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Research Note

Evaluation of User-friendly - Ready-to-use Gelatin-impregnated Filter Paper Strips for Preservation of Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus*

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Abstract

Bacteria are ubiquitous in nature, live on animate and inanimate surfaces playing diverse roles and act as either beneficial or harmful organisms. The present study focuses on developing a simple and cost-effective method for long-term storage of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA). The method involves drying bacterial cultures viz., nine MRSA strains and one MSSA strain, on gelatin-impregnated filter paper strips. A 12% nutrient gelatin medium was used for maintaining and storing the bacterial cultures. The results showed that the bacteria preserved on the gelatin-paper strips remained viable for up to a year at temperatures of 4°C, -20°C, or -80°C. Further, the bacterial cultures survived for over a month at room temperature (30±2 °C). This practical, user-friendly technique offers an affordable solution to store and transport bacterial cultures, making it particularly beneficial for labs with limited resources or storage space.

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Bacteria are ubiquitous in nature and thrive on a variety of environmental conditions, often at high concentrations. Their functions range from beneficial roles, such as aiding in the fight against invasive pathogens, serving as probiotics, and participating in biogeochemical cycles, to harmful roles such as causing infections in humans, animals, and plants. Preserving bacterial cultures, both beneficial and harmful, is essential to meet research and industrial demands. Bacteriology laboratories maintain these cultures for research, academics, transportation, maintaining industrial mother stocks, and other applications (Gherna & Reddy, 2007). The primary challenge faced by bacteriology laboratories is to preserve and store bacteria in their original state, free from contamination and retaining their metabolic properties intact, either in small or large quantities for a long period (Morton & Pulaski, 1938; Moretti et al., 2024). Preservation of bacterial culture dates to the 1880s, when Soyka developed a method for storing cultures in tubes (Morton & Pulaski, 1938). Over time, various preservation techniques have been developed, suited for either short-term or long-term use, including methods such as drying, freezing, freeze-drying etc. (Gherna & Reddy, 2007). These methods aim to preserve bacterial cultures

outside their natural habitat without compromising cellular integrity or metabolic activity, however, many bacteriology laboratories face space constraints for long-term storage. Common preservation methods include maintaining cultures in nutrient media, overlaying with mineral oil, freezing, drying, and combinations of freezing and drying (Liao & Shollenberger, 2003; Alonso, 2016). While freeze-drying and cryopreservation are considered highly effective, they involve significant investments in terms of equipment and operational costs. In this context, there is an imminent need to develop cost-effective and user-friendly alternatives. Drying of bacterial cultures as a preservation method, first introduced in 1905 (Heim, 1905), has undergone various modifications over the years. Paper-based methods, including strips and disks, have been explored for storing *Enterobacteriaceae* (Gherna & Reddy, 2007). However, limited information is available on the use of strip-based methods for the storage of methicillin-resistant *Staphylococcus aureus* (MRSA). To address this gap, a user-friendly gelatin filter paper strip-based storage method was developed and evaluated in the laboratory. This method was evaluated over a one-year period with one MRSA strain and validated for a period of one month using nine MRSA cultures and one MSSA culture, demonstrating its potential as a cost-effective and efficient storage solution for bacterial cultures.

Bacterial strains: A one-year storage study was initiated using MRSA-32 strain from August 2023 to August 2024, and tested for its viability at three different temperatures viz., 4°C, -20°C, and -80°C. After the success of the preliminary study, the work was extended to other strains that included one MSSA (SA-583), and nine MRSA strains (MRSA-5, MRSA-8, MRSA-12, MRSA-13, MRSA-16, MRSA-21, MRSA 28, MRSA-29, MRSA-32). These bacterial strains were isolated in ICAR-CIFT laboratories during 2015 to 2022 and identified morphologically, phenotypically and genotypically (Visnuvinayagam, Joseph, Murugadas, Chakrabarti, & Lalitha, 2015; Murugadas, Joseph, Reshmi, & Lalitha, 2016; Murugadas, Joseph, & Lalitha, 2016, 2017; Murugadas, Toms, Reethu, & Lalitha, 2017; Murugadas, Joseph, Lalitha, & Prasad, 2020; Vaiyapuri, Joseph, Rao, Lalitha, & Prasad, 2019). All the cultures were tested for viability at four different temperatures viz., Room temperature (RT, 30±2°C), 4°C, -20°C, and -80°C for one month. The one-month storage study was initiated in August 2024 and completed in September 2024 as a validation for the previous one year study.

