Recovery status of bacteriophages of different livestock farms of Veterinary College, Adhartal, Jabalpur, India

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Abstract

Study was conducted to know the presence of bacteriophage in sewage material which can play a very important role during therapy against the some antibiotic resistance organisms. During study waste water samples were collected from different depths of the wastewater collection tanks located in livestock farms of different species (Cattle, pig, goat and poultry). These samples were subjected primarily to rapid detection by streak plate method for the detection of lytic activity followed by primary isolation of phage against two most common bacteria of environment, namely, *B. subtilis* and *E. coli* by Double agar layer (DAL) method. Recovery of phages was maximum from pig feces (67%) followed by dairy cattle farm waste (63%), buffalo farm waste (50%), goat farm waste (13%).

Keywords: Double agar layer (DAL), *B. subtilis* and *E. coli,* Bacteriophage, Sewage, Waste Water, Livestock Farm,

Introduction

Bacteriophages are obligate intracellular parasites requiring specific bacteria as host cell for their replication (Carlton, 1999). Phages are most widely distributed and diverse entities in the biosphere and ubiquitously present which can be found in all reservoirs populated by bacterial hosts, such as soil and sea water and the intestine of animals (Mc Grath and Van Sinderen, 2007). Therefore phages to be recovered from these sources. The emergence of resistance in the pathogenic bacteria against the currently available antimicrobial agents has become a critical problem in modern medicine, particularly because of the concomitant increase in immuno-suppressed patients. The emergence of antibiotic resistant bacteria, especially strains that have multiple drug resistance, has generated interest in alternatives to conventional and current system of microbial control. Lytic phages are the possible replacement for antibiotics to treat bacterial infections that do not respond to conventional antibiotic therapy (O'Flynn et al., 2004) therefore the isolation of bacteriophages was performed to use them in treatment of certain clinical conditions.

Material and Methods

Number of Samples: The animal waste disposals consisted of various body excretions from different species of animals *viz.* Cattle, buffalo, goat, pig and poultry were collected in sterile conical flask from animal waste collection tanks in sufficient amount (100 ml) from superficial and deep layers (up to 20 cm) in sterilized conical flask from. Samples (186) were collected from Livestock Farm (Cattle, Buffalo, Goat and Piggery farm), College of Veterinary Science and A.H., Adhartal, Jabalpur, India.

Table-1: Details of wastewater samples collected from different livestock farms

Livestock farm	Number of wastewater samples collected		
	Superficial layer	Deeperlayer	Total
Cattle	25	26	51
Buffalo	20	20	40
Pig	23	22	45
Goat	8	8	24
Poultry	12	12	16
Ganges water	05	05	10
Total	87	89	186

Host bacteria: *Bacillus subtilis* and *Escherichia coli* both are most abundant bacteria present in the environment; therefore they were utilized as primary hosts for the isolation of phages. The known isolates (in-house isolated, characterized and identified) of *B. subtilis* and *E. coli* were used.

Media for phage isolation: Following commercially available media (Lactose broth, Lactose agar (0.7% agar) and Lactose agar (1.2% agar) were used for the preparation of culture media.

Table-2: Percentage of phage recovery

Sr. No.	Species of livestock farm	Isolation of phage				
		Superficial layer	Deeper Layer	Percent of phage recovery		Total Phage (%)
				Superficial layer	DeeperLayer	
1.	Cattle	12 (25)	20 (26)	48	77	63
2.	Buffalo	08 (20)	12 (20)	40	60	50
3.	Pig	12 (23)	18 (22)	55	78	67
4.	Goat	01 (12)	02 (12)	8	17	13
5.	Poultry	00 (08)	00 (08)	00	00	00
6.	Ganges water	00 (05)	00(05)	00	00	00
	Total	33 (92)	52 (94)	36	55	46

Membrane filter: 0.45 µm Cellulose acetate membrane syringe filter

Processing of sample: The collected samples were processed as per the method described by Jothikumar et. al., (2000) with slight modification. Samples collected were processed separately for the isolation of bacteriophage. Briefly, samples were homogenized for one hour, centrifuged at 3000 rpm for 20 min; supernatant was collected and re-centrifuged at 5000 rpm for 20 min in refrigerated centrifuge (Remi C-24). Then supernatant collected was filtered through a 0.45µ syringe filters and filtrate was placed in sterile plastic vials.

Isolation of phage by DAL method: Isolation and propagation of phage was performed by double agar layer (DAL) method as previously describe by Adams (1959). Briefly, lactose agar (10 ml) dispensed into 100 mm sterile Petri dish which was allowed to solidify, further, 2.5 ml of soft agar (mixed properly with the filtrate `and the host bacteria) was poured on basal lactose agar layer to form double agar layer. The procedure is described as under.

