Original Article

Study of Characteristics of Mycobacteriophage – A Novel Tool to Treat *Mycobacterium* spp.

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Abstract

Background: Mycobacteriophages are viruses that infect *Mycobacterium* spp. Till date, 10427 mycobacteriophages have been isolated and 1670 mycobacteriophage genomes have been sequenced https://phagesdb.org/hosts/genera/1/ (cited on 30th December,2018). In the previous study, 10 different mycobacteriophages from 14 soil samples were isolated, by qualitative plaque formation method using *Mycobacterium smegmatis* as host. Among these, three phages were found to infect four different species of *Mycobacterium*, i.e., *Mycobacterium fortuitum* subsp. *fortuitum* MTCC993, *Mycobacterium kansasii* MTCC3058, *Mycobacterium avium* subsp. *avium* MTCC1723, and *Mycobacterium tuberculosis* MTCC300, besides the host *M. smegmatis*. The phage lysates were concentrated by polyethylene glycol (PEG) precipitation. One of the three phages showing host diversity was selected for further study. The various phage growth parameters such as incubation temperature, time of adsorption, host cell density and effect of cations were standardised. **Methods:** The studies were done by qualitative and quantitative plaque assay method. **Results:** The phage selected for further study showed an optimum adsorption time of 15 min. The optimum temperature for propagation was found to be 37°C. The phage was found to be stable at 42°C. In the presence of calcium, the phage showed a higher rate of infectivity. **Conclusion:** Understanding the biology of mycobacteriophages and their host diversity is the key to understanding mycobacterial systems. This could be the first step toward exploiting the potential of phages as therapeutic agents.

Keywords: Cations, host cell density, multiplicity of infection, mycobacteriophage, temperature

Introduction

Worldwide, tuberculosis (TB) is one of the major causes of death. In India, as per the Global TB report of 2017 the estimated cases of TB were approximately 2,800,000 accounting for about a quarter of the world's TB cases (https://tbcindia.gov.in/showfile.php?lid=3314 (cited on 21st November, 2018) (WHO Global TB report 2018).. Drug-resistant TB is a major crisis. Worldwide in 2017, 558,000 people developed rifampicin-resistant TB, and of these, 82% of people had multidrug-resistant TB. A combined strategy, based on improved drugs, accurate diagnostic tests, and better preventive measures is necessary, to eradicate *Mycobacterium tuberculosis* (http://apps.who.int/iris/bitstream/handle/10665/274453/9789241565646-eng.pdf?ua=1 (cited on 21st November, 2018). WHO Global TB report 2018). Phage therapy as an alternative has several advantages over antibiotics such as host-specificity, self-amplification, biofilm degradation and low toxicity to humans.[1] Mycobacteriophages have tremendous potential in the study of mycobacterial genetics and as antimycobacterial agents. In this study, we characterized the growth parameters of a mycobacteriophage showing host diversity.

Methods

Cultivation of host *Mycobacterium smegmatis MTCC* 994

Mycobacterium smegmatis MTCC 994 (IMTECH, Chandigarh); a nonvirulent mycobacterial strain was used as the host for isolation and characterization of mycobacteriophage. The host strain was cultivated at 37°C in Nutrient Broth for 48 h.

Phage isolation

In a previous study, 10 mycobacteriophages were obtained

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from soil and sewage samples. Of the ten phages, three were found to infect four different species of *Mycobacterium,* i.e., *M. fortuitum* subsp. *fortuitum* MTCC993, *Mycobacterium kansasii* MTCC3058, *Mycobacterium avium subsp. avium* MTCC1723, and *M. tuberculosis* MTCC300, besides the host *M. smegmatis*.[2] One of the phages which exhibited host diversity was chosen for further characterization studies.

Phage enrichment

A volume of 5 ml of previously isolated phage sample was added to 25 ml of log phase culture of *M. smegmatis* in Nutrient broth and incubated at 37°C. Additional 10 ml of log phase culture was added to the flask for 3 consecutive days and further incubated for 4 days. The growth medium was centrifuged at 2000 g and the supernatant was filtered using 0.22 µ membrane filter. The filtrate obtained was tested for the presence of phages using qualitative plaque method. The filtrate was stored with chloroform at 4°C and then used for mycobacteriophage propagation studies.

Mycobacteriophage propagation studies

Effect of temperature

The optimum temperature for phage propagation was determined using the quantitative plaque assay. The plates were incubated at three different temperatures −30°C, 37°C, and 42°C for 48 h and examined for visible plaque formation. The control plates consisted of only host cells incubated at 30°C, 37°C, and 42°C.

