Short Communications

Indian Journal of Animal Sciences **82** (10): 1186–1189, October 2012

Physical characterization of bacteriophages isolated against *Salmonella* Dublin

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Key words: Bacteriophage, Physical, pH, *Salmonella*, Sunlight

Received: 24 January 2011; Accepted: 22 January 2012

Salmonella, a member of family Enterobacteriacae, comprises a group of more than 2501 serovars (Popoff 2001). In cattle and buffaloes *Salmonella* Dublin, is a host specific serovar and it causes diarrhea, fever, haemorrhagic diarrhea, drop in milk production and if not treated may lead to death (Wray and Wray 2000). Phages are the organisms which are present in the nature and kill the bacteria and it has also been reported that phages are 10-times more numerous in the environment than bacteria (Brussow 2005). Though, phage therapy was tried against various bacterial diseases but till date various phages against different bacteria have yet not yielded very efficient clinical trials (Chandra *et al*. 2008). From the previous knowledge it is known that phages are present against all the organisms and they survive in the environment (Rohwer and Edwards 2002). Phages also resist various physico-chemical conditions/stresses to which they are normally exposed to under natural conditions for their survival (Hanlon 2007). Thus, in the present study effect of various physical parameters (pH, temperature and exposure to sunlight) on phages are evaluated.

Isolation of bacteriophages against Salmonella *Dublin:* Agar overlay technique was used to isolate bacteriophages against *Salmonella* Dublin (Adams 1959, Chilamban *et al*. 2004). The samples (faecal and sewage samples) were collected and brought immediately to the phage laboratory and processed for the isolation of phage. In brief, to the 50 ml double strength NZCYM broth (NZ amines Casamino acids yeast extract magnesium sulphate broth) 40 ml sewage supernatant and 10 ml of fresh exponential growth of *S*. Dublin (grown in trypticase soy broth (TSB) at 37°C for 6 h) was mixed and incubated on rotary shaker for 18 h at 37°C. Later, 10 ml supernatant from this was centrifuged at 8000g for 15 min to collect the supernatant again which was passed through pre sterilized 0.22µ PVDF filter and the filtrate was aseptically collected and designated as bacteria free filtrate (BFF).

Combinations of BFF and bacteria were used in various dilutions to test for the presence of phages in the samples before discarding. In brief, equal quantity (100 microlitre) of fresh exponential phase *Salmonella* broth and BFF were suspended in a 5 ml soft NZCYM (0.75%) agar which had been previously melted and held at 45°C in a water bath. The test suspensions were mixed by vortex mixer and were dispensed uniformly over the surface of NZCYM with 1.5% agar in a 100-mm diameter plates. After solidification of the soft agar the plate was inverted and incubated at 37°C for 12h to observe plaques.

Concentration of phages: Salmonella broth (100 microlitre) was suspended in a semisolid NZCYM (0.75%) agar at 45°C and was poured onto a fresh NZCYM agar plate. After solidification of the agar, phages were picked from plaque and streaked first as horizontal lines and later dissecting them by vertical lines (checker board style) using a platinum straight wire loop. The plate was incubated at 37°C for 12h to observe plaques along the streak lines. SM (suspension medium) (2 ml) was poured onto these plates and with a platinum streaking loop the agar was disturbed to release phages from the semisolid agar. The liquid was aspirated, centrifuged at 8000g for 15 min and the supernatant collected was passed through presterilized 0.22µ PVDF filter was denoted as concentrated phage preparation (CPP).

Plaque forming unit (pfu) count: CPP was serially diluted 10 fold in PBS (pH 7.4) up to 10^{-11}. Equal quantity of each phage dilution (100 microlitre) and fresh log phase *Salmonella* (6h growth) in broth were mixed in a soft NZCYM (0.75%) agar using vortex mixer at 45°C and later poured onto a fresh NZCYM (1.5% agar) plate. After solidification, the plates were incubated at 37°C for 12h and plaque count was performed.

Effect of pH on phages: The phage preparation was subjected to various pH conditions i.e. (4, 5, 6, 8, 9 and 10) individually. After every 4 h 500 microlitre phage preparation

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was aspirated from the individual vial and pfu count was performed. This procedure was performed up to 48 h.

