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ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES AGAINST CARBAPENEM RESISTANT *ENTEROBACTERIACEAE* FROM SEWAGE

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ABSTRACT

Antibiotic resistant 'superbugs' have become one of the world's most important public health concerns. The increase in Carbapenem-resistant *enterobacteriaceae*[CRE] poses a challenge in administration of effective treatment for the most common infections. In this study, carbapenem-resistant *Klebsiella pneumoniae* was isolated from raw sewage samples and identified by standard methods such as modified Hodge test and biochemical testing. Bacteriophages against this pathogen were isolated by double layer method. The bacteriophage was observed as plaques which were later purified and the potent phage was concentrated by PEG precipitation. The isolated and purified phage was characterized for structural, genomic and growth properties. Transmission electron microscopy revealed that the phage belonged to the family *Myoviridae.* The bacteriophage also showed a narrow host range. One step growth kinetics showed that the latent period was 20 minutes and the burst size was found to be 9-11 virions/cell. The bacteriophage was viable at pH range of 4-9 and a temperature up to 60^0c. Genomic characters were studied by using enzyme technique and dsDNA genome was detected. The objective of this study was to isolate and characterize a bacteriophage which may exhibit properties indicative of potential use in phage therapy and to add new insights of finding alternatives for treatment of infections caused by carbapenem-resistant *enterobacteriaceae.*

KEYWORDS: Bacteriophage, *Klebsiella Pneumoniae*, Phage Therapy, Carbapenem-Resistant Enterobacteriaceae.

1. INTRODUCTION

Phage therapy or the use of lytic phages is a concept with an extensive history. There has been recent resurgence of interest owing to increase in antibiotic resistance. The *enterobacteriaceae* family has gained resistance to even third generation cephalosporin due to production of ESBL[*extended spectrum beta lactamases*] and MBL[*metallo beta lactamases*] leading to difficulty in treatment.[1] Experts fear CRE as the new superbug causing lethal bloodstream infections with mortality rates of up to 60%, which is the reason why it's also known as the "nightmare bacteria".[2]

Klebsiella pneumoniae belongs to the *enterobacteriaceae* family. It primarily affects patients with compromised defenses to cause severe complications. It is among the leading microbial pathogens associated with nosocomial infections.[3] Carbapenem-resistant *Klebsiella spp* produces an enzyme called *Klebsiella pneumoniae carbapenemase*[KPL] causing resistance. The increased incidence of antimicrobial resistance has propelled the need for alternative treatments. Phage therapy forms one of these alternative strategies. Although phage therapy is a century old method, much importance has not been given in its use. Indeed, to date there has been no reports of major side effects or complications associated with phage therapy. This study was carried out to investigate the possibility of use of lytic phages to treat CRE.

2. MATERIALS AND METHODS

2.1 Collection of samples: A total of 10 samples were collected from 2 different sewage treatment plants in Chennai. Both raw sewage and primary treated sewage were collected.

2.2 Isolation of carbapenem resistant enterobacteriaceae[CRE]

The sewage samples were serially diluted in 9ml nutrient broth and made selective for Carbapenem-resistant organisms by adding 10µl of meropenem at the concentration of 10µg/ml. the tubes were incubated for 24 hours at 37^0c and the positive tubes were further streaked on EMB selective media for *enterobacteriaceae*. The colonies were isolated and pure cultured for further processing.[4]

2.2.1 Antimicrobial sensitivity testing[AST]

Once pure culture of isolated organisms were obtained, the standard Kirby Bauer disk diffusion method was used to determine the antibiotic susceptibility profiles of the isolates.[5] Bacterial inoculums were prepared by suspending the bacteria in 4-5 ml sterile broth and turbidity was adjusted to 0.5 Mc Farland standard. The test was performed using Muller

Hinton agar medium against amoxicillin (10µg), gentamycin (10µg), tetracycline (30µg), ampicillin (10µg) and meropenem (10µg) were used. The plates were incubated aerobically at 37^0c for 24 hours. The zones of inhibition were measured and compared with National committee for clinical laboratory standards[NCCLS] guidelines. E.coli ATCC 25922 was Used as control.[6]