A brief description of the method developed, adopted, and evaluated for the storing of MRSA/MSSA cultures: Exactly 5 mL of overnight bacterial culture grown in tryptic soy broth (TSB) (BD Difco, USA) was pelleted by centrifuging at 8000 rpm for 5 minutes and resuspended in 1 mL of TSB supplemented with 12% gelatin (BD Difco, USA). The nutrient gelatin media contained gelatin at a concentration of 120 g/L, previously used in various protocols for maintenance and storage, as well as in gelatin liquefaction tests, where it served as both a substrate and a gelling agent. Around 50 µL of suspension was spotted onto strips of sterilized Whatman filter paper No.4, dried in a biosafety cabinet, and transferred to 4 different 1.5 mL microcentrifuge tubes. The tubes with dried strips were stored at four different temperatures namely 4°C, -20°C, -80°C and RT (30±2°C). The bacterial strains (after 1 year for MRSA-32 and 1 month for all 10 bacterial strains) were revived by dispensing 1 mL of sterile TSB broth into these 1.5 mL tubes and incubated at 37 °C for 24 h (Fig. 1). The bacterial cultures were streaked on Baird-Parker agar (BD Difco, USA) and incubated at 37 °C for 24 h. The plates showed typical grey-black colonies with an outer clear zone in all the plates characteristic of *S. aureus*.

Whatman filter paper No.4 was cut into 2.5 cm x 0.5 cm strips for use in 1.5 mL microcentrifuge tube, and sterilized in a hot air oven (160°C for 1 h) or autoclave (121°C for 15 min at 15 psi pressure). Alternatively, larger strips have been cut to fit into bigger centrifuge tubes viz., 15 mL or 50 mL. More than 50 to 100 strips were impregnated with 50 µL of gelatin-TSB and bacterial culture mix and used for storage in 1.5 mL tubes. For reviving the cultures, either one strip is taken out and added to the suitable culture broth, or sterile culture broth is added to the tubes containing the strips.

All the ten bacterial strains viz., nine MRSA cultures (MRSA-5, MRSA-8, MRSA-12, MRSA-13, MRSA-16, MRSA-21, MRSA 28, MRSA-29, MRSA-32) and one MSSA (SA 583) could be revived after one month at all the four temperatures viz., -80°C, -20°C, 4°C and RT 30±2°C (Fig. 2). The revival of the cultures needs to be further continued. Also, the 1-year storage study done earlier using one host MRSA32 at three different temperatures (-80°C, -20°C, 4°C)

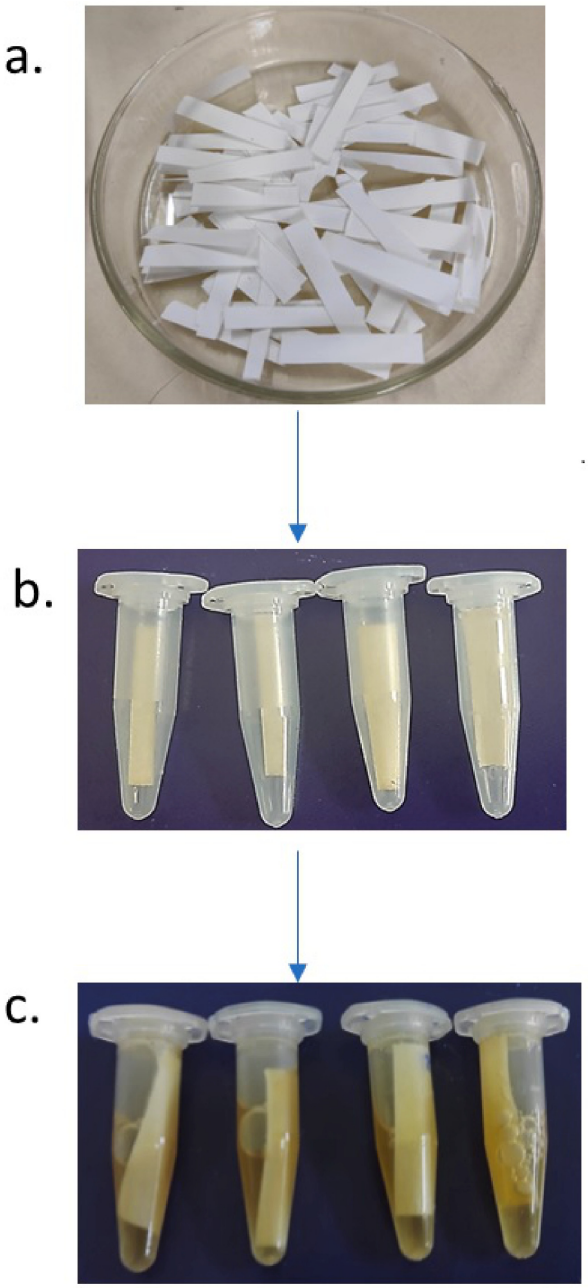

Fig. 1. Depiction on storage and revival of bacterial cultures in gelatin-impregnated strips (a) Whatman filter paper strips cut into required size (b) 1.5 mL microcentrifuge tubes with dried gelatin + culture strips for storage at four different temperatures (4°C, -20°C, -80°C, and RT 30±2°C) (c) The 1.5 mL tubes after the addition of TSB and incubation at 37°C.

indicated that the bacteria could be revived after 1 year, even at 4°C.