Effect of temperature on phages: The phage preparation was subjected to various temperature conditions i.e. (20°C, 50°C and 60°C) individually. After every 4 h 500 microlitre phage preparation was aspirated from the individual vial and pfu count was performed. This procedure was performed for up to 48 h.

Effect of sunlight on phages: The phage preparation was subjected to direct sunlight with ambient temperature ranging from 25–30°C for 9 days continuously and daily pfu count was performed.

Isolation of bacteriophage against Salmonella *Dublin:* Sewage samples (25) were evaluated for the presence of phages against *S.* Dublin and 5 phages were isolated. On the basis of plaque morphology all the 5 phages exhibited plaques which were round, complete and with an average diameter of 3–5 mm. Phages are the viruses which are found wherever a bacterium reside and it was observed that phages are 10-times more numerous in the environment than the bacteria making them most abundant life form on earth (Hanlon 2007). Since there exists a phage corresponding to every bacterium so it offers a potential biological control of bacterial pathogens in human, animal, and plant diseases too (Lederberg 1996, Schuch *et al*. 2002). Isolation of phages from the environment in which a suspected bacterium resides has been a common finding against various bacteria (Salama *et al*. 1989, Xie *et al*. 2005).

Effect of pH: When these phage were subjected to various different conditions of pH (4–10), it revealed that during the first 4h of pH exposure there is a definite reduction in the pfu counts but later on it was observed that the pfu count decreased and were stabilized at 48h at all the pH conditions (Table 1). The above findings indicated that the resistance to withstand varied pH conditions must be an innate mechanism associated with phages, since isolation of phages from sewage/animal wastes is a common finding (Brussow 2005, Sheng *et al*. 2006). For successful survival phages must be

Table 1. Effect of *pH* on the survival of phages

Phage no. (initial phage concentration, log 10)	Time Interval (h)	Phage concentration (Log10)					
		pH4	pH 5	pH 6	pH 8	pH 9	pH 10
P1(9.0)	4	6.70	8.60	8.85	8.78	8.70	8.48
	8	6.30	8.48	8.70	8.48	8.00	8.30
	12	6.30	8.90	8.78	8.70	8.00	8.00
	16	6.70	8.60	8.48	8.30	8.48	8.00
	24	6.48	8.00	8.00	8.00	8.30	8.00
	48	6.30	6.70	6.48	7.00	8.00	8
P2 (8.48)	4	8.30	8.00	8.00	8.30	8.00	8.00
	8	0.00	8.00	6.48	8.00	8.00	8.00
	12	0.00	8.00	6.30	8.00	8.00	8.00
	16	0.00	8.00	6.60	6.48	6.48	8.00
	24	0.00	6.48	6.48	6.30	6.48	6.60
	48	0.00	0.00	6.30	6.00	6.00	6.30
P3 (8.9)	4	8.60	8.78	8.60	8.85	8.00	9.30
	8	8.48	8.60	8.48	8.60	8.00	8.60
	12	8.30	8.70	8.30	8.78	8.00	8.48
	16	8.00	8.48	8.00	8.30	6.60	6.90
	24	8.00	8.30	7.00	6.78	6.48	6.70
	48	0.00	6.30	6.60	6.48	6.00	6.48
P4 (10.16)	4	9.70	10.15	9.78	9.66	9.54	9.90
	8	9.61	9.41	9.36	9.54	9.45	9.80
	12	9.38	9.08	9.20	9.38	9.11	9.56
	16	8.60	8.60	9.52	9.45	9.38	9.45
	24	8.60	8.90	9.51	9.34	8.95	8.85
	48	8.00	8.48	9.00	9.08	8.60	8.30
P5 (10.28)	4	10.20	9.95	10.04	9.72	10.26	9.83
	8	9.86	9.91	9.76	9.89	9.61	9.90
	12	9.68	9.58	9.62	9.11	9.68	9.45
	16	9.51	9.32	9.52	9.18	8.90	8.90
	24	9.20	9.32	9.11	9.15	8.60	9.20
	48	8.60	9.20	8.60	8.78	8.70	7.75

