



**ROLE OF CALCIUM ON THE ADSORPTION RATE OF BACTERIOPHAGE
AGAINST STAPHYLOCOCCUS AUREUS**

^{1,2}Shafeeq-Ur-Rehman, ¹Farhan Rasheed, ¹Ameer Hamza, ²Taimoor Khan and ^{2*}Shahid Raza

¹University of Punjab, Lahore, Pakistan.

²University of South Asia, Lahore Pakistan.

*Author for Correspondence: Dr. Shahid Raza

University of South Asia, Lahore Pakistan.

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ABSTRACT

In treating infection caused by *staphylococcus aureus* which is resistance to antibiotic therapy, Phage therapy presents an alternative therapeutic option. However it is very much necessary to study the external factors that may impact the yield and potency of phage preparations proposed for use in various in vitro and in vivo studies. The present research focuses on the role of calcium on the adsorption rate of a lytic bacteriophage. The presence of calcium increases the adsorption rate of the phage and also participated in the penetration of the phage genome in the cytoplasm of the host. During the phage titration process, a final concentration of 1M calcium chloride supplemented in soft agar, considerably increase the phage titer. However during the isolation of lytic phage against *staphylococcus aureus* and during the high titer active phage preparation, incorporation of such divalent cations would definitely increase the isolation frequency and the final phage yield. This will contribute towards more effective phage preparation for use in treatment against *staphylococcus aureus* infection.

KEYWORDS: *staphylococcus aureus*, cations, infection.

INTRODUCTION

Staphylococcus aureus as gain resistance to most commonly develop antibiotics. (Gottlieb, Fowler et al. 2000; Spence, Thompson et al. 2005) It should be notified that *staphylococcus* is responsible for many diseases like skin and soft tissue infection, osteomyelitis, pneumonia, life threatening bacteremia and mastitis in animals. However there is an urgent need to search for new methods to contest these life threatening infections. Use of lytic bacteriophages and/or there products as therapeutic agents present an effective alternative therapy against the *staphylococcus aureus*. It is very much necessity to enhance the conditions to get the best phage titer. (Kaur, Harjai et al. 2012) In the development of an effective phage preparations. Such a best condition can be incorporated during phage isolation, amplification, maintainance and large scale production and effective phage preparation required for further clenical applications.

The presence of divalent cations is one of the essential factors that affect the phage yield which play an important role at different stages of phage infection cycle. (Gratia 1940; Luria and Steiner 1954) For a particular divalent cation each phage has its own specificity and it is very much important to know which divalent cation at which concentration will maximize the

phage titer of the phage intended for further use. In this research we studied the role of divalent cations, i.e, calcium. Phage was isolated in the lab and charagterized on the basis of morphological, physiological and genetic characteristics.

MATERIAL

The composition of media used was in gram/liter unless otherwise specified according to the requirement. Sterilization was done by autoclaving at 121⁰C and 15lb./ inch² for 15 minutes. Solution was filtered by using syringe filter of 0.2 μm. 0.1 M HCl and 0.1 M NaOH were used to adjust the pH of media. All glass-ware was washed and cleaned with detergent and then sterilized in autoclave, dried variably at 60-100⁰C.

Glass ware used.

Incubater	At 37 ⁰ C
Test tubes	20 ml
Eppendorf	1.5 ml
Micropipette	1000 μl, 500 μl, 100 μl
Micro tips	1000 μl, 500 μl, 100 μl
Centrifuge	11000 G
Filter paper assembly	0.45μl
Flask	25ml

Chemicals and media**Fresh culture of *staphylococcus aureus***

Strain.	<i>staphylococcus aureus</i>
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L-Agar

35 gram L-agar is dissolved in 1000 ml of distilled water and autoclaved it.

Table L – Agar 1000 ml.