Effect of host-cell density

The effect of host-cell density was determined using quantitative plaque assay. 0.3 ml of saline suspension of host cells having different densities (OD_{530}: 0.1, 0.2, 0.3, 0.4, and 0.5) was added to 0.1 ml phage suspension (10^{-4} dilution) and allowed for phage adsorption for 15 min. The phage count was determined by the overlay method using soft Nutrient agar and incubation at 37°C for 48 h.

Effect of adsorption time

A volume of 0.3 ml of host cell suspension (OD_{530}-0.3) was added to 0.1 ml phage suspension (10^{-4} dilution) and incubated for different time intervals: 0 min, 15 min, 30 min and 60 min to allow for phage adsorption. The phage count was determined by the overlay method using soft Nutrient agar and incubation at 37°C for 48 h.

Effect of cations

Effect of calcium ions on phage adsorption by qualitative assay

Nutrient agar plates were made with different concentration of $CaCl_2$ (1, 2, 5, 10 , and 20 mM).The buffers used for diluting phage too had the same concentration of $CaCl_2$. A control plate was maintained without $CaCl_2$. These plates were surface swabbed with host-cell suspension (OD_{530}-0.1). The plates were spot inoculated with 10 µL of phage suspensions (undiluted to 10^{-10} dilution) and incubated at 37°C. The plates were examined for visible plaque formation after 24–48 h incubation.

Effect of calcium ions on phage adsorption by quantitative assay

A volume of 0.3 ml of host cell suspension (OD_{530}-0.3) was added to 0.1 ml phage suspension (10^{-4} dilution) and incubated for 15 min to allow for phage adsorption. The phage count was determined by the overlay method using soft Nutrient agar with different concentration of $CaCl_2$ (1, 2, 5, 10 , and 20 mM). The phage buffer too had same concentration of $CaCl_2$. The control plates were without $CaCl_2$. The plates were incubated at 37°C for 48 h and then examined for the phage plaque count.

Results

A phage isolated in the previous study and showing host cell diversity[2] was selected for further study of growth characteristics.

The temperature stability of the phage was studied at 30°C, 37°C, and 42°C. At 30°C, no visible plaques were seen, whereas at 37°C complete lysis of the host cells was seen. Thus, 37°C was found to be the optimum temperature for the growth of the phage. Plaques were also observed at a higher temperature of 42°C, which implied that the phage is stable at higher temperature [Figure 1].

As shown in Table 1, multiplicity of infection (MOI) obtained with host density of 0.1 at 530 nm (1.7×10^7 cfu/ml) was the highest, namely, 1.7. MOI of 0.04 was obtained using host cell density of 0.2 at 530 nm (7.4×10^8 cfu/ml). However, at both the above-host cell densities, clear distinct plaques were seen though the numbers of plaques were >300 per plate. With further increase in host cell density the MOI and no of plaques

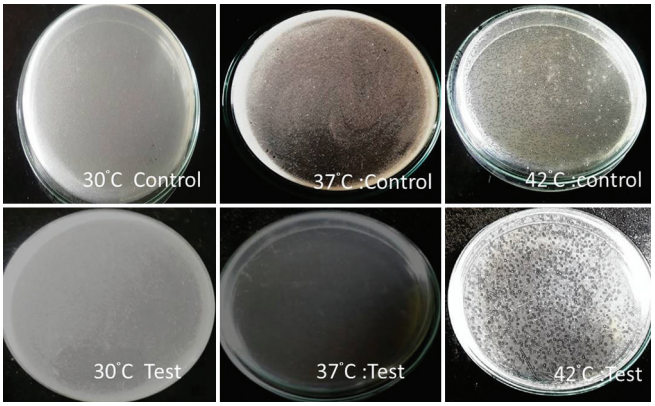

Figure 1: Effect of temperature

Table 1: Effect of host cell density

Host cell density OD at 530 nm	CFU/ml	PFU/ml	MOI
0.1	1.7×10^7	3×10^7	1.7
0.2	7.4×10^8	3×10^7	0.04
0.3	1.0×10^9	2.8×10^7	0.0283
0.4	1.0×10^{12}	2.9×10^7	0.0000295
0.5	1.3×10^{13}	2.0×10^7	0.0000020

CFU: Colony forming units, PFU: Plaque-forming units, MOI: Multiplicity of infection, OD: Optical density

decreased. Hence, for further studies, host density of 0.3 was used to get countable plaques [Figure 2].

