ORIGINAL ARTICLE

ISOLATION AND CHARACTERIZATION OF POTENT LYTIC PHAGE SPECIFIC TO STERNE STRAIN OF *BACILLUS ANTHRACIS*

A Sarje[1]*, S Ahiwale[2]

[1]Department of Microbiology, Dr. Arvind Telang College of Arts, Commerce and Science, Nigdi, Pune
[2]Department of Microbiology, Mahatma Phule College of Arts, Commerce and Science, Pimpri, Pune

Article received:10/05/2022, Revised:15/05/2022, Accepted: 27/05/2022

Address for Corresponding : *Dr. Sangeeta Ahiwale,* Department of Microbiology, Mahatma Phule College of Arts, Commerce and Science, Pimpri, Pune, INDIA. +9199860356226
E-mail- sangeeta.ahiwale2@gmail.com

ABSTRACT

Bioterrorism is a biological attack which involves intentional release of bacteria, viruses in naturally occurring form or a human modified form that sicken or kill people, livestock or crops. *Bacillus anthracis*, is the causative agent of anthrax and is most commonly used agent in a biological attack. Anthrax spores are easily found in nature, can be produce in lab, and can last for a long time in the environment. Infection occurs when the spores, the dormant form of the bacteria, are ingest, inhaled, or come into contact with a lesion on the skin. Some *Bacillus anthracis* strains are antibiotic resistant or not targeted by vaccines. Therefore, to overcome this problem phages specific to the *Bacillus anthracis* species can be isolated as an alternative method to antibiotic treatment. The objective behind this study is isolation, characterization, screening and purification of potent lytic phages specific to the host. The *Bacillus anthracis* (sterne strain) was previously isolated and identified by cultural and biochemical characteristics using Bergey's manual of Determinative Bacteriology. Its morphological and biochemical characters were studied for the identification of host. Isolation and enrichment of phages was carried out which involves mixing environmental samples (source of phage) and specific host strain in enrichment media (Phage broth). Double agar layer plaque assay method was carried out to calculate the phage titer. Potent lytic phages were successfully isolated from the soil sample against *Bacillus anthracis* which will be useful in anthrax therapy or in reducing spore inoculums in combination with vaccines.

Keywords: Bacteriophages, *Bacillus anthrasis,* Double layer agar method, MOI, Spot assay method

INTRODUCTION

Bacillus anthracis, the organism which is causative agent of anthrax caused in humans and animals, derives its name from the Greek word for coal, because of its ability to cause black, coal-like cutaneous eschars. *Bacillus anthracis* belongs to the group *Bacillus cereus sensu lato*, is a Gram positive, spore forming, aerobic or facultatively anaerobic and rod shaped organism, which appears in chains. [1,2] This bacterium exists in two forms, vegetative cells present inside the host and spore form which persist in the soil or environment. This organism has the ability to infect humans by gastrointestinal, cutaneous or respiratory routes. The most common is cutaneous anthrax, which accounts about 90% of all human cases and which is acquired through a lesion on the skin. The other two forms include gastrointestinal anthrax and pulmonary anthrax, also known as inhalation, anthrax. Cutaneous anthrax infections are the least symptomatic and have mortality rate of around twenty percent. Gastrointestinal anthrax has mortality rate of around sixty percent. Whereas, Inhalation anthrax is the most severe form of anthrax with a near eighty percent mortality rate. Serious clinical development is Meningitis, which may follow any of the three form of anthrax [3].

The anthrax spores are easy to store, transport, and disseminate and may survive in soil for many decades. This durable spores can be used as a idea biological weapon for mass destruction. Due to this feature, *Bacillus anthracis* was used as bioterriost weapon against animals in world war I and both animals and man in world war II. Bioterrorism, in the form of letters containing *B. anthracis* spores was used due to which 2 cases of inhalation anthrax and 11 cases of cutaneous anthrax was being identified[1,4].

It is estimated that one deep breath of weaponised aerosol may contain approximately 105 spores and within a week of exposure, inhalation anthrax might develop[5]. Many investigations suggests that *B.anthracis* show resistant to many antibiotics such as cephalosporins, trimethoprim, and sulfomanides[6]. Therefore, society needs an effective weapon to neutralize this threat. Bacteriophages are natural enemies of bacteria and the recent data strongly suggest these phages are effective in treating the bacterial diseases including those caused by antibiotic resistance microbes. It is estimated that abundance of the viruses in the biosphere exceeds 10^{30-31} virions that is ten times more than bacterial cells[1,7]. Therefore phages can be represented as a promising tool in controlling colonizing host bacterium[8].