S. No.	Components	gms/L
1	Tryptone	10.0
2	Yeast Extract	5.0
3	NaCl	5.0
4	Agar	15.0

METHOD**Host Bacterial Strains**

This study included different bacterial strains that are *Staphylococcus aureus* as the host strains for the characterization of bacteriophages against them was kindly provided by Dr. Noman from department of microbiology and molecular genetics university of Punjab.

Phage Titer Determination

Phage titer was determined as plaque-forming units (PFU/ml). Single isolated plaque was enriched according to the previous method and prep was again serially diluted and 10 ul from each dilution was mixed with 140 ul bacterial culture and 850 ul L-broth and over layering was done after mixing with the soft agar on agar plates. Incubation at 37c was given overnight and number of plaques was counted and PFU/ml was calculated according to the following formula (Capra, Quiberoni *et al.* 2006)

PFU/ml = NO. of plaques × dilution factor.

2.4.6 Effect of Calcium Ion on the Rate of Phage Adsorption

Metal ions also have effect on the metabolic activities of microorganisms they can enhance microbial activities or can decrease metabolic activities. To observe the effect of metal ion on the rate of phage adsorption, 1 M CaCl₂ was mixed with phage infected bacterial growth. A host bacterial growth of 25 mL each in two flasks was taken. One flask was supplemented with 500 µl 10⁹ PFU/mL of the phage S.A and 1 ml fresh bacterial culture only, while the second flask was inoculated both with 500 µL of phage S.A and 250 µl CaCl₂ (IM) and 1 ml fresh bacterial culture followed by constant shaking (90rpm) incubation at 37°C. Sampling was done at intervals of 10, 20, 30 and 60 minutes and the number of free phages were calculated in the control without CaCl₂ and the treated mixtures having 1 M CaCl₂(Capra, Quiberoni *et al.* 2006). The percentage free phages were calculated using the double layer agar technique(Capra, Quiberoni *et al.* 2004). The following formula was used.

%age free phages = $(N/N^0) \times 100$, where N=PFU per milliliter of phages at 0 minutes while N⁰ PFU per milliliter at 10, 20 and 30 minutes.

RESULT**3.4.1 Phage count and plaque morphology.**

Plaque forming unit per milliliter (PFU/ml) were calculated along with the observation of plaque morphology were recorded. The plaque morphology of phage was small and circular on double agar layer plates. The size of the plaques varied from 1-5mm the plaque count of isolated phages ranged between 10⁹ – 10¹².

**Picture 3.2 Plaques of *Staph aureus*: PFU/ml was calculated by double layer agar method was 10⁹ – 10¹²****3.5.3 Effect of calcium ion on the phage adsorption rate**

The effect of calcium ion was analyzed on the adsorption rate of the isolated phages to detect whether more adsorption of phages takes place to the host to result in more phages 'production or not. This may help in producing more PFU/ml phages in less time and play significant role in phage therapy. The varying effects of calcium ion on the adsorption of phage was observed and recorded. The PFU/ml of 10⁸ was added at the start of the experiment and the percentage of free phages was detected that indicated the adsorption rate of bacteriophages. The number of free phages was decreased in calcium treated at 10 minutes interval and increased in control but after 20 minutes phage titer was rapidly increased in calcium treated and control and after 30 minutes in calcium treated pfu/ml was increased so rapidly that it was more than non-calcium treated.

Table 3.3. The effect of calcium ion was checked on the adsorption rate of the isolated phages to detect whether more adsorption of phages takes place to the host to result in more phages production or not. With the passage of time PFU/ml is goes on increasing.

Sr #	Time (min)	Phages (with CaCl ₂)	Phages (without CaCl ₂)
1	10	3.2 × 10 ²	4.5 × 10 ⁶
2	20	6.9 × 10 ⁸	5.5 × 10 ⁷
3	30	8.9 × 10 ⁹	6 × 10 ⁸

DISCUSSION

Because the pathogenic potential of staphylococcus is very much aided by its ability to have resistance to antibiotics. So a promising alternative treatment against

staphylococcus is in the form of phage therapy (Han, Kim et al. 2013) (Kwiatk, Parasion et al. 2012) (Garcia, Madera et al. 2009). It has already been used against many pathogens such as Ecoli (Dąbrowska, Skaradziński et al. 2010) (Matsuzaki, Yasuda et al. 2003)..(Capparelli, Parlato et al. 2007),(Dąbrowska, Skaradziński et al. 2010; Kwiatek, Parasion et al. 2012). In current study we have characterized phages against staphylococcus aureus. Phages have lytic activity against pathogenic staphylococcus aureus which were isolated from milk of mastitis infected cow.

