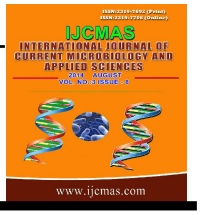
Int.J.Curr.Microbiol.App.Sci (2014) 3(8) 205-210

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 8 (2014) pp. 205-210**
http://www.ijcmas.com



Original Research Article

Isolation of phage from animal waste of different LSF and their utility in phage therapy

Sanjay Kumar Shukla[1*], S.D.Hirpurkar[2], Swatantra K.Singh[3] and R.Rajoria[4]

[1]Department of Veterinary Microbiology, College of Veterinary Science and AH., Rewa, India
[2]Department of Veterinary Microbiology, COV Sc & AH., Anjora Durgh, Chhattisgarh, India
[3]Department of Pharmacology and Toxicology, College of Veterinary Sci. and AH., Rewa, India
[4]Department of Department of AGB, college of Veterinary Science and AH., Rewa, India
**Corresponding author*

Keywords

Biodiversity, Marine Diatoms, Physico-Chemical parameters, India.

A B S T R A C T

Samples collected from collection tanks of animal waste of different Live Stock Farms for isolation, centrifuged at 3000 rpm for one hour for homogenization; supernatant re-centrifuged at 5000 rpm and filter through 0.45µ syringe filter. In a sterile vial, 500 µl of filtrate and 500 µl of 6 hrs old bacterial cultures (*B. subtilis/E. coli* on Nutrient broth and Lactose broth respectively) were mixed along with 20 µl MgCl2 (25mM). It agitated on shaker for 20 min added to a test tube containing 2.5 ml of molten soft agar. Now mixture was poured onto lactose agar basal plates to solidify and then incubated at 37^0C up to 48 hours. Plaques scrapped with lactose broth from surface of agar plate and scrapings pooled and treat with 2-3 drops of chloroform to form phage lysate (purified phage suspension). Lysates of phage used to estimate the therapeutic utility by treating chronic cases of wounds. Wound showing gradually reduction in the swelling and discharge and completely subside at 10th day after phage application. Twenty cases recovered out of twenty four and percent of recoveries was eighty three. Phage therapy has been developed- as an alternative method of therapy against antibiotic resistance organisms- to provide practical solution of the crisis of antibiotic resistance and public health problems. It is also is very effective and economic as compare to existing antibiotic therapy. Response to phage treatment in the chronic wounds in the present clinical trial was highly encouraging.

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). Recovered Phages are categorized on the basis of host range which is very important for the preparation of "Phage Cocktail" for phage therapy. These recovered phage isolates also subjected to

temperature sensitivity to know the survivability of phage lysate on different temperatures so that we can store them for further use.

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 immunosuppressed patients and emergence of antibiotic resistant, 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).

Materials and Methods

Samples of phage isolation

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 (100 ml) from superficial and deep layers in sterilized conical flask from. Samples (186) were collected from Livestock Farm (Cattle, Buffalo, Goat and Piggery farm), NDVSU, Adhartal, Jabalpur (M.P.), India. (Table no. 1).

Isolation of phage

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 phage. 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 machine. Then supernatant collected was filtered through a 0.45µ syringe filters and filtrate was placed in sterile plastic vials. 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.

Six hour old bacterial culture of *B. subtilis /E. coli* were separately $3x10^7$-$1x10^8$ 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 $MgCl_2$ (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 45^0C in a water bath. The mixture was poured onto lactose agar basal plates, allowed to solidify and then incubated at 37^0C up to 48 hours.

Plates were observed at various time intervals (6, 12, 24 and 48 h) for development of plaques. Plates with plaques 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 vials at 4^0C.

Clinical trial

Clinical trial was conducted to observe therapeutic effect of phage lyaste on chronic cases of wound in the large animals at Live Stock Farm (LSF), College of Veterinary Science. & A.H., NDVSU, Jabalpur (M.P.). In the present study twenty four cases of wounds chronic were selected for the clinical trial. Previously these animals were subjected to conventional treatment and did not cure. These animals were subjected to topical application of phage cocktail. Pus samples were collected from the wounds. Antibiotic resistant isolates subjected to phage sensitivity test to know the sensitivity of phage against the pathogenic bacteria. Cocktail was prepared by mixing phage of broad host range and applied topically on wound.

