ORIGINAL ARTICLE

Unveiling the potential of bacteriophages as a novel anti-microbial approach against ESKAPE pathogens: A comprehensive characterization and analysis of bacteriophages isolated from the environment

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ABSTRACT

Context: pH and temperature are essential factors that affect the efficacy of phage therapy. Phages are sensitive to changes in pH and temperature, which can affect their stability, infectivity, and survival rates. Therefore, it is crucial to determine the optimal conditions for phage therapy to maximize its effectiveness. **Objective**: To isolate phages against MDR ESKAPE pathogens and to study their morphological features, antibacterial activity, host specificity, and the effect of different range temperature and pH on their viability. **Methodology**: Bacteriophages cultured from different sources of water were exposed to different temperature (32^0C to 40^0C) and pH of 1-14 and later bacteriophage kinetics were studied in-vitro. **Results:** Bacteriophages isolated showed lytic activity against ESKAPE pathogens. Plaque formation was seen on every bacterial lawn against their respective bacteria, but pinpoint plaques/no plaques were seen against other pathogens owing to their specificity. Most bacteriophages against ESKAPE pathogens were viable at a temperature of 38˚C and a pH of 7, making them the optimum temperature and pH for their isolation. **Conclusion:** Increasing antimicrobial resistance among ESKAPE pathogens has led to the emergence of bacteriophage as novel therapeutic agents and proper standardization of bacteriophage can make phage as stand alone therapy in absence of antibiotics.

Keywords: Bacteriophage, Lytic phage, ESKAPE pathogens, Antibacterial activity, Host specificity, Phage therapy

INTRODUCTION

Phages are small viruses with the ability to kill bacteria without affecting the cells of other eukaryotic organisms. They were first described almost a century ago by William Twort, and discovered shortly after by Félix d'Herelle (considered the founder of bacteriophages)[1]. Since its inception, the use of phages as a therapy to treat acute and chronic infections has been suggested due to its specificity against bacteria.[1-3] It is understandable why there was such initial excitement about the use of phage therapy to treat bacterial infections in the pre-antibiotic period. The idea of using phages therapeutically to treat bacterial infections was very contentious from the start and was not universally accepted by the general public or the medical community. Early studies received a lot of criticism for their contradictory findings and lack of adequate controls. Phage research and therapy were largely consigned to medical history in western nations as the age of antibiotic chemotherapy emerged with the discovery of sulfa medicines in the 1930s and subsequently penicillin in the 1940s. However, the former USSR, Poland, and India, to a lesser extent, continued to be important research and development hubs for phage treatment. Surprisingly, during the past ten years, the advent of multi-drug resistance bacteria has prompted researchers to reevaluate this century-old strategy and give phage therapy a second look as a "new" and possibly effective treatment option for challenging bacterial diseases.[4-5]

According to WHO antimicrobial resistance fact-sheet 2021, high rates of resistance against the major antibiotics used to treat common bacterial diseases, such as urinary tract infections, sepsis, sexually transmitted infections, and various types of diarrhoea, have been documented globally, suggesting that we are running out of effective antimicrobials. For instance, the rate of resistance to ciprofloxacin, an antibiotic frequently used to treat urinary tract infections, ranged from 8.4% to 92.9% for *Escherichia coli* and from 4.1% to 79.4% for *Klebsiella pneumoniae* [6].

Common intestinal bacteria such as *Klebsiella pneumoniae* can result in potentially fatal illnesses. *K. pneumoniae* has developed resistance to carbapenes which is used as a last line of treatment. More than half of patients treated for *K. pneumoniae* infections in some nations do not respond to carbapenem medicines owing to resistance. Fluoroquinolones, which are used to treat urinary tract infections, are widely resistant to *E. coli*.[7] More than half of patients receiving this medication are no longer responding in several nations throughout the world. Colistin is the only therapy left for life-threatening infections caused by *Enterobacteriaceae* that are resistant to carbapenem. Additionally, colistin-resistant bacteria have been found in a number of nations, where they are responsible for diseases that cannot be treated with antibiotics [8].

The bacterium *Staphylococcus aureus* is a frequent source of infections in both the general population and healthcare settings. The mortality rate of Methicillin-resistant *Staphylococcus aureus* (MRSA) infections is 64% compared to infections that respond to treatment[9]. The treatment and control of gonorrhoea have been hampered by widespread resistance in extremely diverse strains of *N. gonorrhoeae*. Sulphonamides, penicillins, tetracyclines, macrolides, fluoroquinolones, and early-generation cephalosporins have all shown a rapid rise in resistance. Currently, the only empiric monotherapy for gonorrhoea available in the majority of nations is the injectable extended-spectrum cephalosporin like ceftriaxone. Among these resistant bacterial infections, ESKAPE pathogens (*Enterococcus spp., Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.*) pose a significant threat in intensive care settings as these emerged as multidrug resistance (MDR), extended drug resistance (XDR), and pan drug resistance (PDR) strains which are challenging to treat[6,10]. World Health Organization (WHO) has recently listed ESKAPE pathogens in the list of 12 bacteria against which new antibiotics are urgently needed.[7,11] Over the last decade, the number of antimicrobials effective against ESKAPE pathogens has declined. So, the need for new therapies made researchers study phage therapy more against ESKAPE pathogens which could be a groundbreaking alternative to antibiotics.

Phage researchers have a revived interest in phage therapy, and practical studies on bacteriophages have intensified recently, along with clinical trials[12]. For phage therapy, the main interest has focused on lytic phages, where following its replication inside the host bacterium, the generated phage offspring is expelled by lysis.[8] This ability of phages to infect and kill only specific bacteria without any lytic effect on the eukaryotic cell provides to the development of their use in humans but, characterizing them by their morphological features, host specificity, evaluating the effect of temperature and pH on their viability is essential before implementing them as the therapeutic agents[12].

MATERIALS AND METHODS

Ethical Clearance

The study protocol was approved by Institutional Ethics Review Board, S. S. Institute of Medical Sciences and Research Centre, Davangere, Karnataka, India.