After isolation and purification of phages their host range was determined and for this purpose phages were allowed to grow on different bacterial strains and their ability to infect those bacteria was determined by observing the formation of plaques in case of each new hosts. It was found that our isolated phages were having narrow host range due to their ability to specifically infect only one bacterial strain which was *Staphylococcus aureus*.

This phage was produced at large scale for further experimental work. Physiological characterization of isolated phages was also done for this purpose it effects of different environmental factors was checked on the ability of the phages to make plaques.

Metal ions show significant effect on the absorption of bacteriophages. CaCl_2 is one of those metals which has effects on the absorption of bacteriophages and this effect varies on the basis of their different types as well as on the stage of life cycle at which that is added. CaCl_2 when add it helps phages to get absorb on the surface of their host rapidly. Results indicates that there were more free phages present in the sample which was not treated with CaCl_2 as compared to that sample which was treated after 10 mints. Later it was found that with the passage of time more bacteriophages were seen in free form at 20 mint and 30 mint interval in sample treated with CaCl_2 as compared to control and this happened because CaCl_2 not only help in the absorption of the phages on the surface of their host but also help them to release of the host rapidly. More they will get absorbed earlier they would complete their life cycle.

Figure

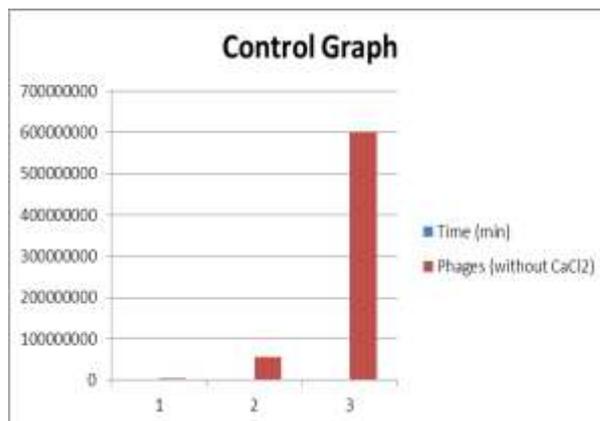
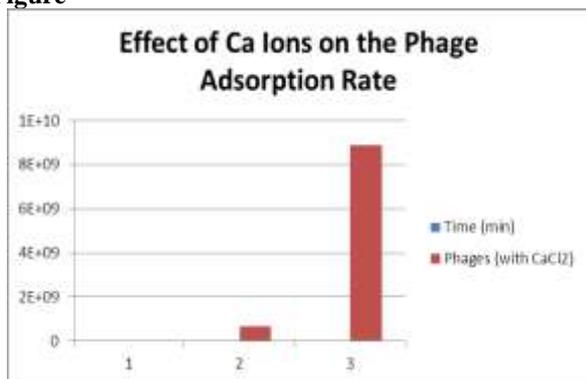


Figure 4.4 Effect of calcium ion on phage adsorption

There were more free phages present in the sample which was not treated with CaCl_2 as compared to that sample which was treated after 10 mints. Later it was found that with the passage of time more bacteriophages were seen in free form at 20 mint and 30 mint interval in sample treated with CaCl_2 .

These factors were determined because it has importance in the overall activity of the phages. End purpose of this study was to use phages in phage therapy and for this purpose phages must be stored at appropriate conditions. So appropriate temperature and ph for the storage and best activity of the phages has great importance and has direct effect on the use of phages in the phage therapy.

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