For the plaque detection in the sample, six hour old bacterial culture of *B. subtilis* / *E. coli* were separately taken. The concentration of bacteria was adjusted to 3x107-1x108 CFU/ml. Nutrient broth and Lactose broth was used for B. subtilis and E. coli respectively. In a sterile vial 500 µl of filtrate and 500 µl of six hour old bacterial culture were mixed then 20 µl MgCl2 (25mM) was added to enhance the adsorption of phage over bacterial surface. After this, it was agitated on shaker for 20 min and then added to a test tube containing 2.5 ml of molten soft agar held at 450C in a water bath. The mixture was poured onto lactose agar basal plates, allowed to solidify and then incubated at 370C up to 48 hours. Plates were observed at various time intervals (6, 12, 24 and 48 h) for development of plaques.

Preparation of phage lysate: Lysate was prepared by the DAL method described by Jothikumar et al. (2000) with slight modification as described above. Plates with plaques were selected and the top agar was scrapped with 5 ml of Lactose broth. The scrapings were pooled and 2-3 drops of chloroform was added to this and held for 10 minutes at room temperature and then centrifuged at 5000 rpm for 20 minute in refrigerated centrifuge. Agar debris settled down and supernatant was filtered through a 0.45µ cellulose syringe filter. lysates (purified phage suspension) were stored in sterile plastic vials at 40C for further use.

Titration of phage lysate: Titration was done by preparing ten fold serial dilution of the phage lysate. For titration, 10-1 to 10-9 dilutions were made in normal saline. Each dilution was subjected to plaque formation by DAL method (Adams 1959). Phage titer was determined in terms of plaque forming units (PFU/ml) with the help of formula given below:

PFU/ml = No. of plaques x Dilution factor

Results and Discussion

Isolation of bacteriophage: Lytic activity in samples was adjudged as criteria for presence of phage in samples and on the basis of clear plaque morphology samples were selected for the isolation of phage with DAL method. For Primary isolation of phage *B subtilus* and *E coli* were used as bacterial host cell. Details of the results are presented in table2. The results of phage screening in the present study reveled that the concentration of phage was greater in deeper layer as compared to the superficial layer of collection tank. Similar findings were reported by Salama et al., (1989) and Carey-Smith et al., (2006). The superficial layer of collection tanks have direct sunlight exposure, have high temperature and most of organic matter settles in the deeper layer, thus providing conditions for the lesser proliferation of bacteria. This may be correlated with our finding. The recovery of phages was maximum in pig feces (67%), followed by cattle farm waste (63%), buffalo farm waste (50%), goat farm waste (13%). Our

findings were supports findings of other workers (McLaughlin et al., 2006 and Jamalludeen et al., 2007) who had reported the abundance of phage in pig farm waste as compared to other livestock farm waste.

Table 3: Number of phage isolated on B. subtilis and/or E. coli by DAL method

Source of sample	No. of samples	Selected Isolated phage *B. subtilis*	*E. coli*
Cattle farm	10	10	2
Buffalo farm	4	4	0
Goat farm	2	2	0
Pig farm	12	12	2
Total	28	28	4

The results showed that *B. subtilis* favored the growth of phage in all samples irrespective of species from which it was isolated. However, *E coli* isolation media showed 20% recovery of phage from cattle farm followed by 17% from pig and none of the samples from buffalo and goat farm yielded phage in *E coli.* There was cent percent growth of phage on *B. subtilis* as compared to 14 % recovery on E. coli as the primary host (Table3). Size of *E. coli* is small therefore the surface area available for attachment of bacteriophage is lesser as compare to the B. subtilis which is large bacterium and provide large area of attachments of bacteriophage. This could be the reason for reduced number of recovered phage isolates against E. coli in the present study.

Titration of phage lysate: Recovered phage isolates were subjected for titration to obtain high titer lysate preparation so as to ensure maximum number of phages in lysate to trigger lytic activity for phage therapy.

Table 4: Average titer of phage lysate (PFU/ml) prepared for clinical trial

Phages used for titration	Average titer of phage (PFU/ml)
BsHR1	$3x10^{11}$
BsHR2	$3x10^{9}$
BsHR3	$5x10^{10}$
BsHR4	$5x10^{11}$
EHR1	$3x10^{12}$
EHR2	$2x10^{11}$

BsHR1, EHR1 and EHR2 phage in equal part were used for preparation of cocktail of phage lysates. Most effective titre of phage lysate is $3x10^{12}$.

Conclusions

- Prevalence of phage was more in deeper layers as compared to superficial layers of collection tanks.
- Maximum number of samples from pig farm waste was found positive for phage while least observed in poultry.
- Coli phage referred as EHR1 and EHR2 had relatively wider host range as compared to Bacillus phages referred as BsHR1 BsHR2 BsHR3 BsHR4.
- Both B. subtilis and E. coli favoured the growth of phage and hence can be used as a suitable host for isolation of phages.

Acknowledgment

Authors are very much thankful to Dr. R. P. S. Baghel, Dean, College of Veterinary Science and Animal Husbandry, Jabalpur to providing all the necessary funds and facilities. Authors also acknowledge with regards to Dr. (Mrs.) E. Joseph, Professor & Head, Department of Veterinary Microbiology who is very much friendly and her scientific thoughts helped me with lots of enthusiasm to work.

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