Bacterial Isolates

The ESKAPE pathogens such as *Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium, Klebsiella pneumonia, Pseudomonas aeruginosa and Acinetobacter baumannii.* were isolated from the clinical specimen of the patients admitted to the Intensive Care Unit (ICU) at the S.S. Institute of Medical Sciences & Research Centre, Davangere, India. The various clinical specimens collected from the patients were processed and bacteria were isolated and identified as per the standard microbiological techniques[7,9]. The VITEK system was used for further identification and confirmation of bacteria isolated and to screen for antibiotic susceptibility. Gram-positive and Gram-negative antibiotics panels were used for evaluating antibiotic susceptibility patterns as per the CLSI criteria[7,9,13].

Isolation of bacteriophage:

The phages were isolated from different sources of water by the method of Smith and Huggins. Collected water (50ml) was added into a sterile conical flask and treated with a few drops of chloroform. To this 5 ml of lactic phage broth and 1 ml of the 24 hrs old broth cultures were added. The sample inoculated with bacterial pathogens was incubated at 37ºC for 12-24hr in a shaker water bath. After 12-24hrs the lysate was shaken with a few drops of chloroform for about 10 min, centrifuged at 10,000 rpm for 10 min and the supernatant was filtered through 0.22m pore size and the filtered phage samples were precipitated using polyethylene glycol (PEG) and NaCl. The overnight precipitated samples were centrifuged at 15,000 × g for 30 min and the pellet is mixed with buffer. The precipitated phages were extracted against chloroform at a 1:1 ratio to remove bacterial debris and stored at -20◦C[14,15].

In-vitro confirmation of bacteriophage activity

The bacterial lawn was prepared on nutrient agar plates employing 1.0ml of 24hr culture by flooding and draining out the excess. Wells were dug into the agar by employing a sterile cork borer and the 20 ml phage suspension was loaded into each of the wells. Sterile distilled water served as the control. The plates were incubated at 37^0 C for 24 hr. Thereafter the zone of inhibition, if any, was recorded. The plaques if obtained were further passaged on the same target bacterial host to reconfirm its activity[14,15].

Phage morphology

To study the morphology of the isolated bacteriophage, the phages were negatively stained using 2% uranyl acetate and visualized under Transmission Electron Microscope. The electron microscopic study was carried out at the National Institute of Mental Health and Neuro-Sciences, Bangalore. Briefly, 5 µL of phage filtrate was placed over the copper grid and allowed to settle for 10 min. To the dried grid surface, 2 µL of uranyl acetate was added and allowed to stain for 1 min. The excess dye was washed using distilled water by placing 5 µL of water on the grid and removed immediately. The copper grid was allowed to dry for 30 min and visualized under a transmission electron microscope[16].

RESULTS

Bacterial isolates

ESKAPE pathogens were isolated from various clinical specimens and AST revealed all the isolates were multidrug resistant.

Bacteriophage isolation

The bacteriophages were isolated from four different sources of water; lake, sewage, paddy field, and an open drain. The phages isolated from different sources against ESKAPE pathogens produced plaques of size ranging from 0.5–11 mm in diameter for Gram-positive bacteria and 1mm to 9mm for Gram-negative bacteria.

Electron microscopy of the phages

The electron microscopy of the phages isolated against *Staphylococcus aureus*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterobacter species* is shown in figure 1;6. All the phages isolated had an icosahedral head, measuring about 70-100 nm in diameter, and a 100-120nm long tail. Based on the morphology and the rules provided by the International Committee on Taxonomy of Viruses (ICTV, Bethesda MD, USA) all the phages are tentatively classified under Siphoviridae family

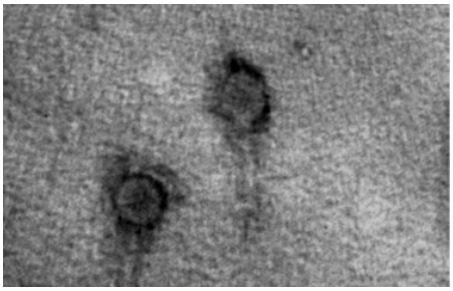

Fig-1: Bacteriophage of Staphylococcus aureus

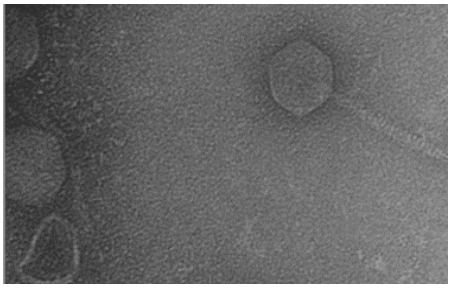

Fig-2: Bacteriophage of Enterococcus faecium

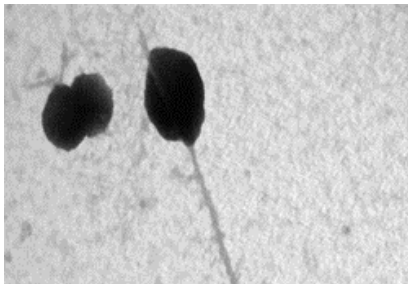

Fig-3: Bacteriophage of Acinetobacter baumannii

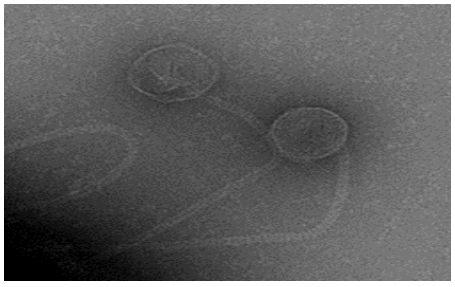

Fig-4: Bacteriophage of Pseudomonas aeruginosa

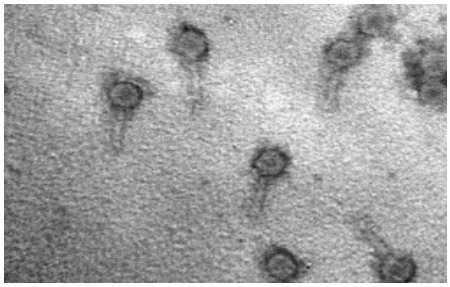

Fig-5: Bacteriophage of Klebsiella pneumoniae

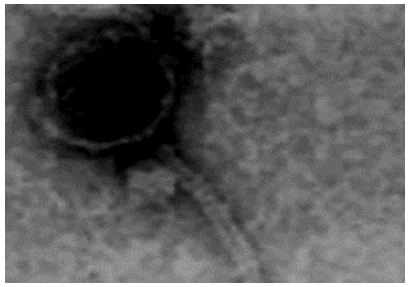

Fig-6: Bacteriophage of Enterobacter species

Staphylococcus aureus phage :

Phages against *Staphylococcus aureus* were isolated from lake water, sewage water and open drainage water.

