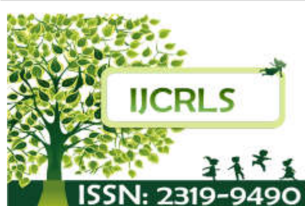

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ISOLATION OF *SALMONELLA* FROM SPOILED FOOD SAMPLES AND ENRICHING PHAGES TO EVALUATE POSSIBLE CONTROL MEASURE FOR *SALMONELLA*

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ABSTRACT

Food borne pathogen, *Salmonella* spp. was isolated and characterized from spoiled food samples. In order to develop control strategy and to eradicate this pathogen, bacteriophages were isolated from river water samples. Isolated bacteriophages showed large clear plaques indicating presence of lytic phages with higher infectivity and burst size of 712 x 10^5 PFU/ml. Genome size of the bacteriophage was approximately 33967 bp with 3 fragments yielded from AluI restriction and 2 fragments from MluI restriction. EcoRI and HindIII endonucleases could not restrict phage DNA. Proteome analysis showed 9 well separated bands on SDS PAGE.

Key words: *Salmonella*, bacteriophage, phage therapy, food-borne disease, pathogenesis, multiple drug resistance, genome, proteome

INTRODUCTION

Most commonly recognized food borne infections are those caused by the bacteria *Campylobacter*, *Salmonella*, and *E. coli O157:H7*. Food products especially of the animal origin are more prone to be contaminated with *Salmonella*. *Salmonella* are known to cause illnesses such as typhoid fever, paratyphoid fever, and food poisoning (Salmonellosis) (Zhao *et al*., 2001). *Salmonella* is rapidly gaining resistance to antibiotics like ampicillin, chloramphenicol, cotrimoxazole, and ciprofloxacin. This increased resistance towards antibiotics makes it difficult to treat the illnesses caused by *Salmonella* (Sanghavi *et al*., 1999). These limitations in the use of present antibiotic therapies lead to emergence of another thought process which theoretically explains means of controlling *Salmonella* infections using bacteriophages. Bacteriophages are bacterial viruses which attack the bacterial cells and kill them. The host specificity of bacteriophages makes them unique. This bacteriophage therapy though is in its developmental stages, holds great promise in future. The potential to use phages as therapeutic agents in controlling human and animal disease has been recognized for some time. More recently the extension of phage biocontrol to food applications has been investigated. Phages have been investigated as a potential means to eliminate pathogens like *Campylobacter* in raw food and *Listeria* in fresh food or to reduce food spoilage bacteria (Wittebole *et al*., 2014).

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In present study we isolated *Salmonella* spp. from spoiled food samples. Isolation and enrichment of bacteriophages specific to *Salmonella* was done from river water samples. These phages were further studied for DNA size, restriction profile and protein profile.

MATERIALS AND METHODS

Isolation of *Salmonella spp.*

Approximately 25 grams of various food samples such as chicken, eggs, milk, curd and butter were spoiled in laboratory by keeping under un-sterile conditions at room temperature for 3 days. These samples were used for isolation of *Salmonella.* Microbial growth on the contaminated food samples was streaked onto Hektone enteric agar plates and plates were kept for incubation at 37˚C for 24 hours. After incubation colony characters, Gram staining and motility test were performed to confirm presence of *Salmonella*.

Antibiotic sensitivity test

Salmonella isolate was screened for antibiotic sensitivity by disc diffusion method. Overnight grown broth culture of *Salmonella* isolate was surface spread on a Hektone enteric agar plate. Antibiotic impregnated discs were placed equidistantly on the agar plate. Discs impregnated with various antibiotics of standard concentrations were used for this analysis. The antibiotics used were Penicillin (10µg/ml), Ampicillin (20µg/ml), Tetracycline (30µg/ml), Streptomycin

(10µg/ml), Erythromycin (15µg/ml), Gentamycin (10µg/ml). After placing antibiotic discs the plates were incubated at 37˚C for 24 hours (Bauer *et al*., 1966).

Enrichment of *Salmonella* phages

Approximately 250ml of river water samples were collected into the sample collecting bottle from different sites and transferred to the laboratory. Enrichment of phages was done by inoculating 100ml of each river water sample separately in 100ml of double strength LB media containing 1ml of overnight grown culture of *Salmonella*. The media was incubated at 37˚C for 24 hours. After incubation medium was centrifuged at 5000rpm for 10 minutes at 4˚C. 10ml Supernatant was collected into a fresh vial. 0.2ml chloroform was added to the supernatant and mixed thoroughly; this was used as the enriched phage lysate (Gwyneth *et al*., 2006).

Isolation of phages using agar overlay method

Enriched Phage lysates were screened by double layer agar method. 25µl of enriched phage lysate was mixed with 0.2ml of overnight grown broth culture of *Salmonella* isolate (host culture) in 5ml of Hektone enteric soft agar media and was poured on top of 10ml Hektone enteric hard agar plate. The plates were incubated at 37˚C for 24 hours. Plaque forming units present per ml (PFU/ml) of the samples were calculated after observing the plates (Gwyneth *et al*., 2006).

