In addition, the use of DMSO solution as a preservation agent also needs to be re-examined, considering that the properties and characteristics of DMSO can change depending on temperature and usage. A preservation agent must support the survival of the preserved bacteria so that the phenotypic and genotypic characteristics are maintained. Based on the preceding explanation, this study aimed to determine the viability of MRSA bacteria stored at different temperatures and times in DMSO solution.

2. RESEARCH METHOD

This research was conducted from July to October 2022 at the Integrated Laboratory of the Teaching and Education Faculty (FKIP), Universitas Muhammadiyah Purwokerto. The sample used was MRSA bacterial culture stock belonging to the D4 Medical Laboratory Technology Study Program, Faculty of Health Sciences, Universitas Muhammadiyah Purwokerto.

The research method used was a pre-experimental design with a one-shot study case research design. This method and design were used because there was only one experimental class without a control class in this study. Data analysis was carried out in the form of quantitative descriptive analysis.

The tools and materials used in this study were MRSA bacterial culture, Tryptic Soy Agar (TSA) media, Plate Count Agar (PCA) media, DMSO solution, spiritus, silica gel, 1.5 ml Eppendorf tube, autoclave, colony counter, incubator, laminar Air Flow (LAF), refrigerator and freezer -20 ℃.

Preparation of MRSA bacterial preservation agents

The preservation agent used in this study was DMSO solution with a concentration of 15%; the method of preparation was based on the dilution formula below (Laia *et al*., 2019).

$$V1 \times M1 = V2 \times M2$$

V1: initial volume
V2: volume after dilution
M1: initial concentration
M2: concentration after dilution

Based on the results of these calculations, to make a 15% DMSO solution, 4.5 ml of sterile DMSO solution is needed and sterile distilled water is added to a total volume of 30 ml.

Preparation of MRSA bacterial culture

MRSA bacterial culture was prepared by rejuvenating the culture stock on new slanted TSA media and then incubating it for 1 x 24 hours at 37 ºC. After growth, the bacterial culture was inoculated on several TSA plates using a spread plate, and then incubated at 37℃ for 2 x 24 hours.

The bacterial culture in the TSA media is then harvested by placing the silica gel into the petri dish and then gently shaking the petri dish so that the silica gel will move in all directions and the bacterial culture can stick evenly on the surface of the silica gel. So, silica gel in this study was used as a carrier medium for the MRSA bacterial culture to be preserved.

Preservation of MRSA bacteria

The 15% DMSO solution sterilized using an autoclave at a pressure of 121 atm is divided into several sterile microtubes measuring 1.5 ml. Silica gel coated using an aseptic work technique with MRSA bacterial culture is put into a microtube containing 15% DMSO solution. Each microtube is filled with 5 grains of silica gel and then stored according to the type of treatment that has been determined.

Storage of MRSA bacteria

Microtubes containing MRSA bacterial culture and 15% DMSO solution were then stored in three boxes. The first box was stored on a storage rack in the laboratory (room temperature). The second box is stored in the lower part of the refrigerator with a temperature of ± 4 ℃, while the third box is stored in the upper part (freezer temperature of 0 ℃). This storage process is carried out for ± 4 months, from July to October 2022.

MRSA bacterial viability test

The MRSA bacterial viability test is carried out routinely once a month by re-growing the bacterial culture on PCA media. Place 1 ml of bacterial suspension from the microtube in each storage box into a sterile petri dish. Furthermore, into the petri dish also poured liquid PCA media (temperature ± 45 ℃) and then flattened by moving to form a figure of eight. After that, the petri dish was left for 30 minutes until it

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completely solidified. PCA media was taken to be incubated at 37 ℃ for 2 x 24 hours. After that, the number of bacterial colonies growing in each petri dish was counted using a colony counter.

3. RESULTS AND DISCUSSION

The bacterial viability test results are presented in Table 1.

Tabel 1. MRSA bacteria viability test results on PCA media

Temperature Storage	Time (months)	The number of bacteria	Desc-cription
Room temperature (15-25 ℃)	1	<3,0 x10^2 (8 x 10)	
	2	<3,0 x10^2 (6 x 10)	
	3	0	not growing
	4	0	not growing
Refrigerator 4 ℃	1	>3,0 x 10^3 (65,6 x 10^2)	
	2	6,0 x 10^2	
	3	3,3 x 10^2	
	4	<3,0 x10^2 (1 x 10)	
Freezer (-20 ℃)	1	<3,0 x10^2 (1 x 10)	large colony
	2	>3,0 x 10^3 (33 x 10^2)	
	3	13,2 x 10^2	
	4	8,0 x 10^2	

Based on the research data, the group of bacteria stored at freezer temperature had the best viability compared to the group at room temperature and 4 ℃. The number of bacterial colonies decreased the least in the freezer temperature group compared to the other temperature groups. The results in this study are consistent with the results of research conducted by Yagüe *et al.* (2021), who stored *S. aureus* bacteria from swab samples at three different temperatures, namely -70℃, 20℃ and 37℃ and showed the results that bacteria stored at the lowest temperature had the best resistance.