Antibacterial activity and Host specificity:

Staphylococcus aureus phage lysed all *Staphylococcus aureus* strains tested. With in this lytic spectrum, clear plaques were produced on all strains except on a few strains of methicillin-resistant *Staphylococcus aureus* which had a mixture of opaque and clear plaques. The inhibition of bacterial growth in strains that the phage could not form plaques is most likely due to partial expression of the phage genome, sufficient for killing but not enough for phage production to a level necessary for plaque formation. Plaque size ranged from 0.5 to 4 mm. The *Staphylococcus aureus* phage was found to form plaques on 62% of the MRSA isolates and inhibited bacterial growth of an additional 12% of the strains, thus exhibiting an antibacterial effect against 74% of the MRSA strains. *Staphylococcus aureus* phage produced pinpoint plaques on CoNS strains that were spotted with high load of phages. No plaques were seen on *Enterococcus faecium.*

Effect of pH and temperature:

The maximum number of plaques obtained at different temperatures and pH are presented in Figures 7. It is evident that with the increase in the concentration of H^+-ions, the possibility of recovering the phages is less when compared to the decrease of H^+-ions. At a pH of 3 to 5, no phages were recovered at different temperatures to which the phages were exposed. At pH 6, the maximum number of phages isolated was less than the initial concentration (9.57 log10 PFU). The maximum phage recovered at temperature 32^{0}C is 7.15 log10 PFU, at 34^{0}C is 7.24 log10 PFU, at 36^{0}C is 7.14 log10 PFU, at 38^{0}C is 7.35 log10 PFU, at 40^0 C is 6.99 log10 PFU and at 42^{0}C is 6.76 log10 PFU (Table-1).

At pH 7, the maximum number of phages (11.916 log10 PFU) was isolated at 38^{0}C. It was more than the initial concentration (9.57 log10 PFU). The phages isolated at other temperatures are; at 32^{0}C is 11.475 log10 PFU, at 34^{0}C is 11.884 log10 PFU, at 36^{0}C is 11.910 log10 PFU, at 40^0 C is 11.866 log10 PFU and at 42^{0}C is 9.964 log10 PFU (Table-1, Fig-7).

At pH 8, the maximum number of phages isolated was less than the initial concentration (9.57 log10 PFU). The maximum phage recovered at temperature 32^{0}C is 7.456 log10 PFU, at 34^{0}C is 7.693 log10 PFU, at 36^{0}C is 7.982 log10 PFU, at 38^{0}C is 8.187 log10 PFU, at 40^0 C is 7.445 log10 PFU and at 42^{0}C is 6.439 log10 PFU (Table-1,). At pH 9 to pH 12 no phages were recovered.

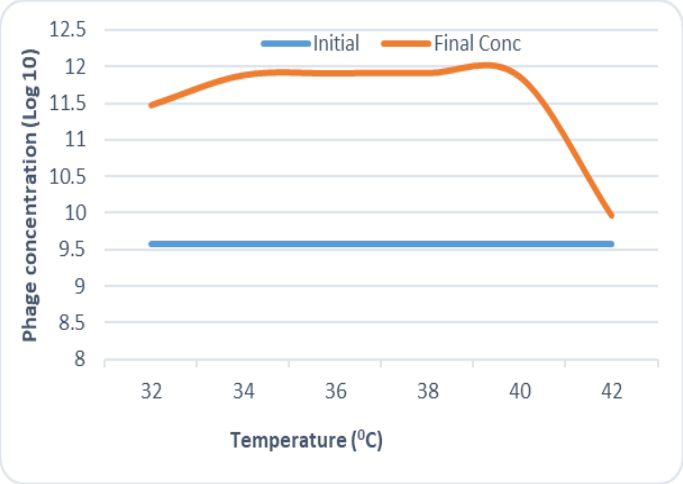

Fig 7: Different concentration of *Staphylococcus aureus* phages produced at different temperature at pH 7. Initial concentration of phage tested is 9.6414 log 10. The maximum concentration of phage obtained at temperature of 36^{0}C is 11.99493 log 10

Table-1 :Initial and the final concentration of *Staphylococcus aureus* phages at pH 6, 7 & 8 at different temperature

Temp (^{0}C)	pH					
	6		7		8	
	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)
32	9.570543	7.15295	9.57054	11.47538	9.57054	7.45682
34	9.570543	7.24303	9.57054	11.88429	9.57054	7.69372
36	9.570543	7.14238	9.57054	11.91025	9.57054	7.98281
38	9.570543	7.35602	9.57054	11.91614	9.57054	8.18752
40	9.570543	6.99025	9.57054	11.86611	9.57054	7.44560
42	9.570543	6.76492	9.57054	9.964731	9.57054	6.43933

Enterococcus faecium phage :

Similar to the phages against *S. aureus* and *E. faecium,* phages against *Enterococcus faecium* were isolated from lake water, sewage water and open drainage water. The size of plaque ranged from 1.0mm to 5mm in diameter

Antibacterial activity and Host specificity :

E. faecium phage (Fig 2) formed plaques on all six isolates. Clear plaques were produced on all strains tested. Pinpoint plaques were seen when *Enterococcus faecalis* was spotted with *Enterococcus faecium* phages.