MATERIALS AND METHODS

Isolation, Enrichment and Purification of Phages:

5 grams of soil sample was mixed with 30ml of phage broth (Hi media) and 3ml culture of *Bacillus anthracis*. These mixtures of different soil sample were shaked vigorously for 12h at 37°C at 150 revolution per min., it was then centrifuged at 10,000 rpm at 4°C for 10min. The supernatant was then filtered through sterile membrane filter (0.2µm). The filtrate was kept at 4°C until use and then used as source of phage. Phages were isolated from the filtrate (lysate) by double agar plaque assay technique[9] in which mid log phase culture (0.5ml) of the host (Previously isolated) and the filtrate (0.5ml) were mixed in 3ml sterile soft agar (0.6% w/v agar) and then poured onto sterile phage agar plates. The plates were incubated at 37°C for 24h and observed for plaques[9,10].

Calculation of phage Titer in the lysate:

To calculate pfu/ml double agar layer plaque assay was performed with the mid-log phase host [Bacillus anthracis: OD_{650}: 0.42 at 7 hrs] and purified lysate of the respective host. Lysate was serially diluted aseptically. These dilutions of the lysates were used for double agar layer plaque assay [9,10].

Characterization of bacteriophage:

Bacteriophage characterization is the mandatory step prior to the practical application in the various field. Phages can be characterized based on the different parameters. The phages were characterized by plaque morphology, host range, stability and infectivity of phages at various pH values and different temperature.

Plaque morphology:

The nature of plaque was studied on various media viz., Nutrient agar (NA), Phage agar (T agar). An aliquot (1ml) of phage lysate was mixed with 1ml of mid log phase culture of respective host. An aliquot of 0.1ml of each mixture was spread onto the surface of the medium. Plates were incubated at 37°C and observed every 3h over a period of 24h for development of plaques.

MOI value of phage:

MOI is the number of infectious virus particles divided by the number of target host cells. The MOI value or the ratio of number of phage to number of host bacteria should not exceed 1 in order to determine one step growth curve. If the multiplicity of infection is 1 or more than 1, that means the number of phages is equivalent to the number of host bacteria. To determine MOI value, specific host cell density is required[10].

Host range:

The phages were test for their ability to infect bacterial strains belonging to different species viz, *Pseudomonas, Klebsiella, Bacillus subtilis, Staphylococcus aureus, Bacillus cereus*. It was performed by spot assay method. 1ml of host was added in 4ml of soft agar and spread on the phage agar plate. Once the over layer was solidified, 20µl of phage lysate was spot inoculated on the plate. Plates were incubated at 37°C and examined for clear zone after 24h[11].

Optimal pH and temperature for phage stability:

The stability was studied at varied pH (4.0 to 12). An aliquot of 1ml lysate was mixed with respective buffer of different PH. Tubes were incubated at room temperature for 1h. Contents of the tubes were serially diluted in T-broth and suitable dilution was used to determine the number of pfu (plaque forming unit) in the lysate by the soft agar over layer technique, Aliquots (1ml) of phage lysate in sterile screw capped tubes were incubated for 1 hr at selected temperature (Temp range: 4^0C, 10^0C, 25^0C, 37^0C, 45^0C, 60^0C, 70^0C). Phages were titrated by the double agar overlayer technique.

Phage stability was calculated using the formula:

Percent (%) Stability = $N/N_O \times 100$

Where,

N: Number of viable phages after 1 hour of incubation.

$N_{O:}$ Initial number of phages.

RESULTS

Characteristics of Isolated Phages:

Phages specific against *Bacillus anthracis* were isolated from the soil sample by double agar plaque assay technique. Several lytic as well as lysogenic phages (clear and turbid plaques) were isolated. The plaques were clear and circular in nature with an average plaque diameter of 3mm on nutrient agar and 2mm on phage agar medium. The average number of phage particles per plaque varied with different media. These phages were selected for further studies.

Fig 1. Phage isolation from soil sample.