2.2.2 MHT[Modified Hodge test]

MHT was used as a confirmatory test for the detection of carbapenemase enzyme production. A suspension of E.coli ATCC 25922 was adjusted to 0.5 Mc Farland standard and inoculated using a sterile swab onto MHA. A 10µg imipenem disk was places at the centre of the plate and test strains were streaked from edge of the disk to the periphery of the plate in three directions. The plate was incubated overnight at 37^0c. The presence of a 'cloverleaf' shaped zone of inhibition by the test strain was considered positive for carbapenemase production.[7]

2.3 Identification of organism

The isolated Carbapenem-resistant enterobacteriaceae was identified by using standard staining procedures such as gram staining and capsule staining, Biochemical testing[IMVIC, Oxidase, catalase] and plating on differential media[Macconkey agar].

2.4 Isolation of bacteriophage

Sewage samples were centrifuged at 10,000 RPM for 10 minutes at 4^0c and the supernatant was filtered to remove debris. 45ml of sewage sample was enriched with 5ml nutrient broth and 5ml of overnight culture of isolated bacteria was added and incubated overnight. (fig.1) Bacteria was removed by centrifugation, supernatant was filter sterilized using Millipore filter and this filtrate was used for plaque assay.[8]

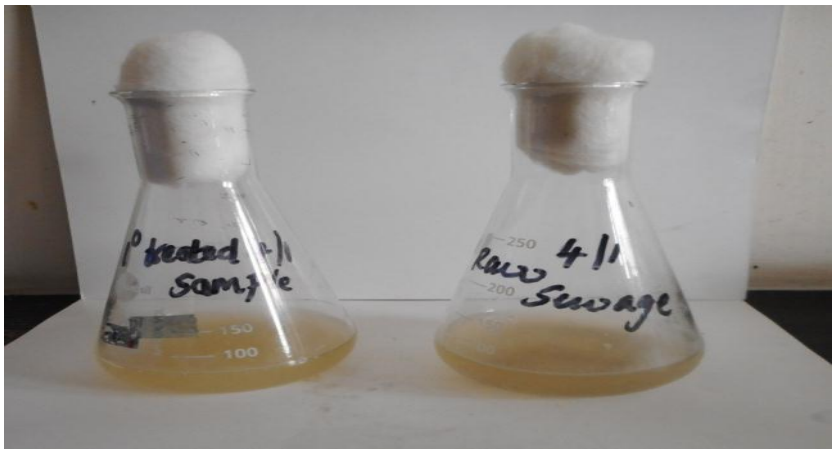

Fig. 1: Sewage culture.

2.5 Plaque assay and selection of potent phage

To detect the presence of phages against isolated CRE, double layer technique was used.[9] 5 drops of the filtrate was added to molten soft agar tubes (Kcl, tryptone, 0.7% agar) and 0.1 ml of overnight host culture suspension was added. The molten agar was poured on set hard agar (Nacl, Cacl, tryptone, 1.5% agar) plates and incubated at 37^0c overnight. The plates were observed for different plaque morphologies and recorded. The potent phage was selected by observing for turbidity after incubation for an additional 24 hours.

2.6 Plaque purification and phage propagation

Isolated plaques were purified successively until single morphology plaques were obtained. A single isolated plaque was picked up with a pipette tip and suspended in 1ml of SM buffer[10] and a drop of chloroform was added to remove bacterial contamination. This suspension was serially diluted and plated again by double layer technique with host organism and incubated for 24 hours. The plaques thus obtained were of uniform morphology. The plates with uniform morphology plaques were selected and 4ml of SM buffer was added to plates and placed on an orbital shaker for 24 hours.[11] The liquid was collected, centrifuged at 10,000 RPM for 10 minutes and supernatant was once again filter sterilized and stored with a drop of chloroform as stock lysate.

2.7 Phage purification and concentration: Phage particles were purified and concentrated by precipitation with PEG 8000.[12] The stock lysate was further cultured in 1000ml nutrient broth with host bacterium and centrifuged for 15 minutes. The supernatant was separated and solid 1M Nacl was added and completely dissolved. Solid PEG 8000 was added at a concentration of 10% and stirred on ice for 30 minutes and left overnight at 4^0c. The solution was centrifuged at 10.000 g for 20 minutes and supernatant was discarded. The pellet (precipitate) was resuspended in 1x PBS buffer and used for characterization.

