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EFFECT OF PH & TEMPERATURE VARIATIONS ON PHAGE STABILITY - A CRUCIAL PREREQUISITE FOR SUCCESSFUL PHAGE THERAPY

A. N. Pradeep [1], S. Ramasamy [2], J. K. Veniemilda [3] and C. S. Vinod Kumar [*3]

Department of Pharmacology [1], Department of General Medicine [2], Saveetha Institute of Medical & Technical Sciences (SIMATS), Saveetha University, Thandalam, Chennai - 602105, Tamil Nadu, India.
Department of Microbiology [3], S. S. Institute of Medical Sciences & Research Centre, Davangere - 577005, Karnataka, India.

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Correspondence to Author:
Dr. C. S. Vinod Kumar

Professor,
Department of Microbiology,
S. S. Institute of Medical Sciences & Research Centre, Davangere - 577005, Karnataka, India.

E-mail: vinodmicro@gmail.com

ABSTRACT: Phage therapy has become a successful alternative to conventional antibiotic therapy due to the increasing number of multidrug-resistant bacteria in most clinical settings. Various factors like phage adsorption to the host site and phage neutralization by the host determine the efficacy of phage treatment. However, external factors like temperature and pH are critical in deciding the success of using phages in therapeutics. The high stability of phages under both favourable and unfavourable conditions guides their stability in phage therapeutic preparations as well as at the site of infection. In the present study, we exposed the phage to different pH and temperature combinations. The phage showed statistically significant activity at pH 7 and 8 compared to pH 6. In terms of different exposure temperatures, the phage showed good antibacterial activity at temperatures between 31 ^{0}C and 40 ^{0}C. A combination of pH 7 with temperature 38^0 C showed the highest recovery of the phage; however, stability was seen in a considerable range of temperature and pH. The lytic activity of phage was insignificant at extremes of temperature and pH. The present study results indicate that phages, in terms of their stability to variations in external factors, can be a promising alternative to antibiotics or can be used in combination with antibiotics for the successful treatment of multidrug-resistant pathogens.

INTRODUCTION: Phage therapy was a popular treatment choice for many infections before the discovery of antibiotics [1]. The recent rise in the number of multidrug-resistant bacteria due to the reduced efficacy of antibiotics has posed a serious challenge for treatment. Due to this and a decline in the number of newer antibiotics in the pipeline, renewed interest has emerged among researchers to revisit the use of phages to treat infections, especially those that are refractory to treatment with most routine and high-level antibiotics [1, 2].

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Phage therapy has several advantages compared to antibiotics in terms of safety, host specificity, and high multiplicity at the infection [2]. Live phages and components of phage proteins are being extensively studied for their therapeutic efficacy in various infections in both *in-vitro* and *in-vivo* models [3, 4]. More importantly, the results of such studies against multidrug-resistant organisms have shown promising results in using phages to treat such infections as an alternative or supplement to conventional antibiotic therapy [1, 4].

Critical factors that affect phage therapy are the initial concentration of phage, adsorption rate, period of latency, burst size and host factors like phage neutralization in the body by the reticuloendothelial system [5, 6]. Other factors like the ratio of bacteria versus bacteriophage and external

factors like temperature and pH, though not critical, play an important role in the success of phage therapy [7]. External factors like temperature and pH can predict phages' incidence, storage viability, and infectivity [8]. The bacteriophage can be subject to structural or genetic damage if exposed to large variations in such factors [9].

Researchers have observed that optimum temperature and pH are critical for phage to grow and survive [10]. While bacteriophage is highly diverse in their ability to survive under unfavourable conditions and is present in all places where the specific host bacteria are present, most phages that lyse human pathogenic bacteria prefer a neutral to a near-neutral condition in terms of temperature and pH, which correspond to the preference of their host bacteria [11-13].

Similar to bacteria, temperature and pH decide the occurrence of bacteriophage [8]. At the right temperature and pH, optimum conditions for infectivity of phage are present [8]. Though numerous studies are available on the efficacy of phage in terms of its antibacterial activity, literature regarding the effect of external factors like temperature and pH are limited. The present study was conducted to analyze the influence of temperature and pH on the stability of phages isolated from environmental sources against Methicillin-resistant *Staphylococcus aureus* ATCC 43300.