Fig 2. Plaque morphology of *Bacillus anthracis* on T-agar

Calculation of phage Titer in the lysate:

The plaques were counted on different dilution plates and recorded. Form those the plaque forming unit was calculated as 1 x 10^9 PFU/ml ~100%

Host range:

Spot assay was carried out to study host range. The activity of the isolated phages were examined against *Pseudomonas, Klebsiella, Bacillus subtilis and Staphylococcus aureus.* The study indicated that the phage showed lysis of *Bacillus subtilis, Bacillus cereus* and *B.anthracis*. A clear zone in the bacterial lawn indicates complete lysis of host due to phage. No clear zone or complete lawn growth indicates no activity of phage lysate on host.

Sr. No	Host	Phage activity
1.	*Pseudomonas*	No Lysis
2.	*Streptococcus aureus*	No Lysis
3.	*Klebsiella*	No Lysis
4.	*Bacillus subtilis*	Lysis
5.	*Bacillus anthracis*	Lysis
6.	*Bacillus cereus*	Lysis

Table 1- Host range of Phages.

Calculation of Multiplicity of infection value for *Bacillus anthracis* phage:

The success of one step growth curve depends upon the standard host cell density that is able to yield maximum plaques on plate. The actual number of phages that will enter any given cell is a statistical process and that requires a mid-log growth phase cells. Therefore, the information about the mid-log growth phase of the host bacteria is necessary. The Multiplicity of Infection (MOI) is the ratio of infectious agent and the host cells and MOI of 0.04 was obtained.

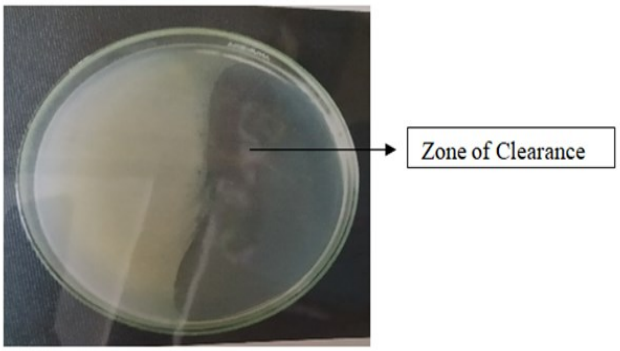

Fig 1: Phage isolation from soil sample

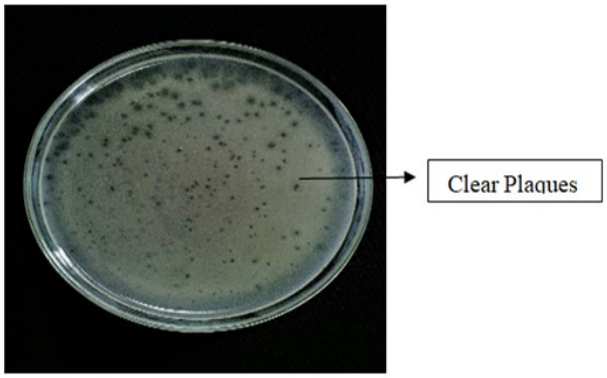

Fig 2: Plaque morphology of *Bacillus anthracis* on T-agar

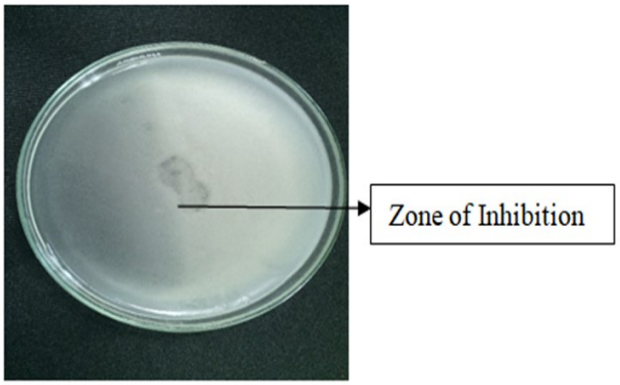

Fig 3: Activity of phage against *B. subtilis*.