From the data presented in Table 2, it was observed that with an increase in adsorption time, there was an increase in the number of plaques. An adsorption time of 15 min was considered as optimum as countable plaques were seen. Hence, for all subsequent quantitative assays, the adsorption time was kept at 15 min [Figure 3].

The results of the qualitative assay to determine the effect of $CaCl_2$ in the propagation of phages are presented in Table 3. In plates without $CaCl_2$, plaques were seen up to 10^{-7} of phage dilution. No plaques were seen with further dilution of the phage. However, in all the plates with every $CaCl_2$ concentration, plaques were seen up to 10^{-10} of phage dilution. The quantitative assay [Table 4] revealed that with increase in concentration of $CaCl_2$ from 1 to 20 mM, the plaque count greatly increased. At 20 mM concentration of $CaCl_2$, there was complete clearance of the host [Figure 4].

Hence, increase in the concentration of Ca^{2+}resulted in amplification of phage yield. This shows that though Calcium is not absolutely necessary for initiating infection, the presence of calcium resulted in a higher rate of infection, complete lysis of host cells, and improved amplification of phage yield.

Discussion

Stella *et al*. isolated 18 mycobacteriophages. All the mycobacteriophages gave clear lytic plaques at 30°C and 10 of them were able to form plaques at 37°C but none propagated at 42°C.[3] The first mycobacteriophage isolated by Gardner and Weiser, in 1947, was completely inactivated at 75°C after 10 min but only partially inactivated at 72°C.[4] Selles and Runnals studied the effect of temperature on phage survival. They diluted the phage to a concentration of 10^7 phage particles per ml in nutrient broth at pH 7 and incubated in a closed water bath at 50°C, 56°C, 58°C, and 60°C. At 10-min interval, samples were withdrawn and assayed for the number of surviving phage particles. Of the three phages they studied, one was found to be heat stable with 0.8% survival for 60 min at 58°C and 60°C.[5]

Thompson and Coates studied the effect of high temperature on animal virus development in tissue culture. They concluded that one of the important limiting factors for growth is temperature and in most acute diseases, including those caused by viruses, fever is a prominent symptom. It is, therefore,

Table 2: Effect of time of adsorption

Time of adsorption (min)	PFU/ml
0	3.9×10^6
15	2.75×10^7
30	4.0×10^7
60	Uncountable

PFU: Plaque-forming units

Table 3: Effect of calcium ions on adsorption - qualitative assay

Phage dilutions	Without calcium	Concentration of $CaCl_2$				
		1 mM	2 mM	5 mM	10 mM	20 mM
Undiluted	+	+	+	+	+	+
10^{-1}	+	+	+	+	+	+
10^{-2}	+	+	+	+	+	+
10^{-3}	+	+	+	+	+	+
10^{-4}	+	+	+	+	+	+
10^{-5}	+	+	+	+	+	+
10^{-6}	+	+	+	+	+	+
10^{-7}	+	+	+	+	+	+
10^{-8}	-	+	+	+	+	+
10^{-9}	-	+	+	+	+	+
10^{-10}	-	+	+	+	+	+