2.8 Spot test: Spot test was performed to confirm the presence of phage particles in purified lysate.[13] A lawn culture of the host organism was made on nutrient agar plates with Cacl the lysate was spotted on the agar. The plates were incubated at 37^0C for 24 hours and observed for plaques.

2.9 Phage characterization

2.9.1 Transmission electron microscopy: The phage morphologic characters were studied by transmission electron microscope.[14]

2.9.2 Host range determination

The ability of the isolated bacteriophage to lyse other organisms was studied to determine the host range and classify as broad or narrow range.[15] 10 different bacterial species such as *E.coli, Pseudomonas aeruginosa, Salmonella typhi, Proteus mirabilis, Serratia marcesens, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Enterobacter aerogenes and Bacillus subtilis* were used to perform a spot test.

2.9.3Effect of pH and temperature: The effect of different temperatures and pH on the viability of the bacteriophage was studied by subjecting the lysate suspension to various temperatures and pH for 30 minutes-1 hour. The lysate was plated by double layer technique with host and results were recorded after 24 hours of incubation.[16]

2.9.4 One-step growth curve[phage kinetics]

Phage growth characteristics were studied using a simple one-step growth curve procedure.[17] Phage titration was determined as per protocol of Jothikumar et al.[18] The multiplicity of infection[MOI] was calculated by dividing the number of bacteria by the number of phage in the sample. 0.1ml of the phage lysate was added to the 'adsorption tube' with 0.9ml of test culture and exact time was recorded. At 10 minutes, 0.1ml was transferred to tubes with 9.9ml broth labeled as 10^{-2} and 10^{-4}. At 20 minutes, 0.1ml was added to soft agar tube from 10^{-4} with 0.1ml of host culture and plated by double layer technique. The previous step was repeated at 25,30,40,50,60,70 and 80 minutes and the plates were incubated at 37^{0}c overnight. The plates were observed for PFU and a graph was drawn with time on the x-axis and PFU/ml on the y-axis. Various characters and kinetics such as latency period and burst size were determined.

2.9.5 Phage Genome characterization

The genome type of the isolated phage was determined by DNase/RNase test.[17] 0.1ml of purified lysate was treated with 10µl of 10µg/ml DNase, RNase and S_1 nuclease enzyme in 3 different tubes. The tubes were incubated for an hour and the sample was used to perform double layer technique with host. Formation of plaques would indicate a positive result.

3. RESULTS

3.1 Isolation of carbapenem-resistant enterobacteriaceae: Positive results were observed in all raw sewage and primary treated samples where turbidity indicated the growth of meropenem resistant organisms. Further when these positive tubes were plated on selective

eosin methylene blue medium for isolation of *enterobacteriaceae*, four different morphological colonies were observed. Each of these colony types were pure cultures and subjected to further testing for identification.

3.2 Antimicrobial susceptibility testing: Among the four different isolates, two were found to completely resistant to various beta-lactam drugs indicating production of ESBL's (fig.2) (table.2).

Fig. 2: Antimicrobial susceptibility test.

Table 1: Antimicrobial susceptibility testing.

Antibiotic	Diameter	Standard			Result
		S	I	R	
Amoxicillin	0mm	≥18mm	16	≤13mm	**Resistant**
Ampicillin	0mm	≥17mm	15	≤13mm	**Resistant**
Tetracycline	0mm	≥15mm	13	≤12mm	**Resistant**
Gentamicin	0mm	≥15mm	13	≤11mm	**Resistant**
Meropenem	0mm	≥23mm	21	≤19mm	**Resistant**

3.3 MHT (Modified Hodge test): The two isolates were further used in MHT to confirm the production of carbapenemases. One isolate was positive and formed a cloverleaf indentation. (fig.3).