MATERIALS AND METHODS:

Study Setting: The present study was conducted at the Departments of Pharmacology and Microbiology, S. S. Institute of Medical Sciences & Research Centre, Davangere, India. The study had the approval of the Institutional Biosafety Committee and Institutional Animal Ethics Committee.

Bacterial Isolate: MRSA ATCC 43300 was used as the bacterial strain. A 10^7 colony-forming unit (CFU)/mL concentration of MRSA was prepared. The colony count was confirmed by spread plate technique [14].

Isolation of Bacteriophage: The phage was isolated from sewage by the procedure mentioned by Smith and Huggins [15]. Raw sewage sample was collected from local sewage treatment sites in Davangere, India. The samples were treated with an equal volume of nutrient broth and incubated at 58°C for 30 min, following which 1mL of an overnight broth culture of MRSA 43300 was added. The culture was incubated for 24h at 37°C. The broth was then centrifuged, and the supernatant was filtered and spot inoculated on a nutrient agar plate with a previously inoculated lawn culture of MRSA 43300. Discrete plaques were selected and treated with overnight broth culture of MRSA.

The process was repeated to obtain pure phage lysate. The lysate was checked for activity and sterility. Plaque forming unit (PFU)/mL was determined using MRSA 43300 by the soft agar overlay method [16]. The phage lysate was purified and diluted, and a concentration of 3×10^9 PFU/mL was selected for further *in-vitro* studies [13, 17].

Effect of Temperature and pH on the Stability and Infectivity of Phages: The phage was exposed to different pH and temperature combinations to analyze the thermal and pH stability. Phage suspension at a titre of 3x10 ^{9} PFU/mL was prepared in sterile nutrient broth adjusted at each pH from 1 to 14. The phage suspension at each pH was incubated at every individual temperature from 15° C to 60° C for a period of 6 h.

After incubation, the phage suspension was serially diluted, and surviving phages were counted by double agar overlay method [16]. 100 µL of the phage and 100 µL of log phase culture of MRSA 43300 were added to 4 mL of semi-solid (with 0.5% agar) nutrient agar, mixed, and poured quickly and uniformly on the surface of a solidified nutrient agar plate, avoiding trapping air bubbles. After incubation at 30°C for 16-18h, the plaques were counted, and PFU/mL was determined by multiplying the dilution factor.

RESULTS: The result of phage thermal and pH stability at various temperature and pH combinations is shown in **Fig 1-3**. The phage either did not survive or lost infectivity or showed minimal activity at temperatures below 21°C and above 45°C and pH <6 and >8 when incubated with the host bacteria in the double agar overlay method. At pH 6, from an initial load of 10^9 PFU/mL, the phages showed a 4 to 6-log reduction at temperatures between 35°C to 40°C **Fig. 1**.

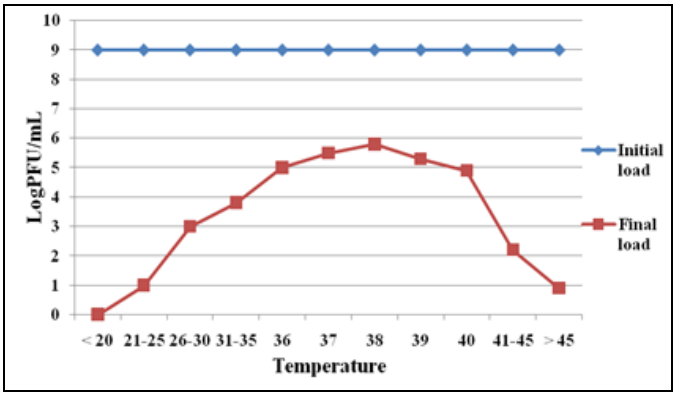

FIG. 1: EFFECT OF DIFFERENT TEMPERATURES ON THE STABILITY AND ANTIBACTERIAL ACTIVITY OF PHAGE AGAINST MRSA 43300 AT pH 6

At extreme temperatures, *i.e.*, at 20°C and 45°C, the phage titre dropped to as low as 10 to 100 PFU/mL. Recovery of phages, though low, was better between 37° C and 39°C. At pH 7, the phage showed good stability at a wide range of temperatures, *i.e.*, 26°C to 45°C **Fig. 2**.