Based on this previous research, bacteria have the highest survival or viability when bacterial cells are stored at the lowest temperature. Fitriana (2019) in her research stated that the lower the storage temperature, the slower the metabolic processes in these bacteria will even stop. The slower the metabolic process, the less toxic it produces.

In addition to the different viability, bacterial colonies at 4 ℃ and freezer (-20 ℃) also showed the same results as the study by Yagüe *et al.*, (2021), where there are variations in size and shape. This suggests that a cold and freezing environment can trigger metabolic changes in *S. aureus* bacterial cells. However, it is necessary to carry out further research on this matter considering several other factors that can influence such as growth media, preservation agents, and the methods of preservation used.

Bacterial growth decreased monthly in the freezer temperature group (-20 ℃). In the first month, the number of bacterial colonies was stated to be one because the number of bacterial colonies was so dense that it was impossible to count.

The second and third months have the same growth pattern. However, the decrease in the number of colonies was quite drastic. This condition occurred as a result of an improper thawing process. In this study, the bacterial culture stock that had been stored was immediately removed from the freezer and allowed to thaw at room temperature. According to Setiaji *et al*. (2015), thawing should be done by immersing the sample in warm water (37 ℃) for 60-90 minutes. This is done to avoid stress on cells due to sudden temperature changes.

The decrease in bacterial viability in this temperature group may also be due to the treatment of bacteria directly stored in the freezer without a gradual decrease in temperature. As a result, the osmosis process cannot run properly, and water remains in the ice. The excess water remaining in the cell can damage the cell resulting in the death of the bacteria.

Mahmmoud (2020) reported that the best temperature for maintaining *S. aureus* bacteria is -20 ℃. Other results were also expressed by Guo *et al*. (2020), where the morphology of the bacteria hardly changes at this temperature. In

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addition to the unchanged morphology at this temperature, the bacteria can be better recovered. Because basically, storage at this temperature can slow down the rate of biochemical reactions, which can be detrimental to stored bacterial isolates.

However, for long-term preservation, previous researchers did not recommend this temperature. To maintain strains of pathogenic bacteria for a long time, one needs to use storage temperatures of -80 °C and -196 °C, considering that at these temperatures, the possibility of DNA mutations can be avoided and is close to 0% (Guo *et al*., 2020; Missiakas & Schneewind, 2018; Prakash *et al*., 2013; Range *et al*., 2012)

In the 4 °C temperature group, the stored MRSA bacterial isolates did not experience freezing. The growth pattern was the same as the freezer group, which experienced a decline. The decrease in bacterial viability is probably due to the less-than-optimal storage temperature and the effect of DMSO as a preservation agent. Bacterial cells have a high intracellular water content in unfrozen conditions, so DMSO cannot freely enter the cell. The increase in fluidity because of using DMSO at an inappropriate temperature will cause cell damage/swelling. With this explanation, DMSO at this temperature is not protective against stored bacteria (Hine *et al*., 2019). Research Tseng *et al*., 2014; Chang and Lin (2019) states that temperature and storage time are essential in the viability and ability of *S. aureus* to be cultured again.

When the storage temperature of MRSA bacteria decreases, these bacteria adapt by developing a mechanism for converting membrane lipids into shorter unsaturated fatty acids. This membrane modification was carried out to overcome stress to maintain the membrane's fluidity (Rosmania & Yuniar, 2021).

In addition to temperature and time, the preservation method is also a factor in decreasing bacterial viability. The research results Ermenlieva *et al*. (2021) showed that *S. aureus* isolates were successfully re-grown after being stored for 6 months at 4 °C. This difference occurred due to differences in the preservation methods used, in which the *S. aureus* bacteria were stored without adding a preservative agent, and only stored using semisolid agar media.

This study used a 15% DMSO solution preservation agent, which according to *Prakash et al*. (2013), DMSO solution does have better penetration ability when compared to glycerol solution. Still, the use of this DMSO solution is limited, considering that it has several weaknesses, such as its properties that can turn toxic in certain circumstances.

MRSA bacterial colonies stored at room temperature showed the lowest viability. This was indicated by the small number of colonies that grew after re-culture and incubation for 48 hours. During the first and second months of storage, the colonies that grew numbered less than 30 colonies. In the third and fourth months, the MRSA bacterial isolates stored at room temperature could no longer be cultured. However, the results of this study indicated a longer storage duration for MRSA compared to previous studies (Yagüe *et al*., 2021), which stored *S. aureus* at room temperature and could be recovered after 11 days of storage. These different results may occur due to differences in storage methods and humidity during storage.

The results of this study are also in line with the research of Chang and Lin (2019), which stated that *S. aureus* stored at 25 °C showed more tremendous changes in the number of cells that could be cultured. Research courtesy of *Soltys et al*. (2012) stated that storage at room temperature is related to using DMSO as a preservation agent. The choice of DMSO solution as a preservation agent has a higher risk than other preservation agents because of its high toxicity to bacteria at room temperature.