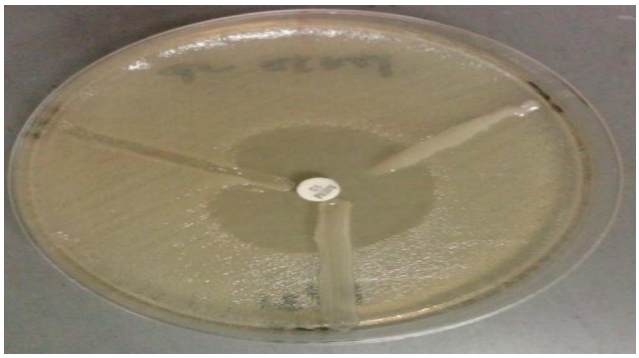

Fig. 3: Modified Hodge test.

3.4 Identification of organism: The isolated CRE was identified as *Klebsiella pneumoniae* (fig.4) after performing various standard tests.

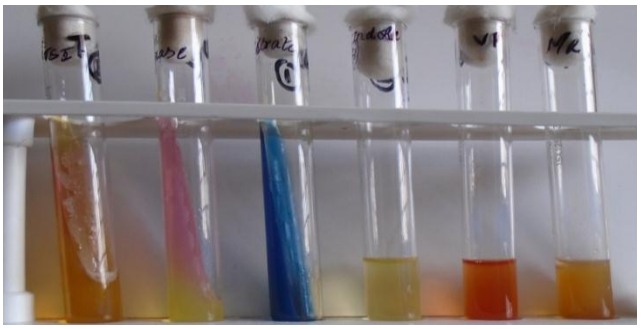

Fig. 4: IMVIC biochemical test.

3.5 Plaque assay and selection of potent phage

Plaques of two different morphologies were observed. One type being large and turbid and the other small, clear and round (fig.5). The potent phage was selected by prolonged incubation after which the large plaques turned turbid indicating lysogeny and the small plaques remained clear. Hence, the small plaques were selected as the potent phage.

Fig. 5: Plate with different morphology plaques.

3.6 Plaque purification and propagation: The plaques were purified until single morphology plaques were observed (fig. 6, 7). Later, stock lysate was prepared using uniform plates.

Fig.6: small plaques.

Fig.7: large plaques.

3.7 Spot test: Spot test was performed for the detection of phages in the purified lysate and phage was detected in all lysate samples. Lysis of host bacterium was observed in all samples. (fig.8).

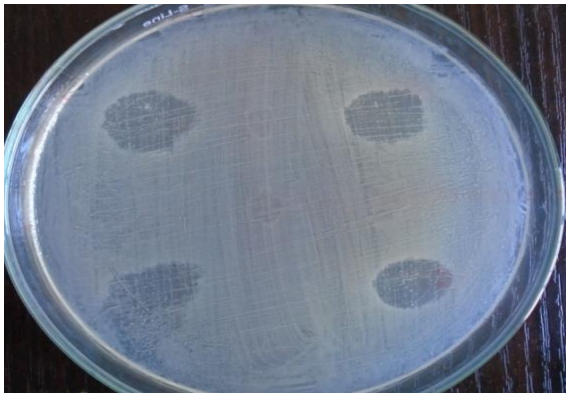

Fig. 8: Spot test.

3.8 Phage characterization

3.8.1 TEM: The complete morphological features of the bacteriophage were studied under TEM. The bacteriophage was observed with a icosahedral head and a tail of intermediate length with contractile sheath typical of the family *Myoviridae* in the order *Caudovirales*. (fig.9).

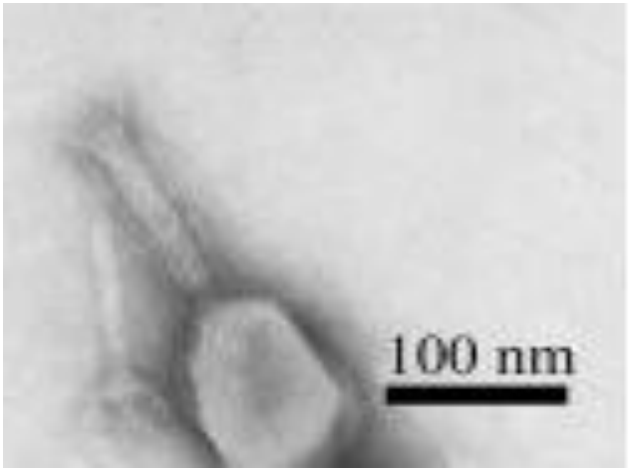

Fig. 9: Transmission electron microscope.