Appropriate follow-up of the case was maintained to avoid the further contamination of wound. Therefore the status of wound recovery was recorded at regular time interval (0, 5^{th} and 10^{th} days) to observe the recovery in the form of physical appearance and microbiological investigation wound to compare the condition and stratus of wound before and after the treatment.

Results and Discussion

In the present study isolation of phage from various sources of farm waste was done in bacterial host. These phage isolates were applied on to chronic septic wounds in livestock and utility of lytic phages was observed over conventionally used antibiotics for effective treatment against antibiotic resistant bacteria.

In the present study, phage was isolated from the animal waste collection tank, constituting various body excretions of different animal species *viz.* cattle, buffalo, goat, pig and poultry. Sampling was carried out from various farms to collect samples of wastewater or sewage from different locations in and around the Jabalpur (mentioned earlier) region as mentioned in table 1. The recovery of phages was maximum in pig feces (67%), followed by cattle farm waste (63%), buffalo farm waste (50%), goat farm waste (13%). There was cent percent growth of phage on *B. subtilis* as compared to 14 % recovery on *E. coli* as the primary host (Table3). This showed that *B. subtilis* favored the growth of phage in all samples irrespective of species from which it was isolated.

These recovered broad host range phage were mixed together for the treatment of chronic septic wounds. Mixing was done after phage sensitivity testing of bacterial organism isolate from septic wound which were showing multiple drug resistance (Table no. 4). Recovery of phage treated cases was recorded on the qualitative clinical examination *viz.* physical appearance of wound and investigation to assess the bacterial load of wound after phage treatment. Microbiological investigation to find out bacterial load of wound was carried out besides the physical recovery from wound infection with respect to presence of organisms in the wound after the treatment. No organism was found at 5^{th} day after topical application of phage over the wound in any of the cases. Twenty chronic cases were recovered completely out of twenty four therefore the recovery percentage was eighty three (Fig 1).

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).

Table.1 Details of wastewater samples collected from different livestock farms

S. No.	livestock farm	Number of wastewater samples collected		
		Superficial layer	Deeper layer	Total
1	Cattle	25	26	51
2	Buffalo	20	20	40
3	Pig	23	22	45
4	Goat	8	8	24
5	Poultry	12	12	16
6	Ganges water	05	05	10
Total		**87**	**89**	**186**

Table.2 Percentage of phage recovery

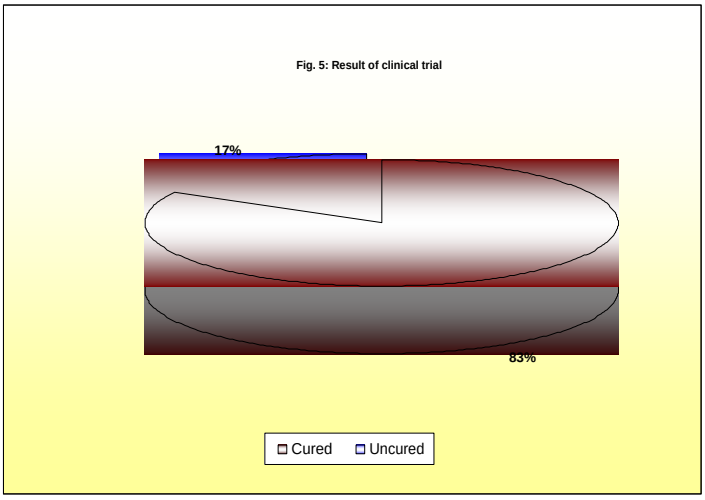
S. No.	Species of livestock farm	Isolation of phage				
		Superficial layer	Deeper Layer	Percent of phage recovery		Total Phage (%)
				Superficial layer	Deeper Layer	
1	Cattle	12 (25)	20 (26)	48	77	63
2	Buffalo	08 (20)	12 (20)	40	60	50
3	Pig	12 (23)	18 (22)	55	78	67
4	Goat	01 (12)	02 (12)	8	17	13
5	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**

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

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

Table.4 Antibiotic sensitivity pattern of pathogen isolated from the wound

S. No.	Name of bacteria	No. of isolate	Number of bacterial isolates found sensitive to the antibiotic discs									
			Am	T	E	Cf	Cp	Cx	Ac	G	P	K
1	***E. coli***	15	0	2	0	5	7	0	0	7	0	4
2	***Klebsiella spp***	12	0	0	8	9	0	0	0	11	0	5
3	***P. aeruginosa***	23	0	1	0	0	3	0	0	2	0	0
4	***Salmonella* spp**	5	0	1	1	2	2	0	2	4	0	3
5	***S. aureus***	21	3	0	5	6	2	2	0	0	0	0



