Evaluating the effect of different temperatures and culture media on the production of methicillin-resistant *Staphylococcus aureus* (MRSA) bacteriophages

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ABSTRACT

Background: *Staphylococcus aureus* is a common pathogen that causes various infections. The emergence of methicillin-resistant *S. aureus* (MRSA) has made treatment more challenging, necessitating the exploration of new therapeutic approaches. In this regard, the study investigated the optimal conditions for phage production and activity. **Objective:** Evaluating the effect of different temperatures and culture media on the production of methicillin-resistant *Staphylococcus aureus* (MRSA) bacteriophages. **Methodology**: This was cross-sectional hospital-based study carried out in Baghdad Governorate, Iraq, from the beginning of October 2022 to November 2024 on *S. aureus* isolates. A total of 3 isolate from *S. aureus* bacteria which were taken from, Al-Kadhimiya Hospital and the isolate of phage (Rih21) Bacteriophage. Three different media used in the experiments (Luria broth, Mueller Hinton, and Nutrient broth). And three different temperatures (37, 40, 45 ˚C). The results recorded by counting plaque number and phage titer calculated. **Results**: In the study, the effect of media composition on *Staphylococcus aureus* phage production was investigated using a double agar overlay method. The plaque morphology of the phage was observed to vary in different culture media, with sizes ranging from small to large and appearances ranging from clear to turbid. Among the tested media, Luria Broth was identified as the optimal medium for phage production. A temperature of 37°C was determined to be the ideal temperature for phage production. Additionally, Phage Rih21 exhibited lytic activity against MRSA isolates in vitro, suggesting its potential as a therapeutic option for combating MRSA infections. These findings support the use of phage therapy as a promising strategy against MRSA infections. **Conclusion**: Storing phages at 4°C in a 50% glycerol medium provided the best preservation and density of phages.A temperature range of 28-37°C was identified as optimal for bacteriophage production.The Rih21 bacteriophage exhibited lytic activity at temperatures ranging from 37-45°C, with the ideal temperature for activity being 37°C.The optimal temperature for phage production varied based on the specific phage and its host.

Keywords: *S. aureus*, temperature, Rih21 phage, bacteriophage.
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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a common cause of a wide range of illnesses, from minor skin infections to severe sepsis and deadly pneumonia. It has a variety of damaging components, including toxins and immune evasion factors, which allow it to colonize and spread within the body during infections (1). According to previous studies, *S. aureus*-induced bacteremia is more deadly than human immunodeficiency virus, tuberculosis, and viral hepatitis combined in terms of death (2). When antibiotics gained widespread use, phage therapy was abandoned, even though it had been used for nearly a century (4). A recent study suggests that phages can be used as a therapeutic treatment by utilizing gene engineering to eliminate prophage-related

genes (5). As a substitute for or in addition to antimicrobial treatment for bacterial infections that are resistant to multiple drugs (6,7). Over time, bacteriophages have developed special proteins that take advantage of the metabolic processes of their bacterial hosts to aid in reproduction. Due to the extensive presence of phages around the world and the identification of many compounds that prevent bacterial growth and DNA synthesis, treatments for infectious disorders are currently being researched (8). This study aimed to investigate the effect of varying temperatures and media on the production of Methicillin-resistant *Staphylococcus aureus* phage. *S. aureus* has advanced resistance to almost all currently used antibiotics, starting with its initial discovery of penicillin resistance in the 1940s (3).

METHODOLOGY

Staphylococcus aureus bacteria from overnight culture was cultivated for 1-2 h, Then the optical density at 600 nm was measured to determine bacterial cell density (1). Three different media were used in the experiment. Luria, Nutrient and Mueller Hinton broth were prepared. Three tubes containing 5ml from each media were inoculated with 500µl bacteria and placed in shaker incubator at 37°C after incubation for 1h, 25µl from phage stock were added to each tube and incubated at three different temp (37, 40, and 45°C). After 5h incubation, 1ml from each bacterial culture was centrifuged to remove bacterial cells and the supernatant containing phage for all culture was kept at 4°C. In order to determine the phage titer, 10 Eppendorf tube containing 900µl SM buffer were prepared and 100µl from supernatant (phage sample) were added and mixed well by vortex. Then another 10 Eppendorf tubes containing 250µl *S. aureus* were prepared and left at incubator for 1h. After that, 20µl from the phage samples was added into each tube and mixed well with bacteria and left in the hood for 10 min. The bacteria and phage mixture were transferred into tube containing 3 ml top- agar mixed well. Finally, it poured over bottom agar plate and the petri dishes were placed in incubator at 37 °C.