Optimal pH and Temperature for phage stability:
Phage is stable over wide range of temperature that is from 4^0C to 70^0C and pH 4.0 to 10

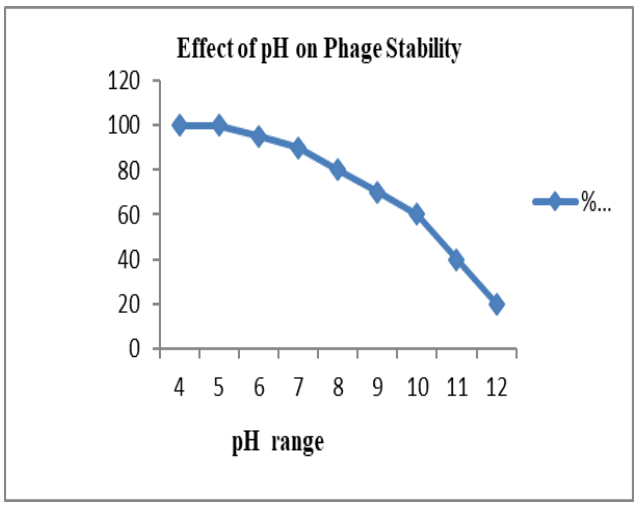

Fig 4: Stability of Phage at Different pH

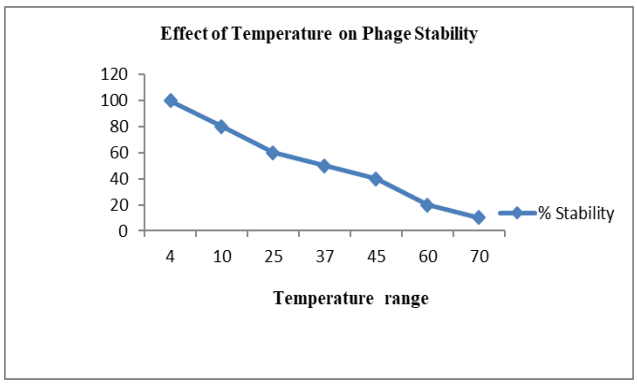

Fig 5: Stability of Phage at Different Temperature

DISCUSSION:

Bacteriophage are omnipresent and there are numerous types of phages appear in the environment. Therefore, phages active against *Bacillus anthracis* (both lytic and lysogenic) are widespread in the environment and have been isolated from soil, carcasses, feces, sewage and the intestinal tract of the earthworm Eisenia fetida[12]. There could be many factors that affect the number and behaviour of phages, the association of phages and bacteria with the organic matter content especially that influences the petabolic activity of host bacteria, pH, temperature, prey-predator relationship. Therefore, characterization of isolated bacteriophage is a mandatory step in order to explore them in various field. pH is one of the most important factor that affect the stability and infectivity of bacteriophage as they are expose to acidic as well as alkaline conditions in environment. Temperature is the another important parameter, where phages are found from freezing to boiling temperatures. The phages isolated in this study was found to be stable at various pH values viz..., from pH 4.0-10, with optimal stability at pH 7. Similarly, the isolated phages showed stability over a wide range of temperature, with optimum stability at 4°C and 70°C. A article by Anna Krasowska and Anna Biegalska suggest that phage isolated against Bacillus species seems extremely stable in the pH range 4 to 10 and temperature at 4^0C to 70^0C[13]. The isolated phage also showed a narrow host range and showed activity against *Bacillus subtilis* species. The literature contains many descriptions of experiments under different conditions and hence varying result. For example, some *Bacillus subtilis* phages have higher percent of adsorption than other phages while on the other hand, they have same burst size as other phages.

CONCLUSION:

The newly isolated phage of *Bacillus anthracis* has many unique features such as short generation time and high stability over a wide range of pH and temperature. It was observed that plaque morphology was similar on NA plates as well as on T-agar plates that is lytic as well as lysogenic plaques were observed on both the medium. The isolated phage showed activity against *Bacillus subtilis* species therefore the phage has a narrow host range and is specific to *Bacillus* species only. It shows stability over a wide range of temperature and pH. The results of this study suggest that some naturally occurring phages may be suitable for various applications such as phage-based technologies and also useful in anthrax treatment if used as vaccines in combination with antibiotics.

ACKNOWLEDGEMENT:

The authors would like to acknowledge the Principal, Mahatma Phule College, Pimpri, allowing access to Microbiology Laboratory and provision of resources relevant for phage isolation.

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How to cite this article:
A Sarje, S Ahiwale. Isolation and characterization of potent lytic phage specific to sterne strain of Bacillus anthracis . Int J Bacteriophage Res 2022:2:13-17.