

AN INSIGHT INTO BACTERIOPHAGES' RESPONSE TO VARIOUS PHYSICAL AND CHEMICAL CONDITIONS: TEMPERATURE, pH, SALTS

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Abstract

Bacteriophages can survive under unfavorable conditions and their abilities of survivability are highly diversified. The influence of different physical and chemical conditions, such as temperature, pH and salts on the survivability of coliphages and shigaphages isolated from local clinical sewage samples have been summarized. Temperature at 45°C for 30 min caused 53.13% decrease in the number of coliphages but rather increased the number of shigaphages in present study. Temperature at 56°C for 30 min caused 91.41 and 100% fall in the number of coli- and shigaphages, respectively. pH 4 mediated 96.88% and pH 11 caused 100% decline in the number of plaques for coliphages. On the other hand, number of plaques of shigaphages was diminished by 100% at pH 4 and 91.67% at pH 11. MgCl₂, MgSO₄ and Na₂SO₄ salt solutions increased the number of coliphage plaques by 287.5, 125 and 37.5%. On the contrary, the decrease in the number of shigaphages was 79.31, 10.34 and 44.83%, respectively through the treatment. The various physical and chemical conditions investigated in this research have affected the test bacteriophages diversely. Temperature while applied at higher level (56°C) affected the growth of both coliphages and shigaphages. Acidic and alkaline pH modified coli- and shigaphages' survivability and growth negatively. The three salts in question augmented the growth of isolated coliphages profoundly. However, the same three salts failed to do so for the isolated shigaphages.

Introduction

Bacteriophages are viruses which interact with individual bacterial cell surface components and show specificity for particular bacterial species^(1,2), but harmless to human, animals and plants. Bacteriophages represent perhaps the most ubiquitous of all biotic drivers⁽²⁻⁴⁾. They are the most numerous form of life on earth; ten times more numerous than bacteria^(5,6). They can be found in all environments where bacteria grow: in the Sahara, hot springs, the North Sea, and polar inland waters⁽⁷⁻¹⁰⁾.

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Bacteriophages were first discovered in 1915 by William Twort, and in 1917 Felix d'Herelle realized that they had the potential to kill bacteria⁽²⁾. Thereby the opportunity of utilizing bacteriophages in human and veterinary medicine, wastewater treatment as well as in various agricultural settings has been exploited^(11,12). Phages can keep their promises to decontaminate carcasses and other raw products, to prevent or reduce bacterial colonization in livestock, to extend the shelf life of perishable man-made foods and to disinfect equipments and contact surfaces⁽¹³⁾. The demand for natural and biological tools rather than the usage of chemical preservatives in foodstuff and food processing surfaces has recently been enhanced.

More and more people are dying every year because of the multi drug-resistant strains of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and wild strains of *Salmonella typhi* and *E. coli*⁽¹⁴⁾. The recent emergence of antibiotic-resistant pathogenic bacterial strains has motivated researchers to look for means of disease control^(15,16). Phages are thus being preferred because, unlike broad spectrum antibiotics, phages are highly specific and do not illicit resistance from untargeted bacterial strains⁽¹⁷⁾. The use of bacteriophages for the treatment of diseases that are potentially life threatening is called phage therapy. Phage therapy has been explored for the treatment of diverse diseases, like diarrhoea, cholera, dysentery on several occasions with promising results^(18,19).

Earlier study on phages suggested that the phages have cured 90% of the chronic suppurative bacterial infections (emphysemas, peritonitis, osteomyelitis etc.) in humans caused by antibiotic resistant pathogen such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *E. coli*⁽²⁰⁾. Outside the host cell, the phages are almost non living and they always need a host cell for replication and other metabolic processes⁽²⁰⁾. So there is no side effect of phage therapy on treated hosts. Therefore, researches focused on isolating and understanding phages that could provide an additional tool to tackle the global vulnerability to antimicrobial resistance.

Bacteriophages can also cause great economic losses due to fermentation failure in dairy plants⁽²¹⁾. Hence, physical and chemical treatments of raw material and/or equipment are mandatory to maintain phage levels as low as possible. Phages are difficult to eliminate because they rapidly disseminate in dairy plants⁽²²⁾. As phage infections lead to economic losses in dairy factories, the development of control measures require study of bacteriophages.