+: Plaques present, -: Plaques absent

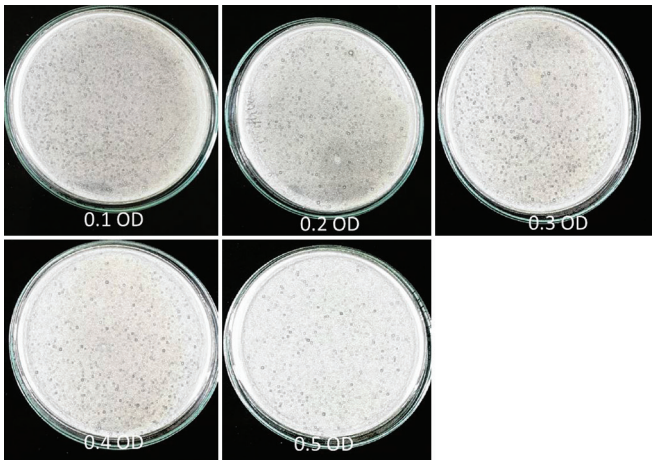

Figure 2: Effect of host cell density

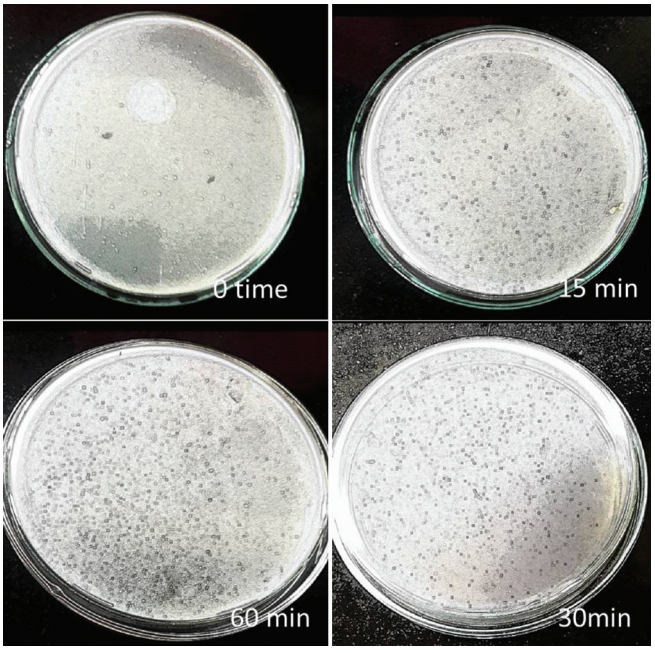

Figure 3: Effect of adsorption time

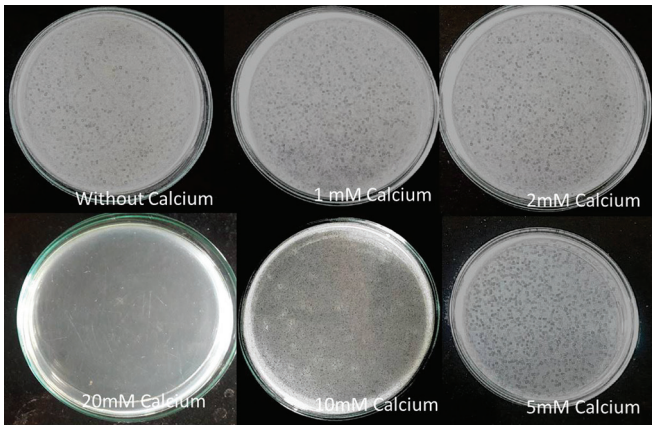

Figure 4: Quantitative effect of calcium ions

Table 4: Effect of calcium ions on adsorption - quantitative assay

Concentration of $CaCl_2$	PFU/ml
Without calcium	2.8×10^7
1 mM	$>3.0\times10^7$
2 mM	$>3.0\times10^7$
5 mM	Uncountable
10 mM	Uncountable
20 mM	Complete clearance

PFU: Plaque-forming units

important to determine the effect of temperature on the growth and survival of infective agents, namely phages.[6] Increase of temperature and drop in pH are the two nonspecific responses of an infected animal. Viral development is influenced by a variation in temperature and an increase in temperature of the animal body can drastically modify the course of a viral disease.[7,8]

Froman and Scammon studied the effect of different incubation temperatures on the susceptibility of *M. avium* to mycobacteriophage and found that incubation of strains of *M. avium* at 42°C, prior to exposure to phage profoundly affected their susceptibility to lysis. When incubated at 37°C, all nine strains were phage resistant; at 42°C, six of nine strains were phage susceptible.[7]

McNerney *et al*. observed that the efficiency of phage infection was found to increase with increase in concentration of phages up to 10^7 PFU/ml. They suggested that abortive infection might be occurring at the higher concentration of phages. Phage infection of mycobacteria will also be influenced by the aggregation of host cells and although the distribution of colony forming unit may be predicted to follow a Poisson distribution, variations in the size of clumps may influence the variation in the number of progeny phages produced. They investigated the development of a bacteriophage replication assay for the detection of *M. tuberculosis* by using mycobacteriophage D29. They demonstrated that the efficiency of phage infection improves at higher MOI. The number of *M. tuberculosis* bacilli found in the sputum from infected patients varies widely and may range from <10 to $>10^5$. Thus, to maximize the sensitivity of the assay a high concentration of D29 would be required. However, reduced infection rates were observed when bacterial suspensions were inoculated with phage concentrations of $>10^8$ PFU/ml.[9]

Hagihara *et al*. studied the adsorption-invasion time of Mycobacteriophage B-1; using seven different strains of *Mycobacterium*. They found that number of plaque-forming units increased logarithmically and showed the maximal value in 5–10 min after mixing the phages and the host cells.[10] The ratio of the phage number to host cell number influences the number of bacteria that will get infected. Poisson's equation predicts that the number of infected bacteria will increase exponentially with the MOI. At an MOI of 1, nearly 60% of the cells get infected.[11]

