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Full Length Research Paper

Isolation of a bacteriophage against *Salmonella* Dublin and determination of its physical resistance under varied *in vitro* conditions

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In the present study a phage against *Salmonella* Dublin was isolated using agar overlay technique. On physical characterization of the phage (pH, temperature and sun light exposure) it was found that the phage could survive at varied pH conditions with reduction in its numbers. A temperature of above 50°C and direct sunlight beyond 5 days was found to be deleterious for survival of the phage.

Key words: *Salmonella*, bacteriophage, physical parameters, pH, sunlight.

INTRODUCTION

Salmonella is an important bacterial organism causing enteric infections in humans as well as in animals (Domniguez et al., 2002). Ever since its discovery, it is a big problem affecting various species of animals. 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). Various antibiotics have been used to combat the disease but due to the development of drug resistance in the bacteria it some-time result in failure (Singh et al., 2009). Moreover, with the development of multiple drug resistance it is more alarming because *Salmonella* affects humans too because it being zoonotic in nature (Pereira et al., 2007). Phages are the organisms which are present in the nature and which kill the bacteria.

Phage therapy has been 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). The use of host specific bacteria phages has been promoted as cost effective and adaptable approach to control zoonotic bacteria (Sulkvelidze and Barrow, 2005). Phages survive in the environment where the bacteria are present (Boca et al., 2002). Phages have the ability to resist various physical conditions or stress which they are normally exposed to under natural conditions. Thus, in the present study, the effect of physical parameters on phage indicating its survival ability during adverse environmental conditions is evaluated.

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MATERIALS AND METHODS

Isolation of Bacteriophage against *Salmonella* Dublin

Agar overlay technique was used to isolate bacteriophage against *Salmonella* Dublin (Adams, 1959; Chilamban et al., 2004) .The samples (faecal as well as sewage samples) were collected and were brought immediately to the phage laboratory and processed for the isolation of phage. In brief, to the 50 ml double strength NZCYM broth (Life Technologies) 40 ml sewage supernatant and 10 ml of fresh exponential growth of *Salmonella* Dublin (grown in Trypticase Soy Broth (TSB), (Himedia, Mumbai) 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 8000 g for 15 min to collect the supernatant which was passed through presterilized 0.22 µ PVDF filter (Axiva, Mumbai) 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 µl) 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 a hard (1.5% agar) NZCYM in a 100 mm diameter plates. After solidification of the soft agar the plate was inverted and incubated at 37°C for 12 h to observe plaques.

Table 1. Effect of pH on the survival of P2 phage.

S/N	Time interval (h)	Phage concentration (Log10)					
		pH 4	pH 5	pH 6	pH 8	pH 9	pH 10
1	4	9.544068	9.477121	9.477121	9.39794	9.447158	9.477121
2	8	9.20412	9.39794	9.176091	9.39794	9.146128	9.544068
3	24	8.30103	8.30103	8.30103	8.30103	8.477121	8
4	48	8	8.477121	8.30103	8	8	8.30103

Concentration of plages

Salmonella broth 100 µl 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 12 h to observe plaques along the lines. SM buffer (2 ml) was poured on to these plates and with a loop the agar was disturbed to get phages released out from the semisolid agar. The supernatant collector was centrifuged at 8000 g for 15 min to collect the supernatant and named as concentrated phage preparation (CPP).

Plaque forming unit plaque-forming unit (pfu) count

CPP was serially diluted 10 fold in PBS (pH 7.4) up to 10^{10}. Equal quantity of each phage dilution (100 ul) and fresh exponential *Salmonella* (6 h 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 inverted and incubated at 37°C for 12 h to observe and count for plaques.

***Salmonella* counts**

Salmonella counts were determined using a standard spread plate technique. Briefly serial dilution (1:10) of the culture was made in PBS (pH 7.4). 1 ml of the samples from each dilution was plated onto TSA. Plates were incubated at 37°C for approximately 18 to 24 h and colonies counted thereafter.

Enrichment of phages

Plaques were enriched by further streaking across sectionally onto a NZCYM plate on which bacterium has been embedded in the semisolid medium.

Effect of pH on phages

A 10 ml phage preparation having ($100*10^8$pfu/ml) was subjected to various pH conditions that is (4, 5, 6, 8, 9 and 10) individually. After every 4 h 500 ul was aspirated from the individual vial and pfu count was performed as per the procedure mentioned above. This procedure was performed up to 48 h.