Statistical Analysis:

The Statistical Analysis System (SAS) program (2018) was used to detect the effect of different factors on study parameters. The least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significantly compare between means in this study.

Figure (1): Plaque formation of Rih21 phage on culture media at 37 °C and 45°C.

RESULTS

The best temperature is 37◦C and using Luria Broth, which is the best media for phage growth, with an incubation period of less than 24 h. These conditions are optimal for bacterial phage production. Rih21 bacteriophage showed lytic activity at temperatures ranging from 37- 45°C, with the ideal temperature for activity being 37°C. The optimal temperature for phage production varies depending on the specific phage and its host. Phage therapy offers a promising alternative to combat Methicillin- resistant *S. aureus* (MRSA) infections as show in Table (1).

Table (1): Phage production at different temperatures and media after 5 and 24 hours of incubation

Temperatures °C	**Media types**								
	Luria broth Phage titer (PFU/ml) after 5 h			**Mueller Hinton broth Phage titer (PFU/ml) After 5 h**			**Nutrient broth Phage titer (PFU/ml) After 5 h**		
37	15 x10^7	14.2 x10^7	14 x10^7	11 x10^7	10.5 x10^7	10 x10^7	9x 107	8.6 x10^7	8 X10^7
40	7.3 x10^7	7.1 x10^7	6.8 x10^7	6.4 x10^7	6.2 x10^7	6 x10^7	5.8 x10^7	5.3 x10^7	5 x10^7
45	4.5 x10^7	4.3 x10^7	4.1 x10^7	3.8 x10^7	3.6 x10^7	3.2 x10^7	2.9 x10^7	2.7 x10^7	2.4 x10^7
Mean	14.4			10.5			8.5		

As the results showed, the 37°C is the ideal temperature for phage production. The study showed as in figure (1) the higher the temperature, the lower the production of phage. Therefore, the temperature is inversely proportional to the production of the virus per unit time. The phage production experiment revealed a strong relationship between temperature, media type, and phage titer after 5 and 24 hours. The optimal temperature for phage production was 37°C, with Luria broth yielding the highest titer of 15 x 10^7 PFU/ml after 5 hours. As temperatures rose to 40°C and 45°C, phage production decreased significantly, indicating that higher temperatures adversely affect phage stability and *S. aureus* viability**.**Luria broth consistently outperformed Mueller Hinton and Nutrient broths due to its nutrient-rich composition, with mean titers of 14.4 x 10^7 PFU/ml versus 10.5 x 10^7 PFU/ml and 8.5 x 10^7 PFU/ml, respectively. This highlights the importance of nutrient media in phage replication.