3.8.2 Host range determination: Among the 10 other bacteria that were used, the bacteriophage lysed only one bacterium. (table.2) This is presumptive evidence that the phage may be of narrow lysis spectrum or host range.

Table. 2: Host range determination.

Host Organism	Results
Pseudomonas aeruginosa	No lysis
Salmonella typhi	No Lysis
Enterococcus faecalis	No lysis
Esherichia coli	No lysis
Proteus mirabilis	No lysis
Enterobacter aerogenes	No lysis
Serratia marcescens	No lysis
Staphylococcus aureus	No lysis
Staphylococcus epidermidis	No lysis
Bacillus cereus	lysis

3.8.3 Effect of pH and Temperature

The optimum temperature was found to be 37^0c which yielded the most number of plaques and temperature between 60^0c to 70^0c and above were unfavorable which caused inactivation of phage particles. (fig.10). The optimum pH was found to be 7 with maximum yield in plaques and no lysis was seen in acidic pH 1-3 and alkaline range from 12-14. (fig.11).

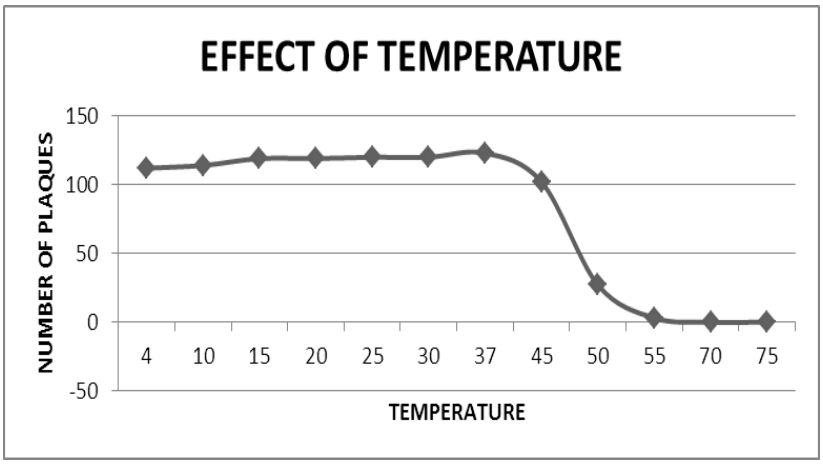

Fig. 10: Effect of temperature.

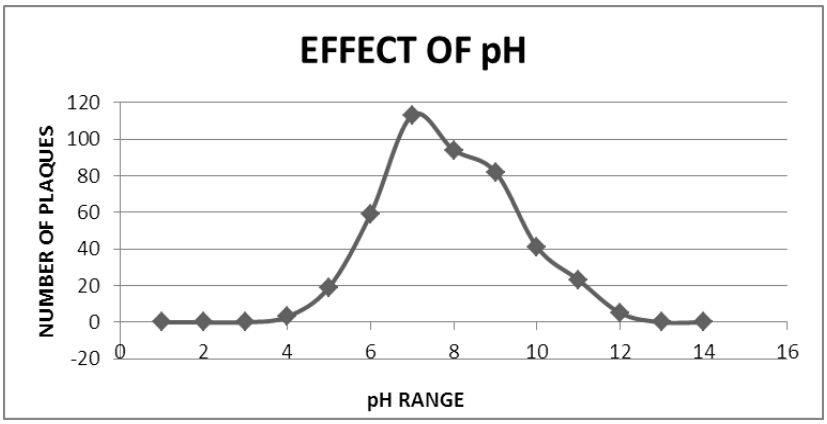

Fig.11: Effect of pH.

3.8.4 One step growth curve: To further characterize the isolated phage against the CRE, one step growth curve analysis was performed. The phage had a latent period of 20 minutes and burst size of 9-11 pfu/infected cell. The adsorption time was found to be 5 minutes. (fig.12).