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. There was cent percent growth of phage on *B. subtilis* as compared to 14 % recovery on *E. coli* as the primary host (Table3). This 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*. Size of *E. coli* is small therefore the surface area available for attachment of phage is lesser as compare to the *B. subtilis* which is large bacterium and provide large area of attachments of phage. This could be the reason for reduced number of recovered phage isolates against *E. coli* in the present

study. In the present study twenty cases were cured out of twenty four, therefore phage therapy showed 83 percent of efficacy. It is very economic and highly effective as compare to the existing antibiotic therapy. The prospects of the phage therapy are bright particularly in the antibiotic resistance crisis.

Thus infusion of lytic phage in single dose proved to be innovative and effective therapy for treatment of chronic mastitis. These findings are similar with the findings of Gill *et al.,* (2006) who treated bovine mastitis (staphylococcal mastitis) effectively with phage therapy**.** However, our findings showed agreement with the findings of Slopek *et al.,* (1987), Alisky *et al.,* (1998) and Wills *et al.,* (2005) who reported rate of success of phage therapy as 75-100% against suppurative infections. Thus, clinically phage cocktail can be prescribed in wound management.

References

Adams, M. H., 1959. Discovery of bacteriophages and methods of study of bacterial viruses. Bacteriophages. Interscience Publ., Inc., New York, Pp. 447-448.

Alisky, J., Iczkowski, K. Rapoport, A. and Troitsky, N. 1998. Bacteriophages show promise as antimicrobial agents. J. Infect., 36**:** 5–15.

Carey-Smith, G.V., Billington, C., Cornelius, A.J., Hudson, J.A. and Heinemonn, J.A. 2006. Isolation and characterization bacteriophges and infecting Salmonella spp Microbiol. Lett., 258: 182-186.

Carlton, R.M. 1999. Phage Therapy: Past History and Future Prospects. Arch. Immunol. et. Therapiae Experimentalis, 47: 267–274.

Gill, J.J., Pacan, J.C., Carson, M.E. Sabour, P.M., Leslie, K.E. and Griffiths, M.W. 2006. Efficacy and Pharmacokinetics of Bacteriophage Therapy in Treatment of Subclinical *S. aureus* Mastitis in Lactating Dairy Cattle. Antimicrob. agents chemother., 50**:** 9 2912-2918.

Jamalludeen, N., R.P. Johnson, R. Freindship, M. Andrew, E. Knopinski, J. Lingohr and G.L. Carlton (2007). Vet. Microbiol., 124**:** 47-57.

Jothikumar, N., Reddy, C.G., Sundaria, R.B. and Saigopalb, D.V.R. 2000. Isolation of coliphages specific to enterotoxigenic *E. coli*. J. Environ. Monit., 2: 372-374.

Mc Grath, S. and van Sinderen, D. 2007. Bacteriophage: Genetics and Molecular Biology (1st ed.). Caister Academic Press. Irelend, Pp 343-344.

Mc Laughlin, M.R., M.F. Balaa, J. Sims and R. King. 2006. Isolation of *Salmonella* Bacteriophages from swine effluent lagoons. J. Environ. Qual., 35**:** 522–528.

O'Flynn, G., Ross, R.P., Fitzgerald G.F. and Coffey A. 2004. Evaluation of a cocktail of three phages for biocontrol of *E. coli* O157:H7. Appl. Environ. Microbial., 70**:** 3417–3424.

Salama, S., Balton, F.J. and Hutchinson, D.N. 1989. Improved method for the isolation of *C. jejuni* and *C. coli* phges. Lett. Appl. Microbiol., 8: 5-7.

Slopek, S., Weber-Dabrowska, M., Dabrowski, M. and Kucharewicz-Krukowska, A. 1987. Results of bacteriophage treatment of suppurative bacterial infections. Arch. Immunol., 35: 569–583.

Wills, Q.F., Kerrigan, C. and Soothill J.S. 2005. Experimental phage protection against *S. aureus* abscesses in a rabbit model. Antimicrob. Agents Chemother., 49(3): 1220–1221.