Referring to Bhattacharya, (2018), DMSO at certain temperatures can reduce electrolyte concentrations during preservation. However, the increase in cell changes due to DNA methylation and histone changes is a weakness of this preservation agent. DMSO bond strength with water will decrease with increasing storage temperature. The cell membrane is more easily hydrated by water than DMSO, and this is reported to cause stress on the membrane surface. The hydrophobicity and concentration of DMSO in the lipid bilayer will decrease with increasing storage temperature, and this mechanism can explain the increased toxicity of DMSO at high temperatures (room temperature in this study)

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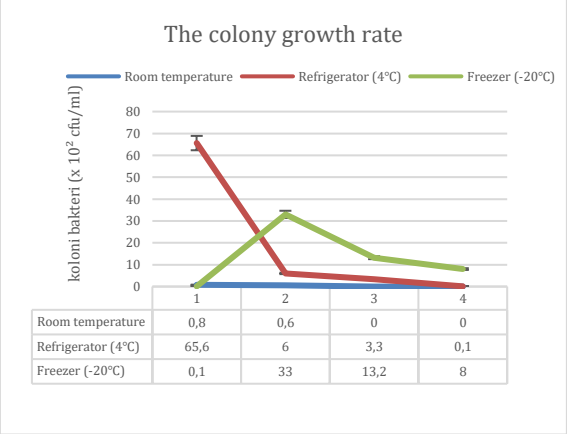
because DMSO will be localized around the polar head groups of bacterial cell membranes (Best, 2015).

In addition to the toxic properties of DMSO as a preservation agent, this is also related to bacterial metabolic processes. At this temperature, metabolic processes occur faster than in other temperature groups. As a result, the results of metabolism in toxicity are also more. These toxic compounds resulting from metabolism kill the surrounding bacterial cells.

There was also contamination in this group. The contamination occurred due to the lack of sterility of the silica gel used as the carrier medium. According to Fitriana (2019), using silica gel as a carrier medium has positive values, such as low and straightforward prices. Previous research Kim-Farley *et al.* (1987) also revealed this statement, recommending silica gel as a transport medium for *C. diphtheria* bacteria. Apart from the possibility that the carrier media was less sterile, bacterial contamination was thought to have occurred due to the busy laboratory conditions when the research was conducted. In addition, working at room temperature also increases the potential for contamination and chemical reactions that may occur during the preservation process compared to low temperatures (refrigerator/freezer) (Ali *et al.*, 2021).

The results of this study indicate that the preserved MRSA bacteria have different viability due to several factors. In addition to choosing the storage temperature, storage time also affects the viability of MRSA bacteria. This result is like that of Adhikari *et al.* (2018), which proved that storage time significantly affected the survival of the test bacteria.

In the graph (see Figure 1), the 4°C-storage group with one month of storage has the highest graph. However, the freezer storage group was the highest because the growth of the colonies was so dense that they could not be counted. Storage time in this study is thought to decrease the number of bacterial colonies. The decrease in the number of bacterial colonies increased with the length of storage time. This is thought to be caused by chemical reactions between DMSO and bacterial cells, such as lipid oxidation during preservation, which results in changes in the structure and function of cell membranes that cause cell death.



	1	2	3	4
Room temperature	0,8	0,6	0	0
Refrigerator (4°C)	65,6	6	3,3	0,1
Freezer (-20°C)	0,1	33	13,2	8

Figure 1. The colony growth rate of MRSA bacteria in each group

DMSO is generally non-toxic at concentrations below 10% (Summer *et al.*, 2022). Because of its widespread use, the optimal concentration of DMSO has not been reported, and the biological effects caused by low concentrations of DMSO (usually below 2%) are ignored.

DMSO is widely known and utilized as a cell preservation and/or freezing agent through the freezing storage method. This study used DMSO because DMSO is more manageable to penetrate cells and can make cell membranes more plastic so that they can bind water in cells which in turn prevents cell dehydration, reduces the toxic nature of salt, and prevents the formation of ice crystals in cells (Dhiani *et al.*, 2021). Although the best concentration of DMSO as a preservation agent has never been reported, the results of this study indicate that 15% DMSO can maintain the viability of MRSA bacteria.

Siddiqui *et al.* (2017) researched DMSO at concentrations of 2.5%, 5%, 10%, and 15% for Vero cell preservation, where DMSO showed a viability of 53% at a concentration of 15%. Indeed, DMSO concentration of 15% in this study cannot be the optimal concentration for a preservation agent due to the influence of several factors such as temperature, time, thawing process, contamination, and laboratory conditions.

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4. CONCLUSION

Based on the results of research and discussion, it can be concluded that the best viability of MRSA bacteria during preservation is at freezer temperature (-20°C) with a storage time of 1 month.

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