Effect of pH and temperature :

No phages were recovered at pH 1, 2, 3, 4, and 5

The maximum phage recovered at temperature 32^{0}C is 7.8273 log10 PFU, at 34^{0}C is 7.8876 log10 PFU, at 36^{0}C is 7.9658 log10 PFU, at 38^{0}C is 8.7275 log10 PFU, at 40^0 C is 8.4312 log10 PFU and at 42^{0}C is 7.6981 log10 PFU (Table-2)

At pH 7, the maximum number of phages (9.7745 log10 PFU) was isolated at 38^{0}C. It was more than the initial concentration (9.2355 log10 PFU). The phages isolated at other temperatures are; at temperature, 32^{0}C is 7.9148 log10 PFU, at 34^{0}C is 8.2837 log10 PFU, at 36^{0}C is 8.5682 log10 PFU, at 40^0 C is 9.0042 log10 PFU and at 42^{0}C is 8.4623 log10 PFU (Table-2, Fig-8).

At pH 8, the maximum phage recovered at temperature 32^{0}C is 7.6801 log10 PFU, at 34^{0}C is 7.7160 log10 PFU, at 36^{0}C is 7.9740 log10 PFU, at 38^{0}C is 8.7625 log10 PFU, at 40^0 C is 8.4596 log10 PFU and at 42^{0}C is 7.0041 log10 PFU (Table-2).

At pH 9 to 12, the phages were not recovered

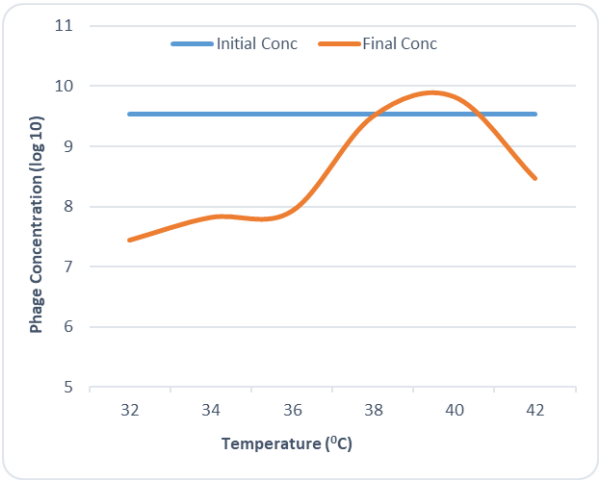

Fig-8: Different concentration of *Enterococcus faecium* phages produced at different temperature at pH 7. Initial concentration of phage tested is 9.6414 log 10. The maximum concentration of phage obtained at temperature of 36^{0}C is 11.99493 log 10

Table-2 :Initial and the final concentration of *Enterococcus faecium* phages at pH 6, 7 & 8 at different temperature

Temp (^{0}C)	pH					
	6		7		8	
	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)
32	9.23552	7.82736	9.23552	7.91487	9.23552	7.69019
34	9.23552	7.88761	9.23552	8.28375	9.23552	7.71600
36	9.23552	7.96581	9.23552	8.56822	9.23552	7.97405
38	9.23552	8.72751	9.23552	9.77451	9.23552	8.76258
40	9.23552	8.43120	9.23552	9.00423	9.23552	8.45969
42	9.23552	7.69815	9.23552	8.46239	9.23552	7.00414

Klebsiella pneumoniae phage :

Phages against MDR *Klebsiella pneumonia* were isolated from lake water, sewage water and open drainage water. The size of plaque ranged from 1.0mm to 7.0mm in diameter. The phage isolated from sewage water had the highest plaque diameter.

Antibacterial activity and Host specificity :

Klebsiella pneumoniae phage lysed all the strains of *Klebsiella pneumoniae tested.* The phage was found to form plaques on 89.2% of the MDR *K. pneumoniae* isolates and inhibited bacterial growth of an additional 5.4% of the strains, thus exhibiting an antibacterial effect against 94.6% of the MDR *K. pneumoniae* strains. But no plaques were seen when *Klebsiella pneumoniae* phage was treated with *Pseudomonas aeruginosa* or *Acinetobacter baumannii,* accounting for its host specificity.

Effect of pH and temperature :

At a pH of 3 to 5, no phages were recovered at different temperatures. At pH 6, the maximum number of phages isolated was less than the initial concentration (9.5705 log10 PFU). The maximum phage recovered at temperature 32^{0}C is 8.7387 log10 PFU, at 34^{0}C is 8.8243 log10 PFU, at 36^{0}C is 9.8030 log10 PFU, at 38^{0}C is 9.9376 log10 PFU, at 40^0 C is 6.8311 log10 PFU and at 42^{0}C is 6.7752 log10 PFU (Table-3).

At pH 7, the maximum number of phages (11.9949 log10 PFU) was isolated at 38^{0}C. It was more than the initial concentration (9.5705 log10 PFU). The phages isolated at other temperatures are; at temperature 32^{0}C is 11.5731 log10 PFU, at 34^{0}C is 11.9182 log10 PFU, at 36^{0}C is 11.9477 log10 PFU, at 40^0 C is 10.7911 log10 PFU and at 42^{0}C is 10.0863 log10 PFU (Table-3, Fig-9).

At pH 8, the maximum number of phages isolated was less than the initial concentration (9.5705 log10 PFU). The maximum phage recovered at temperature 32^{0}C is 7.4517 log10 PFU, at 34^{0}C is 7.8705 log10 PFU, at 36^{0}C is 8.2105 log10 PFU, at 38^{0}C is 8.5705 log10 PFU, at 40^0 C is 7.1072 log10 PFU and at 42^{0}C is 6.9921 log10 PFU (Table-3). At pH 9 to 12, the phages were not recovered