Bacteriophages as well as animal viruses can be vulnerable to unfavorable physical and chemical factors, such as low and high temperatures, pH, salinity, and ions. So far, anyone who is going to preserve phages should acquire adequate information about their relevant susceptibilities⁽²³⁾. High bacteriophage resistance for external factors is important for stability of phage preparations. However, this feature is highly

disadvantageous for research and commercial purposes when maintenance of the structural integrity and particular activities of viral strains become crucial. Inactivation of phages in different environments is multi-factorial. It is known that the variation of one factor that influences phages may change phage sensitivity to others⁽²⁴⁾.

A better understanding of the complex problem of phage sensitivity to external factors and new data may be useful not only for those interested in pharmaceutical and agricultural applications of phages, but also for others working on preservation of phages⁽²⁵⁾. The aim of the present work was to isolate coli- and shigaphages from nature, determine their titre and explore their comparative vulnerabilities towards some physical agents such as temperature, pH and salt solutions.

Materials and Methods

The prevalence of large populations of pathogenic bacteria existing in close proximity in sewage water makes it a relevant source for the isolation of various bacteriophages^(14,34). Thus raw sewage sample was collected from the outer ward unit of an urban based hospital where the whole hospital's sewage effluents were collected and later passed to the municipality drainage system⁽¹⁴⁾. The samples were dark ash in color and had strong odor.

The sewage sample was passed through the Whatman No. 1 filter paper two times to remove most of the suspended debris. Filtrate (1.5 ml) was collected into sterile Eppendorf tube and centrifuged at 12000 rpm for 15 minutes. The supernatant was filtered through 0.22 μm syringe filter (Millipore USA) to remove the bacterial population. The final filtrate was collected into a sterile vial containing viruses only.

Two bacterial species (*E. coli* and *Shigella flexneri*) were employed to isolate the bacteriophages to be used for the determination of the effect of various chemical treatments on them. Luria-Birtani (LB) medium, (Sigma Chemical Company, St. Louis, MO, US) was used for the cultivation of the two bacterial population.

Loopful of bacterial culture (*E. coli* and *Shigella flexneri*) were inoculated into separate tubes containing LB broth. The inoculated broth was incubated for four hours in a shaking incubator at 37°C.

Prepared (centrifuged and milipore-filtered) sewage sample (0.5 ml) with 0.5 ml of test chemical (double strength chemical) was used to make the working concentration exact. The mixture was incubated at room temperature for 30 minutes. For control, 0.5 ml of prepared (centrifuged with Hettich Mikro 120 centrifuge machine, membrane filtered) sewage sample was mixed with 0.5 ml of sterilized distilled water in it. This mixture was also incubated at room temperature for 30 minutes. Then 100 μl of treated sewage sample and 100 μl of young culture of specific bacteria were added into 5 ml of LB soft agar

(0.8%). After mixing gently by hand, this mixture was poured on LB agar plate and briefly swirled by gentle sidewise movement for even and uniform distribution of the top agar over the LB agar in the Petri dish. The top agar was allowed to be solidified. The same process was done for the control. After incubating the plate at 37°C overnight, the cleared zones/plaques on the bacterial lawn were observed. The plaque forming units/ml (pfu/ml) of the supplied treated and untreated sewage sample was counted and calculated for each treatment. The average pfu/ml was calculated from duplicate tests for each physical treatment. Comparison between the counts obtained for the treated and untreated sewage sample was done to understand the effect of the treatments on the viability and propagation of the specific bacteriophage that have been tested in the experiment.

Table 1. The treatments performed in this study.

Treatment	Working concentration	Incubation temperature and time
Temperature: 45°C	Not needed	30 min in waterbath
Temperature: 56°C	Not needed	"
pH 4.0	50 µl of 1N HCl solution in 1.0 ml of processed sewage sample	30 min at room temperature on bench (covered with aluminium foil)
pH 11.0	25 µl of 1N NaOH solution in 15 ml of processed sewage sample	"
MgCl ₂	(1 mol/l) in H ₂ O	MgCl ₂ + 50°C, 1 hr 50°C, 1 hr (control)
MgSO ₄	(1 mM/l) in H ₂ O	MgSO ₄ + 50°C, 1 hr 50°C, 1 hr (control)
Na ₂ SO ₄	(1 mol/l) in H ₂ O	Na ₂ SO ₄ + 50°C, 1 hr 50°C, 1 hr (control)