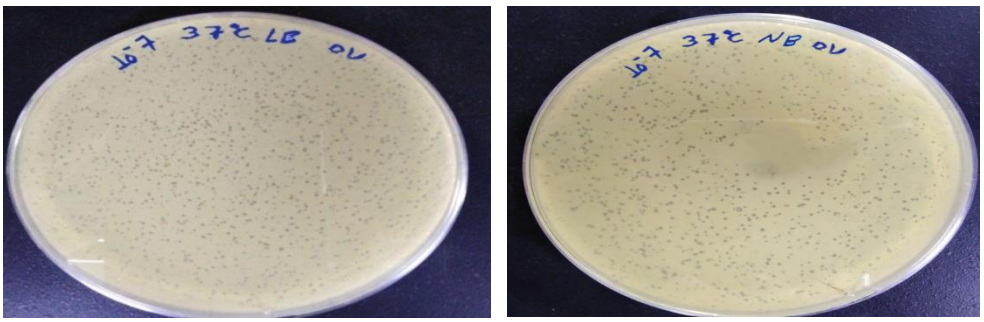

Figure (2): Phage production at 37°C on culture media

Discussion

The influence of media composition on the life cycle of bacteriophages to exhibit diverse plaque morphology on various bacteriological media was investigated by a double agar overlay method (18). *Staphylococcus aureus* phage showed altered plaque morphology from small to large and from clear to turbid, in different culture media used for the double agar overlay method. The findings from the current phage production experiment align well with previous studies examining the effect of media composition on the bacteriophage life cycle, specifically in the context of *Staphylococcus aureus* Rih21. The earlier research noted that phage plaque morphology varied significantly across different culture media, reflecting the influence of nutrient composition on phage activity and replication. Similarly, our results indicate that Luria broth, a nutrient-rich medium, significantly enhances phage production compared to Mueller Hinton and Nutrient broths. Both studies identify 37°C as the optimal temperature for phage activity, supporting the conclusion that this temperature maximizes lytic activity and phage replication. In the current experiment, phage titers decreased sharply as temperatures rose to 40°C and 45°C, consistent with the observations for the Rih21 bacteriophage, which also demonstrated lytic activity predominantly at 37°C. This reinforces the notion that the physiological conditions at this temperature favor optimal viral replication. The relevance of these findings extends to the broader context of phage therapy, particularly against antibiotic-resistant strains like Methicillin-resistant *Staphylococcus aureus* (MRSA). The resurgence of interest in phage therapy, especially in light of rising antibiotic resistance, highlights the need for a deeper understanding of phage-bacterial interactions. Our results suggest that optimizing growth conditions—both in terms of temperature and nutrient mediacan enhance the efficacy of phage therapies. Moreover, the synergy observed when combining phages and antibiotics in therapeutic settings could be further explored by understanding how different media compositions and temperatures affect phage dynamics. The earlier study noted a temperature range of 28-37°C as optimal, indicating that further research is needed to elucidate the underlying mechanisms. This could inform the development of more effective phage therapy protocols, ultimately improving treatment outcomes for bacterial infections. In summary, both studies emphasize the critical role of environmental factors such as temperature and media composition in phage production and activity. Understanding these interactions not only enhances our knowledge of phage biology but also contributes to advancing phage therapy as a viable alternative to combat bacterial infections in an era of increasing antibiotic resistance.

Conclusions

1. The Ideal temperature for phage production ranging between 28-37 °C.
2. The Phage therapy offers a promising alternative to combat Methicillin-resistant *Staphylococcus aureus* (MRSA) infections.

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تقييم تأثير درجات الحرارة المختلفة ووسط الزراعة على إنتاج العاثيات العنقودية الذهبية المقاومة للميثيسيلين(MRSA)

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الخلاصة:

خلفية البحث: المكورات العنقودية الذهبية هي مسببات الأمراض الشائعة التي تسبب الالتهابات المختلفة. أدى ظهور المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA) إلى جعل العلاج أكثر صعوبة، مما يستلزم استكشاف أساليب علاجية جديدة. وفي هذا الصدد، بحثت الدراسة في الظروف المثلى لإنتاج العاثيات ونشاطها.**المواد وطرق العمل:** وجد أن استخدام وسط لوريا مرق والحفاظ على درجة حرارة 37 درجة مئوية يوفر أفضل الظروف لإنتاج العاثيات. بالإضافة إلى ذلك، أظهر Phage Rih21 نشاطًا تحلليًا ضد عزلات MRSA ضمن نطاق درجة حرارة 37-45 درجة مئوية، مع درجة حرارة مثالية تبلغ 37 درجة مئوية. تسلط هذه النتائج الضوء على إمكانات العلاج بالعاثية كاستراتيجية واعدة لمكافحة عدوى.MRSA **النتائج:** في هذه الدراسة، تم دراسة تأثير تكوين الوسائط على إنتاج عاثية المكورات العنقودية الذهبية باستخدام طريقة تراكب الأجار المزدوج. وقد لوحظ أن شكل البلاك للعاثية يختلف في الوسائط الثقافية المختلفة، حيث تتراوح الأحجام من الصغيرة إلى الكبيرة والمظاهر تتراوح من الواضح إلى العكر. من بين الوسائط التي تم اختبارها، تم تحديد Luria Broth باعتبارها الوسيلة المثالية لإنتاج العاثيات. تم تحديد درجة حرارة 37 درجة مئوية لتكون درجة الحرارة المثالية لإنتاج العاثيات. بالإضافة إلى ذلك، أظهر Phage Rih21 نشاطًا غنائيًا ضد عزلات MRSA في المختبر، مما يشير إلى إمكاناته كخيار علاجي لمكافحة عدوى .MRSA تدعم هذه النتائج استخدام العلاج بالعاثية كإستراتيجية واعدة ضد عدوى MRSA .**الاستنتاج:** تخزين العاثيات عند درجة حرارة 4 درجات مئوية في وسط جلسرين 50٪ يوفر أفضل حفظ وكثافة للعاثيات. تم تحديد نطاق درجة حرارة يتراوح بين 28-37 درجة مئوية باعتباره الأمثل لإنتاج العاثيات. أظهرت العاثيات Rih21 نشاطًا تحلليًا في درجات حرارة تتراوح من 37-45 درجة مئوية، مع درجة حرارة مثالية للنشاط هي 37 درجة مئوية. تختلف درجة الحرارة المثالية لإنتاج العاثيات بناءً على العاثيات المحددة ومضيفها.

الكلمات المفتاحية: s.aureus ، درجة حرارة ،عاثية Rih2 ، البكتيريا.