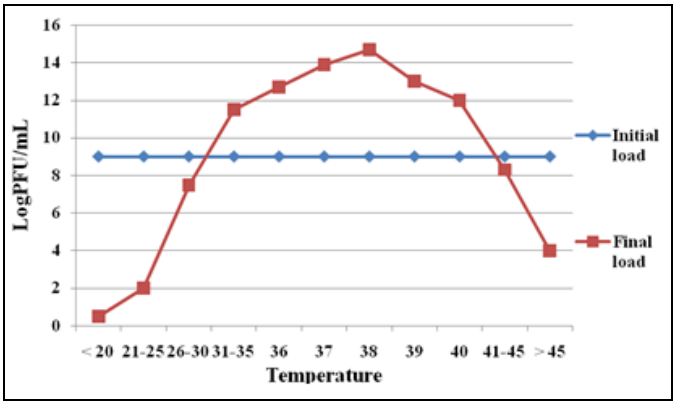

FIG. 2: EFFECT OF DIFFERENT TEMPERATURES ON THESTABILITY AND ANTIBACTERIAL ACTIVITY OF PHAGE AGAINST MRSA 43300 AT pH 7

Phages were recovered at a titre as high as 10^{14} PFU/mL on the double agar overlay plate, with highest recovery at 37°C and 38°C. Phage titres were significantly low ($p<0.05$) at temperatures below 26°C and above 45^0 C compared to those incubated between 26° C and 45°C. Phage count dropped by 1 to 2-log at temperatures 26°C-30°C and 41°C-45° C. There was a 2 to 5-log increase in the final phage titre isolated between 31° C and 40° C. At pH 8 **Fig. 3**, the results of phage stability were similar to that observed at pH 7, with good recovery at temperatures between 26°C and 45°C. Still, the titres were lower at pH 8 (statistically insignificant, $p>0.05$) compared to that at pH 7. There was a 4-log reduction in the phage titre at temperatures 26°C-30°C and 41°C - 45°C. At temperatures between 31°C and 40^0 C, the phage titres were either same or 1 to 3-log higher than the initial load but significantly lesser ($p<0.05$) than the phage load at pH 7.

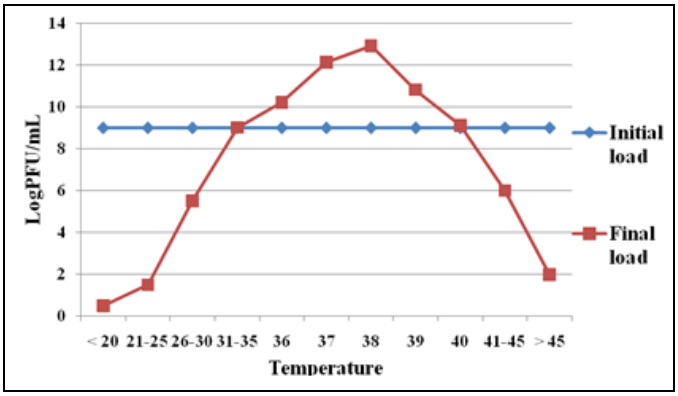

FIG. 3: EFFECT OF DIFFERENT TEMPERATURES ON THE STABILITY AND ANTIBACTERIAL ACTIVITY OF PHAGE AGAINST MRSA 43300 AT pH8

DISCUSSION: Therapeutic failure, especially in the case of multi-drug resistant organisms, is more than often life-threatening. The recent increase in multidrug-resistant pathogenic bacteria has further increased the barrier of a challenge for treating such infections. Numerous studies have been conducted to understand the efficacy of phages on different bacteria. Treatment of human infections with phages that have shown encouraging experimental results is being approached worldwide due to increasing and successive therapeutic failures using antibiotics. Phage therapy is natural, low cost, host-specific - normal flora is not disturbed, dose favourable - single dosing, auto dosing when compared to antibiotics [2].