Effect of temperature on phages

A 10 ml phage preparation having ($100*10^8$pfu/ml) was subjected to various temperature conditions that is (20, 50 and 60°C) individually. After every 4 h, 500 ul was aspirated from the individual vial and pfu count was performed as per the above mentioned procedure. This procedure was performed for up to 48 h.

Effect of sunlight on phages

A 10 ml phage preparation having ($100*10^8$pfu/ml) was subjected to direct sunlight with ambient temperature ranging from 25 to 30°C for seven days continuously. 500 ul of phage preparation was aspirated after every 24 h to detect pfu count as per the protocol mentioned above.

RESULTS

Isolation of bacteriophage against *Salmonella* Dublin

Out of five sewage samples tested from various places (dairy farm sewerage) which were harboring discharge contents from cattle and buffaloes, one sample (20%) yielded phage against *Salmonella.* Dublin. Based on the plaque morphology the plaque was round, complete with a diameter ranging from 3 to 5 mm having clear zone of lysis.

Effect of pH

When this phage was subjected to various different conditions of pH (4 to 10), it revealed that during the first 4 h 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 1 to 2 phages at 48 h at all the pH conditions (Table 1).

Effect of temperature

Phage preparation when exposed to 20°C indicated that initial phage count decreased to approx 50% it kept on decreasing to get stabilized by 24 h and was almost same at 48 h (Table 2). At 50°C after initial 4 h of exposure the phage count decreased drastically and was reduced to 5% from initial count and the count decreased to one 24 h later and was reduced to 0 at 48 h after the exposure. However, at 60°C no phage was detected during the first 4 h of exposure and same was observed till the completion of the study that is 48 h later.

Table 2. Effect of temperature on the survival of P2 phage.

S/N	Time interval (h)	Phage concentration (Log 10)		
		20°C	50°C	60°C
1	4	9.322219	8.69897	Nil
2	8	9.255273	8	Nil
3	24	9.113943	8	Nil
4	48	9	Nil	Nil

Table 3. Effect of exposure to sunlight on P2 phage.

S. No.	Time interval (h)	Phage concentration (Log10)
1	24	8.954243
2	48	8.69897
3	72	8.477121
4	96	8.60206
5	120	8.30103
6	144	Nil
7	168	Nil
8	192	Nil

Effect of sun radiation

When the effect of sun radiation 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 from 100 to 9 during the first 24 h of exposure and thereafter the count was stabilized for next four days to diminish to 0 by the 6^{th} day.

DISCUSSION

Phages are the viruses which eat or kill bacteria for its survival. In the present study out of 5 sewerage samples tested one phage (P2) specific against *Salmonella* Dublin was isolated. It has been observed that for almost all the bacteria there exists a phage corresponding to that bacterium and so phages offer potential for targeted biological control of bacterial pathogens in human, animal, and plant diseases (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 (Xie et al., 2005; Salamaf et al., 1989).

The isolated phage when characterized on the physical resistance parameter, the temperature revealed that the phage P2 survives below 50°C and as the temperature increases beyond 50°C the activity of phages decreases thus exhibiting an inverse relationship between the temperature. The reason might be because the phage might be enveloped and with the excess heat there is irreparable damage to the envelope thereby it rendering it ineffective against bacteria at higher temperature. Thus the study indicates that phages against mesophilic bacteria cannot withstand higher temperature and the exact cause for it requires further study.

The effect of pH when examined gave the insight that the phage can remain viable, surviving a wide variation of pH (2 to 10) . Though, initially there was a reduction in phage count but the activity was not lost completely, indicating that the mechanism of resistance to withstand pH variation is innate with bacteriophages thus letting them survive in sewage where pH varies greatly.

The effect of sunlight indicated that the phage was able to survive direct sun radiation at 30°C for 5 days continuously but on the 6^{th} day the activity was reduced to 0 as was evident from phage count. This indicated that sunlight is lethal to phages and is keeping a check on phage number in the sewage and outside environmental conditions.

Thus from the study it could be concluded that a temperature of above 50°C and direct sunlight beyond 5 days is deleterious to the phage survival. Whereas, pH conditions do not inhibit the phage activity much indicating that phages can tolerate pH variation easily.

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