Results and Discussion

In recent years considerable attention has been focused on bacteriophages, which are widely distributed in the environment. They play important roles not only in treating systemic and enteric diseases but also in preventing bacterial contamination of food products and packaging. Their surprisingly diverse ability of survival under unfavorable conditions has inspired, scientists to characterize them. In this experiment, the prevalence of large populations of pathogenic bacteria existing in close proximity in sewage water made it a relevant source for the isolation of various bacteriophages. They were found to have bactericidal properties as they largely feed on specific bacteria. Various external physical and chemical factors, such as temperature, acidity, salinity, and ions, determine the occurrence, viability, and storage of bacteriophages. These versatile applicants can inactivate a phage by destabilizing its structural integrity by affecting capsid or envelope, facilitating lipid loss, and/or initiating NA structural deviation⁽¹⁴⁾. A few of these attributes may also positively interfere with the endurance and thus enhance its survival.

Bacteriophages infecting *E. coli* and phages specific for *S. flexneri* sustaining in clinical sewage were isolated in summer (April, 2014) and subsequently were enumerated by host assessment. Most (89%) members of the isolated phage population were specific for *S. flexneri*. Only 11% of the isolated viruses had infected *E. coli*. Proportion of various bacteriophages in nature varies widely with time and place.

The phages obtained in this experiment were lytic. The development of clear zones of lysis against specific host bacteria using phage lysate indicated that all the phages isolated were lytic phages.

While heat treatment was applied at different degrees, the test coli phages reacted differently. Higher temperature made viruses more vulnerable as they were reduced by 91.41% at 56 DC compared to 53.13% decrease at 45°C (Fig. 1). Temperature is a crucial factor for bacteriophage survivability⁽²⁷⁻³⁰⁾ as it plays a fundamental role in attachment, penetration and multiplication. Thermal treatments produce morphological changes on phage particles. The effects of such treatments were monitored in transmission electron microscopy⁽²⁷⁾ by Atamer *et al.* (2010). Release of phage DNA from viral capsids, decomposition of phage into head and tail structures, and aggregation of phage tails were the most frequently seen phenomena, principally for the heat sensitive phage studied. For heat resistant ones, changes in morphology were less evident.

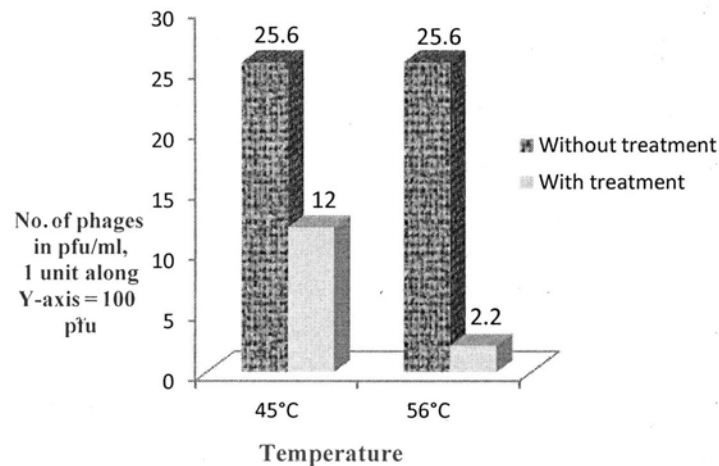


Fig. 1. Susceptibility of isolated coliphages at stated temperature for 30 min.

However, for shigaphages 45°C did not have any negative effect on their plaque numbers at all. But 100% decrease in the number of plaque occurred when the phages were incubated at 56°C for 30 minutes (Fig. 2).

Another important factor affecting phage stability was the acidity and alkalinity of the environment. In a previous study, Kerby *et al.* (1949)⁽³¹⁾ suggested that irreversible

coagulation and precipitation of bacteriophages according to environment might be the factors limiting phage activity. Feng *et al.* (2003)⁽³²⁾ investigated the survivability of coliphages in water and wastewater with regard to the effects of different pH. In his study, coliphages presented lowest inactivation rate in the range of pH 6 to 8. Langlet *et al.*⁽³³⁾ showed that hydrogen ion concentration influenced phage aggregation. Their aggregation could be up to 6 μm in diameter. Acidity may cause a decline in phage count and may subsequently eliminate aggregates through their adsorption on host cell membranes than single virion alone.