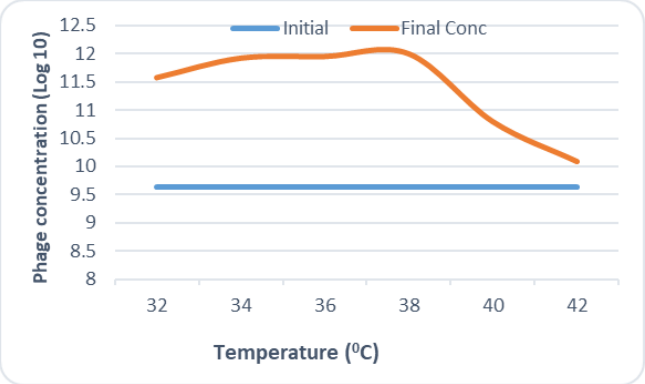

Fig 9: Different concentration of *Klebsiella pneumoniae* phages produced at different temperature at pH 7. Initial concentration of phage tested is 9.6414 log 10. The maximum concentration of phage obtained at temperature of 36^{0}C is 11.99493 log 10

Table-3: Initial and the final concentration of *K. pneumoniae* phages obtained at pH 6, 7 & 8 at different temperature

Temp (^{0}C)	pH					
	6		7		8	
	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)
32	9.57054	8.73878	9.57054	11.5731	9.57054	7.45178
34	9.57054	8.82438	9.57054	11.9182	9.57054	7.87052
36	9.57054	9.80303	9.57054	11.9477	9.57054	8.21058
38	9.57054	9.93765	9.57054	11.9949	9.57054	8.57054
40	9.57054	6.83110	9.57054	10.7911	9.57054	7.10721
42	9.57054	6.77524	9.57054	10.0863	9.57054	6.99211

***Pseudomonas aeruginosa* phage :**

Bacteriophages against MDR *Pseudomonas aeruginosa* were isolated from all the water samples (lake, sewage, field, open drain). The phage had the plaque size ranging from 0.5mm to 11 mm in diameter. Highest diameter was observed among the phage isolated from sewage and open drain water

Antibacterial activity and Host specificity *:*

Pseudomonas aeruginosa phage lysed all *Pseudomonas aeruginosa* strains, but no plaques were seen when it was treated with *Klebsiella pneumoniae* or *Acinetobacter baumannii.* The *Ps. aeruginosa* phage formed plaques on all 31 MDR *Ps. aeruginosa* thus exhibiting an antibacterial effect of 100% against MDR *Ps. aeruginosa*.

Effect of pH and temperature :

At a pH of 3 to 5, no phages were recovered at different temperatures. At pH 6, the maximum phage recovered at temperature 32^{0}C is 8.6512 log10 PFU, at 34^{0}C is 8.8850 log10 PFU, at 36^{0}C is 9.7286 log10 PFU, at 38^{0}C is 9.9850 log10 PFU, at 40^0 C is 7.6700 log10 PFU and at 42^{0}C is 6.7555 log10 PFU (Table-4).

At pH 7, the maximum number of phages (11.9993 log10 PFU) was isolated at 38^{0}C. It was more than the initial concentration (9.6148 log10 PFU). The phages isolated at other temperatures are; at temperature 32^{0}C is 11.6572 log10 PFU, at 34^{0}C is 11.8906 log10 PFU, at 36^{0}C is 11.9173 log10 PFU, at 40^0 C is 10.8562 log10 PFU and at 42^{0}C is 9.3424 log10 PFU (Table-4, Fig-10).

At pH 8, the maximum number of phages isolated was less than the initial concentration (9.6148 log10 PFU). The maximum phage recovered at temperature 32^{0}C is 7.5599 log10 PFU, at 34^{0}C is 7.6939 log10 PFU, at 36^{0}C is 7.9973 log10 PFU, at 38^{0}C is 8.7649 log10 PFU, at 40^0 C is 7.9273 log10 PFU and at 42^{0}C is 7.8313 log10 PFU (Table-4, Fig-10). At pH 9 to 12, the phages were not recovered.

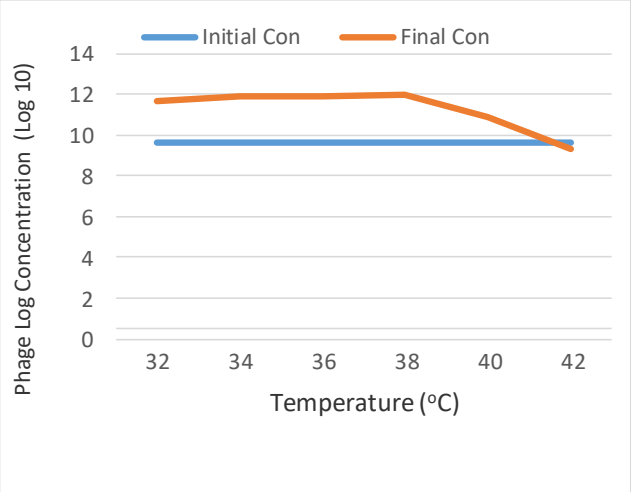

Fig-10: Different concentration of *Pseudomonas aeruginosa* phages produced at different temperature at pH 7. Initial concentration of phage tested is 9.6414 log 10. The maximum concentration of phage obtained at temperature of 36^{0}C is 11.99493 log 10

Table-4: Initial and the final concentration of *Ps. aeruginosa* phages obtained at pH 6, 7 & 8 at different temperature

Temp (^{0}C)	pH					
	6		7		8	
	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)
32	9.61489	8.65127	9.61489	11.6572	9.61489	7.55990
34	9.61489	8.88502	9.61489	11.8906	9.61489	7.69390
36	9.61489	9.72866	9.61489	11.917	9.61489	7.99738
38	9.61489	9.98509	9.61489	11.999	9.61489	8.76492
40	9.61489	7.67004	9.61489	10.8565	9.61489	7.92737
42	9.614897	6.75557	9.614897	9.342423	9.614897	7.831358

***Acinetobacter baumannii* phage :**

Phages against MDR *Acinetobacter baumannii* were isolated from lake water, field water and open drainage water. The plaque size of the phage ranged from 0.5mm to 8.0mm and highest size was seen among the phages isolated from the lake water, field water and open drainage water.