Table 2. Effect of temperature on the survival of phages

Phage no. (initial phage concentration, log 10)	Time interval (h)	Phage concentration (Log10)		
		20°C	50°C	60°C
P1 (9.15)	4	9.08	8.85	0.00
	8	8.95	8.30	0.00
	12	9.00	6.78	0.00
	16	8.70	0.00	0.00
	24	8.48	0.00	0.00
	48	8.30	0.00	0.00
P2 (8.7)	4	8.60	6.30	0.00
	8	8.48	6.30	0.00
	12	8.30	0.00	0.00
	16	8.30	0.00	0.00
	24	8.30	0.00	0.00
	48	6.30	0.00	0.00
P3 (9.11)	4	9.08	8.00	0.00
	8	8.90	8.00	0.00
	12	8.85	6.00	0.00
	16	8.95	0.00	0.00
	24	8.95	0.00	0.00
	48	6.78	0.00	0.00
P4 (10.90)	4	10.70	8.48	0.00
	8	10.30	7.41	0.00
	12	10.36	6.78	0.00
	16	10.36	0.00	0.00
	24	10.36	0.00	0.00
	48	10.30	0.00	0.00
P5 (10.60)	4	10.54	8.85	0.00
	8	10.30	8.70	0.00
	12	10.60	6.85	0.00
	16	10.60	6.00	0.00
	24	10.61	6.00	0.00
	48	10.30	6.00	0.00

Table 3. Effect of exposure to sunlight on phages

Phage No.	Phage concentration (Log10)									
	Time interval (h)									
	0	24	48	72	96	120	144	168	192	216
P1	9.04	8.9	8.48	8.3	8	6	0	0	0	0
P2	8.6	8.48	8.48	8	6	0	0	0	0	0
P3	8.48	8.3	8	8	6	0	0	0	0	0
P4	9.67	9.53	9.28	8.85	8.48	8.30	6	0	0	0
P5	9.99	9.61	9.51	9.23	8.48	7.04	6.60	0	0	0

able to withstand varied pH, however, the exact mechanism as to how phages thrive well in the sewage is still not fully known and needs further studies.

Effect of temperature: Phage preparation when exposed to 20°C indicated that initial phage count decreased slightly and with slight variation among different phage preparations the survival of phages could be observed up till 48h (Table 2). At 50°C after initial 4h of exposure the phage count decreased drastically and phages could be observed only till 12h post exposure. However, except one phage preparation all were reduced to zero after 12h post treatment indicating that the phages can withstand heat up to 50°C and thereafter are inactivated. However, at 60°C no phage was detected even after the first 4h of exposure and indicating that phages could not with stand this temperature. It was reported earlier that phage preparation when subjected to 80°C for 20 min rendered phages non viable indicating phages sensitive to heat at higher temperature (Vinodkumar *et al*. 2008). The probable reason for not withstanding higher temperature might be due to enveloped nature of phages in the study and with the excess heat it must have lead to irreparable damage to the phage making it non viable. However, the exact mechanism needs further studies.

Effect of sunlight: When the effect of sunlight was evaluated on the phages it was observed that phage count decreased when exposed directly to sunlight at almost constant ambient temperature (Table 3). The number decreased after initial exposure in all the phage preparations. Most of the phages exposed to direct sunlight continuously could withstand the exposure only up to 5 days, however, 2 phages could survive the exposure up to 6 days but after that none of the phage preparation had any survivors indicating that sunlight do have a lytic effect on phages.

Thus from the study it could be concluded that phages against *S*. Dublin could withstand great variations in pH and can withstand temperature variation up till 50°C beyond which phages are non viable. Also, phages can withstand direct sunlight exposure maximum up to 6 days continuously and beyond that phages become non viable.

SUMMARY

In the present study a total of 5 phages were isolated against *Salmonella* Dublin using agar overlay technique from 25 samples of dairy effluent. These phages were purified, enriched and subjected to various physical (pH, temperature and sun light exposure) to evaluate their survival ability. On physical characterization of the phages it was found that the phages could survive varied pH conditions with reduction in its numbers from the initial concentration. Upon exposure to various temperature conditions it was observed that the phages can withstand a maximum 50°C and above this temperature it was deleterious for almost all the phages. Exposure to direct sunlight indicated that phages can withstand maximum continuous exposure up to 6 days and beyond that it was found to be deleterious for survival of the phage.

ACKNOWLEDGEMENT

The authors are grateful to the Department of Biotechnology, Government of India for providing the necessary funds to conduct the study. The authors are also thankful to the Director of Research, GADVASU, Ludhiana, Punjab for providing laboratory facilities to complete this study.

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