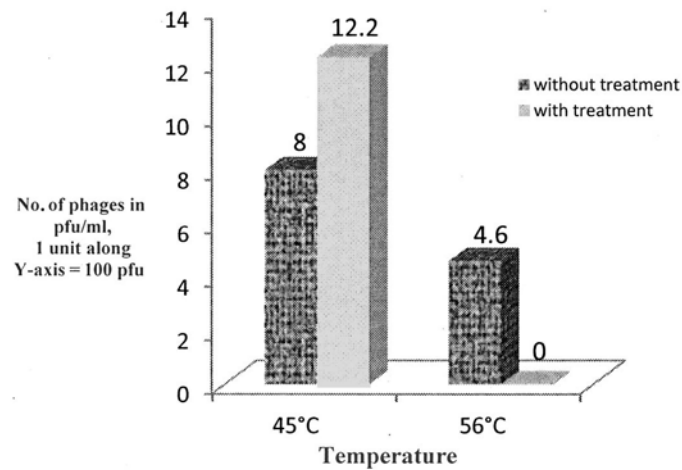


Fig. 2. Response of isolated shigaphages at stated temperature for 30 min.

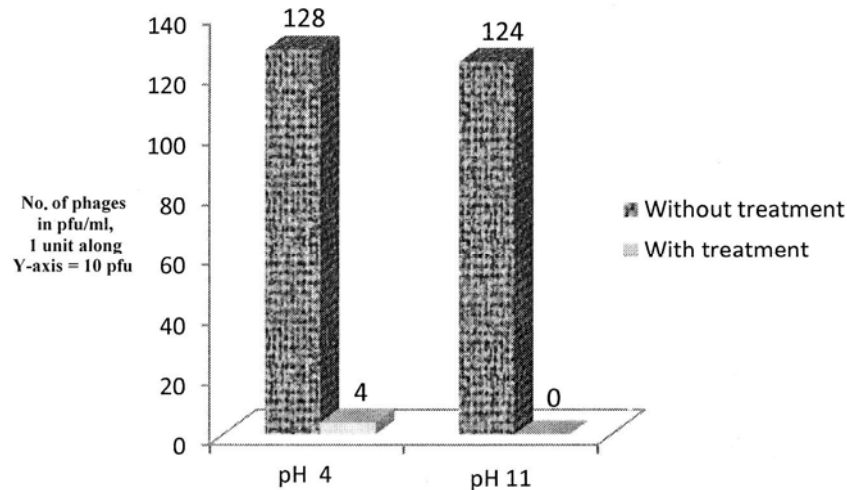


Fig. 3. Vulnerability from isolated coliphages at different pH exposure.

pH 4 mediated 96.875% and pH 11 caused 100% reduction in the number of plaques for coliphages (Fig. 3). On the other hand, the number of plaques for shigaphages was declined by 100% at pH 4 and 91.67% at pH 11 (Fig. 4). So it could be deduced that, acidic environment presented higher inactivation rate to shigaphages than coliphages. On the contrary, alkaline condition provided higher destabilization to coliphages than shigaphages.

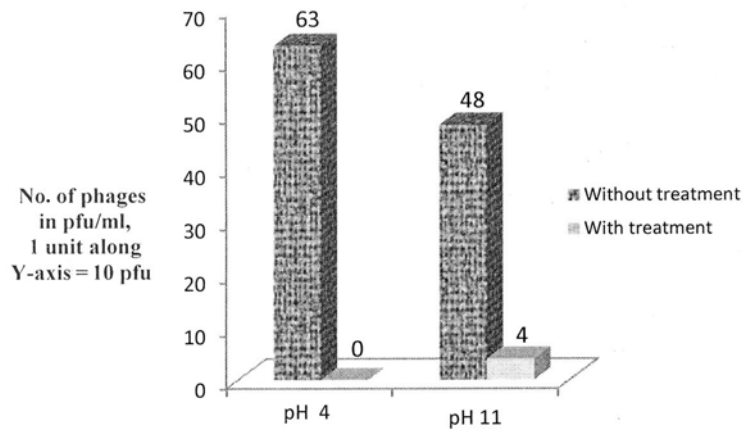


Fig. 4. Survivability of isolated shigaphages at different pH values.