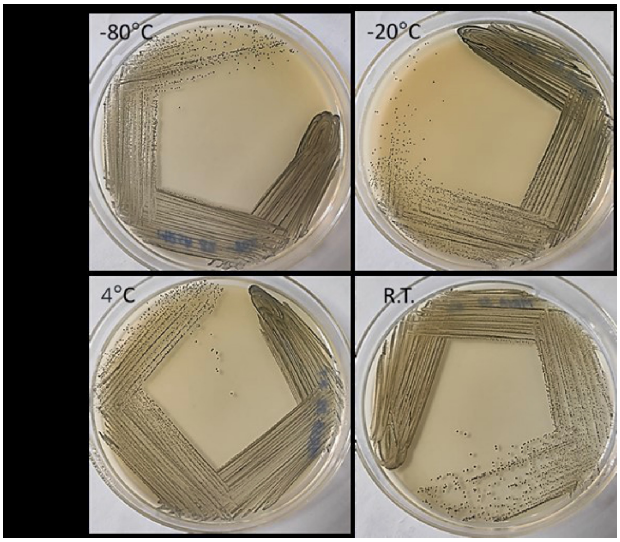

Fig. 2. Revival of MRSA-32 culture preserved in gelatin-impregnated strips at different temperatures (-80°C, -20°C, 4°C, and RT 30±2°C) by streaking on Baird-Parker agar.

It is reported that bacterial cultures could be preserved by gelatin based drying method (Obara, Yamai, Nikkawa, Shimoda, & Miyamoto, 1981). However, there was a lack of data on the drying process using gelatin strips to preserve MRSA. The results from the current study presents the suitability of using gelatin-impregnated strips as a promising method to store MRSA culture at RT for a minimum of one month (further studies must be carried out for an extended period of storage at RT) and for a year at 4°C, -20°C and -80°C (data is evaluated for one year for one culture-MRSA32). Glycerol, dimethyl sulfoxide, non-permeable additives like polysaccharides, starch, whey proteins, maltodextrins, lactose, sucrose, trehalose, and inositol are commonly used as cryoprotectants (Whaley et al, 2021; Oluwatosin, Tai, & Fagan-Endres, 2022). Skim milk, betaine, sodium glutamate, and various other combinations can also be used in the drying process specifically in the freeze-drying studies for various bacterial species (Alonso, 2016). Limited studies were available on the use of gelatin as a preservative agent in drying-based culture preservations in particular for MRSA and MSSA (Heckly, 1978; Kirsop & Doyle, 1991). Gherna and Reddy (2007) suggested the use of sterile Whatman disk or strip for the storing of bacterial culture. A number of bacterial species were preserved as gelatin-mixed cultures in the dried state viz., *Enterobacteriaceae, Neisseria, Branhamella, Pseudomonas, Flavobacterium*

etc. (Obara et al, 1981). However, there are limited studies on the use of Whatman paper strips with gelatin impregnation for storing bacterial cultures. Leal et al. (2016) investigated the suitability of agar stabs for preserving *Yersinia pestis* for a period of up to 40 years. Liao and Shollenberger (2003) evaluated the use of water and phosphate-buffered saline as preservation agents for pathogenic bacteria in plants and animals and recommended screw-capped tube and paraffin membrane sealed storage of gram-negative cultures. However, gram-positive bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus* showed a decline in their viability. In the present study, the gelatin-impregnated strip was evaluated for storage of MRSA/MSSA at different temperatures for a period of one year and there is a possibility of evaluating for a further extended period. Gelatin at 12% was used as the nutrient gelatin media was used for several protocol for maintenance and storage (Heckly, 1978; Kirsop & Doyle, 1991; Gherna & Reddy, 2007).

Few studies conducted on the drying of cultures in the dried gelatin for *Enterobacteriaceae* have recommended a storage period of 1-3 years (Obara et al, 1981). However, there are no reports regarding preserving MRSA cultures using gelatin-impregnated strips. This article outlines a quick and affordable way to preserve MRSA/MSSA making it particularly helpful for laboratories without an ultra-low freezer or lyophilizer, and without high investment. This method can be used for not only storing of MRSA/MSSA but also would be useful for the transportation of cultures, and also will help in accommodating several strains in a limited space. Future studies may focus on validating this approach for various foodborne pathogens and spoilage bacterial strains. Additionally, research needs to be extended to explore the use of alternative cryoprotectants to further enhance the storage duration.

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