Antibacterial activity and Host specificity :

A. baumannii phage formed plaques on 57.1% of MDR *A. baumannii* isolates. *Acinetobacter baumannii* phage in concordance with the above phage specificity, lysed all the *Acinetobacter baumannii* without any phage activity when treated with *Klebsiella pneumoniae* or *Pseudomonas aeruginosa.*

Effect of pH and temperature :

At a pH of 3 to 5, no phages were recovered at different temperatures. At pH 6, the maximum number of phages isolated was less than the initial concentration (9.5415 log10 PFU). The maximum phage recovered at temperature 32^{0}C is 8.3579 log10 PFU, at 34^{0}C is 8.7180 log10 PFU, at 36^{0}C is 9.2562 log10 PFU, at 38^{0}C is 9.0539 log10 PFU, at 40^0 C is 8.4186 log10 PFU and at 42^{0}C is 8.2504 log10 PFU (Table-5).

At pH 7, the maximum number of phages (10.4966 log10 PFU) was isolated at 38^{0}C. It was more than the initial concentration (9.5415 log10 PFU). The phages isolated at other temperatures are; at temperature 32^{0}C is 8.8115 log10 PFU, at 34^{0}C is 8.9882 log10 PFU, at 36^{0}C is 9.9447 log10 PFU, at 40^0 C is 9.6569 log10 PFU and at 42^{0}C is 8.4639 log10 PFU (Table-5, Fig-11).

At pH 8, the maximum number of phages isolated was less than the initial concentration (9.5415log10 PFU). The maximum phage recovered at temperature 32^{0}C is 7.4487 log10 PFU, at 34^{0}C is 7.8269 log10 PFU, at 36^{0}C is 7.9304 log10 PFU, at 38^{0}C is 9.5051 log10 PFU, at 40^0 C is 9.8325 log10 PFU and at 42^{0}C is 8.4742 log10 PFU (Table-5). At pH 9 to 12 the phages were not recovered.

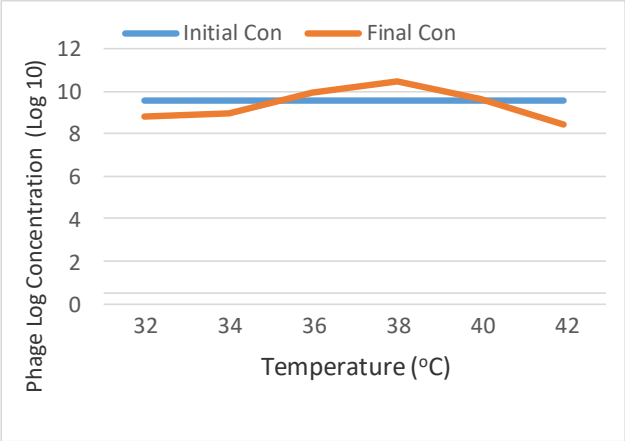

Fig-11: Different concentration of *Acinetobacter baumannii* phages produced at different temperature at pH 7. Initial concentration of phage tested is 9.6414 log 10. The maximum concentration of phage obtained at temperature of 36^{0}C is 11.99493 log 10

Table-5: Initial and the final concentration of *A. baumannii* phages obtained at pH 6, 7 & 8 at different temperature

Temp (^{0}C)	pH					
	6		7		8	
	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)
32	9.541579	8.357935	9.541579	8.811575	9.541579	7.448706
34	9.541579	8.718003	9.541579	8.988291	9.541579	7.826981
36	9.541579	9.256208	9.541579	9.944713	9.541579	7.930440
38	9.541579	9.05395	9.541579	10.49661	9.541579	9.505164
40	9.541579	8.418633	9.541579	9.656998	9.541579	9.832509
42	9.541579	8.250435	9.541579	8.463902	9.541579	8.474225

***Enterobacter* phage :**

Phages against MDR *Enterobacter species* were isolated from lake water, sewage water and open drainage water. The size of the plaque ranged from 0.5mm to 5.0mm and highest size was seen among the phages isolated from the sewage waster.

Antibacterial activity and Host specificity :

Enterobacter phage was found to form plaques on 79% of the MDR *Enterobacter species* and inhibited bacterial growth of an additional 8% of the strains, thus exhibiting an antibacterial effect against 87% of the strains in our collection. Clear plaques were produced on all strains

Effect of pH and temperature:

At pH 6, the maximum number of phages isolated was less than the initial concentration (8.9322 log10 PFU). The maximum phage recovered at temperature 32^{0}C is 7.945 log10 PFU, at 34^{0}C is 7.9523 log10 PFU, at 36^{0}C is 8.010 log10 PFU, at 38^{0}C is 8.9322 log10 PFU, at 40^0 C is 8.695 log10 PFU and at 42^{0}C is 8.02433 log10 PFU (Table 5, Fig 6).

At pH 7, the maximum number of phages (9.7808 log10 PFU) was isolated at 38^{0}C. It was more than the initial concentration (9.2355 log10 PFU). The phages isolated at other temperatures are; at temperature, 32^{0}C is 7.9982 log10 PFU, at 34^{0}C is 8.3780 log10 PFU, at 36^{0}C is 8.6974 log10 PFU, at 40^0 C is 9.3615 log10 PFU and at 42^{0}C is 9.0179 log10 PFU (Table-6, Fig-12).

At pH 8, the maximum number of phages isolated was less than the initial concentration (8.999 log10 PFU). The maximum phage recovered at temperature 32^{0}C is 7.822 log10 PFU, at 34^{0}C is 7.8627 log10 PFU, at 36^{0}C is 8.078 log10 PFU, at 38^{0}C is 8.999 log10 PFU, at 40^0 C is 8.827 log10 PFU and at 42^{0}C is 7.5563 log10 PFU (Table-6). At pH 9 to 12, the phages were not recovered