Since phages have the ability to multiply and kill target bacteria, they have been termed self-replicating antibacterial agents. In order to use bacteriophages as a pharmaceutical treatment, it is necessary to determine the mechanism by which they bring about reduction in the bacterial population. To derive a quantitative relationship between phage growth and bacterial growth, it is necessary to take into account various factors, including the number of phages and bacteria present initially, the growth rate of the bacteria, the phage adsorption rate, the length of the phage latent phase, and burst size.[11]

Tokunaga and Sellers studied the effect of cations on phage D29 replication and observed that none of the cations tested namely Mg^{++}, Na^+, K^+, or Mn^{++} caused an increase in number of plaques. Apparently, only Ca^{++} has the capacity to promote the entry of phage DNA into the host cell and initiate an infection.[12] In 1966, Paranchych observed that divalent cations are required, for the penetration of phage nucleic acids into the host cell.[13] Fullner and Hatfull's group[14] noted enhanced adsorption, stability and productive infection in the presence of Ca^{2+} cations. They observed that phage L5 showed a strict dependence on Ca^{2+} ions (1 mM in both bottom and top agar) for infection of slow-growing mycobacteria. Ca^{2+} is important for optimal plaque formation for both L5 and D29. However, the concentration of Ca^{2+} required for L5 infection is far greater than the concentration required for D29 infection. In contrast, TM4 showed a distinct infection pattern compared with both L5 and D29 and did not show any dependence on $CaCl_2$ for infection of *M. smegmatis*. The authors concluded that TM4 infects both slow and rapid growing mycobacterial species using a mechanism different from the L5 family.[14] Divalent cations may be necessary for the functioning of enzymes involved in the penetration of phage nucleic acid into the host cell.[15] Such enzymes might readily form complexes with various metal cations but become activated only when the complex with specific divalent cations such as Ca^{2+}.[5]

In the case of D29 the role of Ca^{+2}, is assumed to be either through ionic or covalent bonding, or both. Calcium ions may function using any of the following mechanisms: (i) forming a bridge between the negatively charged DNA and

host cell, (ii) chelating with DNA and an enzyme located on the cell surface, suitably orienting both enzyme and substrate, and (iii) activating an enzyme concerned with getting the DNA through the cell wall barrier.[12]

Conclusion

Many factors affect host cell infection and viral multiplication. In order that mycobacteriophages may be eventually used for the diagnosis or treatment of TB; it is essential that the parameters affecting viral replication be well standardized. Temperature effects are generally studied on virus-host complexes, and there is little information regarding the effect of temperature on the host and its susceptibility to viral invasion.[7] An increase in temperature and a decrease in pH markedly depresses the multiplication of certain viruses.[8] The phage in the current study brought about complete lysis of host at 37°C, which is normal body temperature but was also found to be stable and was able to bring about lysis of host at a higher temperature of 42°C though to a lesser extent. This can be considered as a favorable trait for its potential use in the treatment of *M. tubcerculosis* infections.

Diagnostic tools for detecting *M. tuberculosis* in clinical specimens need to be robust and sensitive. To maximize the sensitivity of bacteriophages for the detection of *M. tuberculosis*, it is necessary to optimize phage infection and replication. Optimization of phage inoculum, incubation time and temperature will help achieve a highly sensitive detection system for *M. tuberculosis* by a bacteriophage replication assay.[9]

Bacterial killing can be brought about by two phenomena: Infection by phage, resulting in the lysis of a fraction of infected cells and secondary killing, which is the result of secondary factors such as superoxide radicals released on bacterial lysis. The extent of interaction of secondary factors with susceptible bacteria depends on MOI. At a high MOI, the secondary factors interact with a lesser number of susceptible bacteria than at low MOI. The demonstration that mycobacteriophages can kill their hosts through mechanisms other than lysis could lead to new methods to combat TB.[11] Killing of *M. tuberculosis* using mycobacteriophages has been demonstrated using the lytic mycobacteriophage TM4, which was introduced into a macrophage cell line harboring *M. tuberculosis* and *M. avium*.[15]

The efficiency with which the bacteriophages will reduce bacterial counts will depend on the number of phages present, their adsorption efficiency, average life cycle, and finally, the growth rate of the host.[11] Many of the phage-encoded products, such as lysins, are highly toxic to bacteria; therefore, instead of using phages directly, their products could also be used to eliminate the bacteria.[16] Finally, it is important to note that phages have the ability to inhibit the metabolism of their hosts (host inactivation).[17] Hence, by understanding this mechanism, it may be possible to develop new strategies to kill bacteria and not necessarily use the phage directly for intervention against bacterial diseases.[11]

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Conflicts of interest

There are no conflicts of interest.

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