A successful phage therapy relies on various phage factors, host factors, and external factors [8]. Phage stability at various favourable and unfavourable conditions is crucial for considering phage for therapeutic applications. Bacteriophages are generally resistant to extreme physical and chemical conditions, however, optimum pH and temperature are important for attachment, penetration, and multiplication, *i.e.*, for the survival of phages [18]. At extreme temperatures, the number of phages involved in extension and multiplication can be significantly lesser, or an increased latency can result in delayed-release. Similarly, the pH of the environment decides the occurrence and

stability of phages [19]. We exposed phage to various pH and temperature combinations in the present study. There was no phage activity at extreme conditions of temperature (<21°C and >45°C) and pH (<6 and >8). Studies have reported that viability at pH<4 and >9 and temperatures < 15°C is one of the most common limiting factors for phage activity [20, 21]. Phages are generally more temperature and pH-resistant compared to their host bacteria [22]. In the present study, since the phage was isolated against MRSA 43300, the phage showed temperature and pH tolerance similar to its bacterial host.

We observed better phage stability at pH 7 and 8 compared to pH 6 ($p< 0.05$). There was no statistically significant ($p>0.05$) difference in the phage stability between pH 7 and pH 8, though there was a small difference of 0 to3-log of recovered phages at various temperatures. When phage stability at pH 6 was compared with pH 7 and 8, the difference was less or insignificant at lower (<25°C) and higher (>40°C) temperatures. However, at temperatures between 26° C and 40° C, there was a significant difference ($p<0.05$) of 7 to 15-log PFU/mL of phages. At temperatures between 26°C and 45°C, the phage showed a higher rate of reduction (20% to 60%) in viability at pH 6 compared to pH 7 and pH 8. Researchers have reported extremely good phage stability between pH 5 to pH 9 [23]. It is observed that, at extremely low pH (< 5), phage undergoes irreversible coagulation and precipitation resulting in inactivity [23]. Studies have also suggested the lowest phage inactivation at near-neutral pH (pH 6 to8) and temperatures around 37°C [24].

The right temperature and pH guide the optimum antibacterial activity of phages [25]. Similar to pH, temperature plays a major role in the viability and stability of phages. At pH 6, there was no significant difference ($p>0.05$) in the recovery of phages at temperatures <25°C and >40°C when compared with pH 7 and pH 8. Similar results ($p>0.05$) were observed when phage viability was compared at the same temperatures between pH 7 and pH 8 indicating that temperature below 25°C and above 40°C is less favourable for phage viability/stability irrespective of a favourable pH (pH 7 and pH 8). At temperatures between 31°C and 40°C, there was a significant difference ($p<0.05$) in the log of phages recovered when the three pH (pH6, pH 7 and pH 8) levels were compared. The number of phages recovered at pH 7 was significantly higher when compared to pH 8 and pH 6; similarly, pH 8 showed a higher degree of phage recovery compared to pH 6 between the given temperatures. The difference between the three pH levels was also highly significant ($p<0.05$) between 36°C to 40°C. It is suggested that the thermal resistance of phages is due to the disulfide bonds linking the protein coat [26].

In the present study, the phage showed a higher degree ($p<0.05$) of stability at temperatures between 26°C and 45° C at all the three pH levels (pH6, pH 7 and pH 8) compared to <25° C and >45°C. Optimum phage activity was seen at 38° C at pH 7. Similar results were observed by previous researchers who observed that T4 phage isolated against diarrheagenic *E. coli* showed good stability between 15° C and 45° C with optimum activity at 37°C[27]. There was excellent antibacterial activity of phage on exposure to temperatures between 31°C and 40°C and pH 7 and pH 8. Though the phage was viable at all three pH levels, the highest phage recovery was seen at neutral pH (pH 7). Phage resistance to variations in external factors like temperature and pH is a prerequisite for phage therapy. The present study results show that since these conditions are easier to maintain, phage requirement in terms of pH and temperature is less challenging than antibiotics, which further helps in employing phages for the treatment of common and multidrug-resistant infections.

CONCLUSION: External factors like temperature and pH are important for phage therapy. Phage should be able to remain stable, survive and show its bacteriolytic activity under a wide range of physical and chemical conditions, especially those specific to its host bacteria which are pathogenic to humans. Understanding the optimum temperature and pH of phage stability and activity is one of the mainstays for using phages to treat human infections.

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CONFLICTS OF INTEREST: None

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