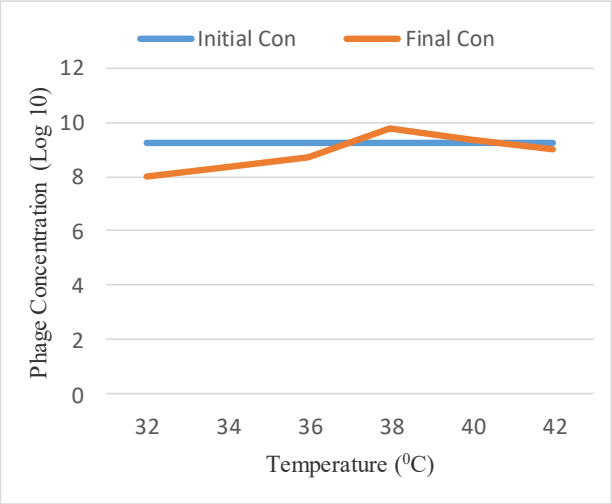

Fig-12: Different concentration of *Enterococcus faecalis* phages produced at different temperature at pH 7. Initial concentration of phage tested is 9.6414 log 10. The maximum concentration of phage obtained at temperature of 36^{0}C is 11.99493 log 10

Table-6: Initial and the final concentration of *Enterobacter spp* phages obtained at pH 6, 7 & 8 at different temperature

Temp (^{0}C)	pH					
	6		7		8	
	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)
32	9.235528	7.945469	9.235528	7.998259	9.235528	7.822168
34	9.235528	7.952308	9.235528	8.378034	9.235528	7.862728
36	9.235528	8.010427	9.235528	8.697421	9.235528	8.078928
38	9.235528	8.932205	9.235528	9.780805	9.235528	8.999770
40	9.235528	8.695657	9.235528	9.361501	9.235528	8.827499
42	9.235528	8.02533	9.235528	9.017954	9.235528	7.556375

DISCUSSION :

The World Health Organization (WHO) created a list of antibiotic-resistant, top global priority infections in 2017 to help with research and the creation of novel, powerful antibiotic therapies. The list was created using multi-criteria assessments and divided into three priority categories: medium, high, and critical. The multidrug-resistant Gram-negative ESKAPE pathogens, including *Acinetobacter baumannii* (carbapenem-resistant), *Pseudomonas aeruginosa* (carbapenem-resistant), *Klebsiella pneumoniae* (third generation cephalosporin-resistant), and *Enterobacter spp*., are included in the "Priority 1: critical". The Gram-positive ESKAPE pathogens such as *Enterococcus faecium* (vancomycin-resistant) and *Staphylococcus aureus* (methicillin-resistant, vancomycin-intermediate and resistant) are included in the "Priority 2: High" pathogen group. These ESKAPE are frequent pathogens in several deadly infections like meningitis, sepsis, pneumonia, and others, especially in intensive care units, which are constant sources of mortality and morbidity. These pathogens have also been isolated from common environmental water sources such as drinking water, surface runoff, ponds, rivers, and sewage water[6-11]. The presence of ESKAPE pathogens in these niches has been attributed to contamination via hospital wastes and improper sewage treatment and disposal.

Even though there are many alternatives to antibiotics available, none have proved as effective as bacteriophage for the treatment when no alternatives are available. Bacteriophages against bacterial pathogens have failed as therapeutic agents because the antibiotics were cheaper and also the difficulty in selecting, isolating, and characterizing the bacteriophages against these pathogens made them less attractive alternatives. Bacteriophages used in phage therapy need to be able to target pathogenic variants of bacteria in a human. In addition to this, they should be virulent phages i.e., they should kill the targeted bacteria when they infect, and phages should be stable and virulent throughout the storage. So, testing specific storage conditions for each phage is very difficult. Because some are more stable at lower temperatures such as around 4^{o}C (these include phages against *Shigella* and Gram-negative bacteria like *Haemophilus, Pasteurella, Pseudomonas, Rhizobium, Vibrio*) and even lower temperatures of -80^{o}C (which include *Aeromonas, Bacteroides, Brucella*, and some phages against *Vibrio*). Some exhibit stability at room temperatures (phages against ESKAPE pathogens). pH also has an impact on the survival and persistence of bacteriophages[14,17]. In response to outside variables, the phage population is often steady. Some phages may be kept in solution or dry form for an extended period of time at neutral pH (6 to 8)[14]. With change in pH, phage titers often decline gradually. For instance, when the pH dropped from 6.19 to 5.38 between 4 and 6 hours, the phage titer of *S. aureus* was lowered by 2 log. Below pH 4.5, there is often less of a chance of harmful bacteria contaminating food as well as a restriction on the growth of numerous phages[18,19]. For instance, the Myoviridae family's phage T4 is unstable at pH of <5. At pH 5.0 and 37°C, phage PM2 (Corticoviridae family) completely loses activity after 1 hour. However, stomach acid might negatively affect the survival of phage in the event of phage oral injection, which could result in therapeutic failure[20]. Finally, these phages should be resistant to patients' immune responses. So, the routes of administration in different diseases vary.

This research was intended to highlight the importance of bacteriophage therapy in treating multidrug-resistant infections caused by ESKAPE pathogens. Many parameters such as antibacterial activity, host specificity, and viability of phages in different pH and temperatures have been assessed for the purpose of knowing the efficacy of the treatment against multidrug-resistant infections. Present findings are in accordance with earlier reports of Tiwari et al[14]. and Shukla[20] and Hirpurkar[21] who observed that phage viability was maximal between pH 5 and 9 and all phages were completely inactivated at pH of 3 and 11. Likewise, Ibrahim et al[22]. observed stable lytic activities at pH 6-8. Low pH reportedly affects phage aggregation and reduces their abilities to penetrate the host cells [11]. Our findings are contradictory to those reported by Krasowska et al.[17] who found that phages of Bacillus were resistant to high temperatures (80°C for 1 min).

In the present study, phages were exposed to various pH and temperature combinations. There was no phage activity at extreme conditions of temperature (45°C)[15,22] and pH. Studies have reported that viability at pH 9 and temperatures < 15°C is one of the most common limiting factors for phage activity[14,] . Phages are generally more temperature and pH-resistant compared to their host bacteria [16] . In the present study, since the phage was isolated against ESKAPE pathogens infecting man, 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 to 3-log of recovered phages at various temperatures. When phage stability at pH 6 was compared with pH 7 and pH 8, the difference was less or insignificant at lower (40°C) temperatures. However, at temperatures between 26° C and 40° C, there was a significant difference ($p< 0.05$), phage undergoes irreversible coagulation and precipitation resulting in inactivity [17]. Studies have also suggested the lowest phage inactivation at near-neutral pH (pH 6 to 8) and temperatures around 37°C[21]. The right temperature and pH guide the optimum antibacterial activity of phages [19]. 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 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. 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 [22-25]. There was an 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).