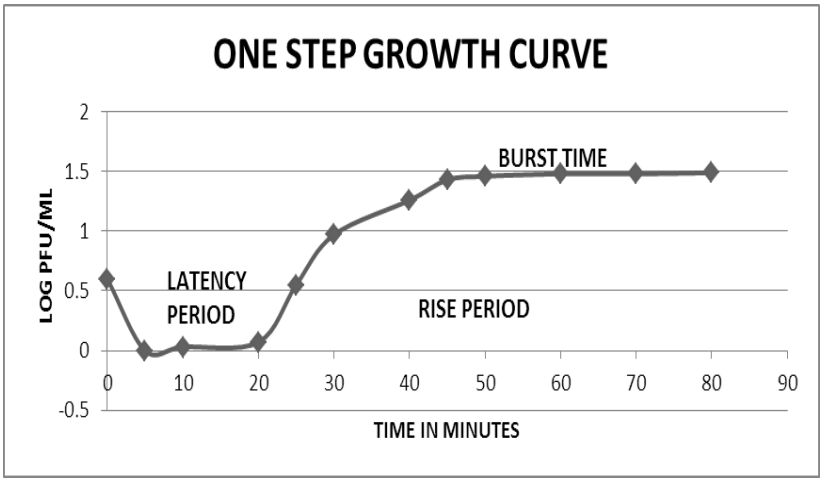

Fig. 12: One step growth curve.

3.8.5 Phage genome characterization

The phage genome was found to be dsDNA as there was formation of plaques in samples subjected to DNase enzyme. There was formation of plaques only in samples exposed to RNase and S1 nuclease enzymes denoting that the genome was neither RNA nor ssDNA.

4. DISCUSSION AND RESULTS

The search for potential therapeutic phages against pan-resistant bacteria has gained momentum due to the increasing prevalence of ESBL and carbapenemase production in strains associated with human infections. Although, phage therapy has along and controversial history, lytic phages are still not explored enough. In the present study, one strain of carbapenem resistant *Klebsiella pneumoniae* was isolated from sewage samples which gave insights on sewage and waste water treatments plants being a possible reservoir of antibiotic resistant genes.[4]

Sewage also serves as a major rich source of bacteriophages and a single potent phage was isolated against the isolate using double layer technique. The less potent and possible lysogenic phage appeared as turbid plaques. Turbidity represents lysogeny which is not helpful in phage therapy.[19] Usage of such phages could lead to antibiotic gene transfer and reverse effects. Hence, the clear, small plaques were chosen as the potent lytic strain.

The isolated phage was purified and concentrated by PEG precipitation for further studies of various characters and the presence of phage in the lysate was confirmed by spot test. Morphological characters were studied using TEM analysis and the isolated bacteriophage was identified as *Myoviridae.*

The host range was also studied against various other bacterium as host.[20] The phage isolate lysed one other organism indicating a narrow host range. Although, this specificity maybe beneficial for administration in single infections, this phage may not be used in case of multiple infections. In which case, a cocktail of lytic phages maybe required.

According to various studies, pH and temperature plays an important role in viability of the phage.[16] In this study, the phage was viable at temperature up to 60^0c and pH range of 4-9. The phage was completely inactivated at high temperatures and low pH below 3 and high ph above 10. This may pose a difficulty while using in oral administration and a stomach acid neutralization maybe required before hand. The one-step growth curve revealed the phage kinetics where the latency period was found to be 20 minutes and burst size was 9-11 virions/cell which is relatively low compared to the family of *Myoviridae* phages in other hosts. Phage genome characterization was done as per the protocol of (Ishnaiwer et al., 2013) by DNase test. The genome type was detected as dsDNA. This method was useful in quick detection using enzymes.

5. CONCLUSION

This study isolated carbapenem-resistant *Klebsiella pneumoniae* from sewage and lytic bacteriophages against this pathogen from same samples. The isolated phage was purified, concentrated and characterized. The phage was of dsDNA genome, belonged to the family *Myoviridae* and exhibited narrow host range. Thus this study indicates that lytic phages could be used as a potential alternative in treating carbapenem-resistant organisms.

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