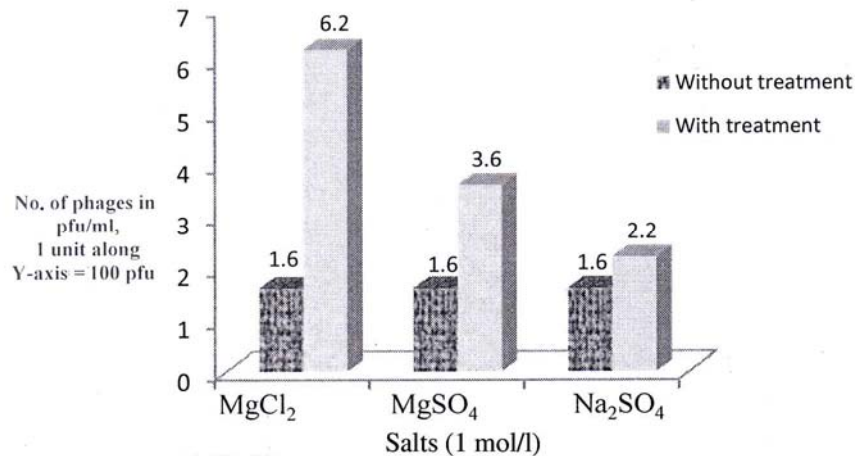


Fig. 5. Effects of various salts on thermal stability of isolated coliphages.

In the present study three inorganic divalent salts (MgCl₂, MgSO₄ and Na₂SO₄) were investigated to understand their degree of influence on the thermal stability of bacteriophages. While treated with each of the three salts, coliphages showed significantly elevated persistence at 50°C. MgCl₂, MgSO₄ and Na₂SO₄ caused the increase

in viral plaque count by 287.5, 125 and 37.5% respectively (Fig. 5). However, shigaphages had lost their activity at 50°C after an hour long treatment with each of the three test salts. The number of shigaphages was reduced by 79.31, 10.34 and 44.83% through treatment with MgCl₂, MgSO₄ and Na₂SO₄, respectively (Fig. 6).

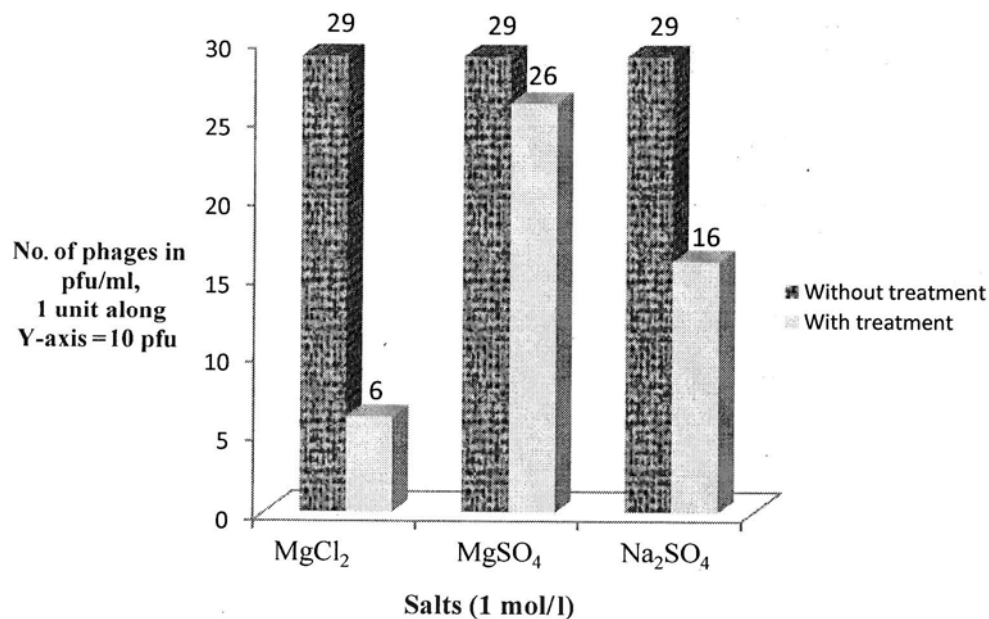


Fig. 6. Effects of various salts on thermal stability of isolated shigaphages.

Viruses are the most diversely structured groups of entities among the microorganisms. The data presented here constitute an important piece of information regarding the variability of sensitivity of bacteriophages to different physical and chemical conditions. The diversity observed among phages to different inactivating and stabilizing conditions is necessary to be assessed prior to using phages and phage-derivative products to ensure the maintenance of standard biosafety level⁽³⁴⁾. Temperature, pH and salts seem to be very important factors for shifting the count of bacteriophages collected from nature. The results demonstrated here could be applied in generating new data for the pharmaceutical and agricultural industries working on phage survivability and methods of their further preservation.

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