In conclusion, pH and temperature are critical factors that impact the success of phage therapy. To maximize the effectiveness of phage therapy, it is crucial to maintain optimal pH and temperature ranges that suit the specific phage strain used in the therapy.

REFERENCES :

1. d'Herelle F. Bacteriophage as a treatment in acute medical and d'Herelle F. Bacteriophage as a Treatment in Acute Medical and Surgical Infections. Bull N Y Acad Med. 1931;7(5):329-48.
2. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. Bacteriophage. 2011;1 (2):66-85.
3. Chanishvili N. Phage therapy--history from Twort and d'Herelle through Soviet experience to current approaches. Adv Virus Res. 2012;83:3-40
4. Eaton MD, Bayne-jones S. Bacteriophage therapy: Review of the principles and results of the use of bacteriophage in the treatment of infections. JAMA. 1934;103 (23):1769–1776.
5. Sommer MOA, Munck C, Toft-Kehler RV, Andersson DI. Prediction of antibiotic resistance: time for a new preclinical paradigm? Nat Rev Microbiol. 2017 ;15:689-696.
6. World Health Organization. 2021. Antimicrobial resistance:Fact sheets 17 Nov 2021. https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance.
7. Veeraraghavan B, Shankar C, Karunasree S, Kumari S, Ravi R, Ralph R. Carbapenem resistant Klebsiella pneumoniae isolated from bloodstream infection: Indian experience. Pathog Glob Health. 2017;111(5):240-246.
8. Majid Taati Moghadam, Mehrnaz Mirzaei, Mozhdeh Fazel Tehrani Moghaddam, Sajad Babakhani, Omid Yeganeh, Sajad Asgharzadeh, Hamed Eraghiye Farahani, and Shahla Shahbazi.The Challenge of Global Emergence of Novel Colistin-Resistant Escherichia coli ST131.Microbial Drug Resistance 2021;11:1513-1524.
9. Kourtis AP, Hatfield K, Baggs J, Mu Y, See I, Epson E, Nadle J, Kainer MA, Dumyati G, Petit S, Ray SM; Emerging Infections Program MRSA author group; Ham D, Capers C, Ewing H, Coffin N, McDonald LC, Jernigan J, Cardo D. Vital Signs: Epidemiology and Recent Trends in Methicillin-Resistant and in Methicillin-Susceptible Staphylococcus aureus Bloodstream Infections - United States. MMWR Morb Mortal Wkly Rep. 2019;68:214-9.
11. World Health Organization. 2017. Publications: Prioritization of pathogens to guide discovery, research, and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis 4 September 2017. https://www.who.int/publications/i/item/WHO-EMP-IAU-2017.12.
12. Llaca-Díaz JM, Mendoza-Olazarán S, Camacho-Ortiz A, Flores S, Garza-González E. One-year surveillance of ESKAPE pathogens in an intensive care unit of Monterrey, Mexico. Chemotherapy. 2012;58:475-81.
13. Lin DM, Koskella B, Lin HC. Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. World J Gastrointest Pharmacol Ther. 2017;8(3):162-73.
14. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 22th informational supplement. Wayne, PA; 2022.
15. Tiwari, Ruchi & Hirpurkar, s.D. & Shakya, S. Isolation and characterization of lytic phages from natural waste material of Livestock 2010;87:644-6.

16. Adriaenssens EM, Sullivan MB, Knezevic P, et al. Taxonomy of prokaryotic viruses: 2018-2019 update from the ICTV Bacterial and Archaeal Viruses Subcommittee. Arch Virol. 2020; 165:1253- 1260.

17. Krasowska A, Biegalska A, Augustyniak D, Łoś M, Richert M, Łukaszewicz M. Isolation and Characterization of Phages Infecting Bacillus subtilis. Biomed Res Int. 2015; , Article ID 179597, 10 pages doi: 10.1155/2015/179597

18. P. García, C. Madera, B. Martínez, A. Rodríguez, J. Evaristo Suárez, Prevalence of bacteriophages infecting Staphylococcus aureus in dairy samples and their potential as biocontrol agents, Journal of Dairy Science 2009;92:3019-26

19. Nurhafizah W. W. I., Alia S. A., Azna N. S., Lee K. L., Nadirah M., Laith A. R., Danish-Daniel M., Zainathan S. C., Shariff M., Mazlan A. G., Najiah M. In-Vitro characterization of lytic bacteriophage PhVh6 as potential biocontrol agent against pathogenic Vibrio harveyi. AACL Bioflux 2017;10(1):64-76.

20. Hirpurkar, S.D. Recovery status of bacteriophages of different livestock farms of Veterinary College, Adhartal, Jabalpur, India. Veterinary World. 2011;4:117-9.

21. Shukla S, Hirpurkar S.D. Recovery status of bacteriophages of different livestock farmsf Veterinary College, Adhartal, Jabalpur, India. Vet. World. 2011;4(3):117–119.

22. Ibrahim W.N.W, Aznan A.S, Saari N.A, Leong L.K, Musa N, Razzak L.A, Danish-Daniel M, Zainathan S.C, Din M.S.M, Ghaffar M.A, Musa N. In-vitro characterization of lytic bacteriophage PhVh6 as potential biocontrol agent against pathogenic Vibrio harveyi. AACL Bioflux. 2017;10(1):64–76.

23. Ackermann H.W., Tremblay D., Moineau S. Long-term bacteriophage preservation. WFCC Newslett. 2004;38:35 –40.

24. Taj MK, Ling JX and Bing LL: Effect of dilution, temperature and ph on the lysis activity of T4 phage against E.coli BL21. The Journal of Animal & Plant Sciences 2014; 24: 1252-55.

25. Fernández L, Gutiérrez D and García P: The perfect bacteriophage for therapeutic applications-a quick guide. Antibiotics Basel 2019; 8:126.

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