One step growth curve of Bacteriophage

5ml of the overnight grown *Salmonella* culture and 5ml of enriched phage lysate were mixed and incubated for adsorption at 37˚C for 20 minutes. After the incubation 90ml of sterile LB broth was added to the mixture. Immediately 0.1ml of suspension was drawn from the mixture and centrifuged at 5000rpm for 10 minutes at 4˚C and supernatant was collected into a fresh vial. 2µl chloroform was added to the supernatant and mixed thoroughly; this was used as the phage lysate and labeled as 0 minute lysate. The 0 minute phage lysate was further diluted to 10^{-4}. Double layer agar method was performed for the phage lysate to obtain PFU/ml of sample for 0 minute incubation. Similarly phage lysates were prepared after interval of every 30 minutes and PFU/ml calculated by double layer agar method. A graph of relative titers Vs time was plotted based on PFU/ml readings for various time interval phage lysate samples (Kokjohn *et al*., 1991).

Genome analysis

Bacteriophage DNA was extracted by Phenol:chloroform method (Radhakrishnan & Ananthasubramanian, 2012). Restriction analysis of isolated bacteriophage DNA was carried out using EcoRI, HindIII, AluI and MluI endonucleases. Whole DNA and restricted DNA were analyzed on 0.6% agarose gel with standard marker lane ranging from 20000bp to 1000bp.

Proteome analysis

Phage structural proteins were analyzed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970). 100µl of high titer phage suspension from the enriched phage lysate was boiled for 5 minutes with sample loading dye and was then electrophoresed on discontinuous SDS-PAGE gel (5% stacking, 12% resolving gel) by using the vertical slab gel electrophoresis system (BioEra, India). Phage protein samples along with the standard molecular weight marker were loaded on gel. Gel was stained using Coomassie blue staining solution. Molecular weight of phage proteins were estimated by comparing with standard molecular weight markers (Ngangbam & Nongmaithem, 2012).

RESULTS AND DISCUSSION

Isolation of *Salmonella*

Salmonella was isolated from spoiled food samples. Black centered, circular colonies with smooth margin and soft consistency were observed on Hektone enteric agar. Gram negative rods were observed after Gram staining and the culture was found to be highly motile with hanging drop technique under microscope (King & Metzger, 1968; Downes & Ito, 2001).

Image 1. *Salmonella* colonies on Hektone enteric agar

Antibiotic Sensitivity Test

Antibiotic sensitivity test was performed for *Salmonella* isolate obtained from spoiled food samples. It was found to be Resistant to Penicillin, Ampicillin and Erythromycin. It was intermediately resistant to Tetracycline and Streptomycin. The isolate was Sensitive to Gentamicin. Results were concluded as per Standard Kirby-Bauer chart (Mandal *et al*., 2009).

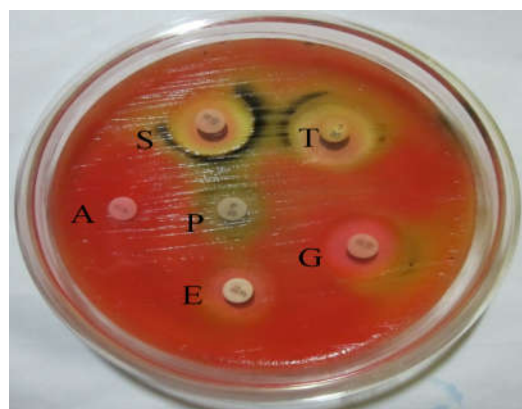

Image 2. Antibiotic Sensitivity Test

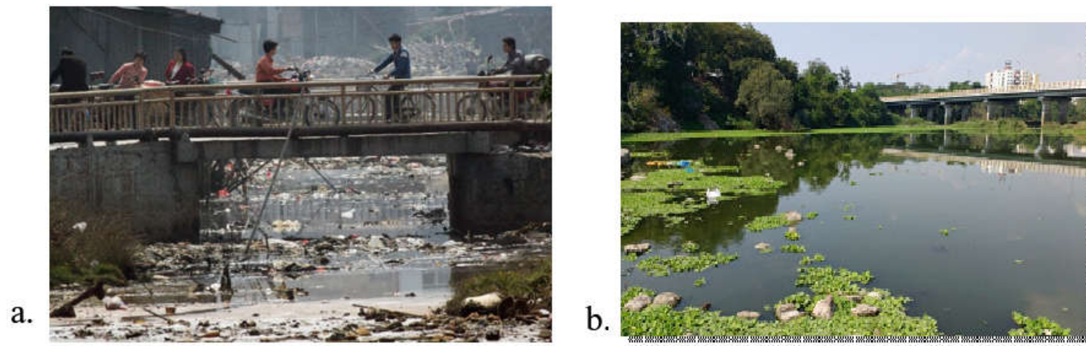

Image 3. Phage sample collection sites (a) Ambil odha, Pune (b) Mula River, Baner

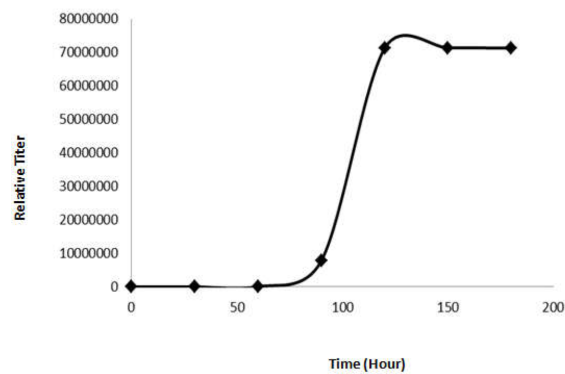

Image 4. One step growth curve of isolated *Salmonella* phage

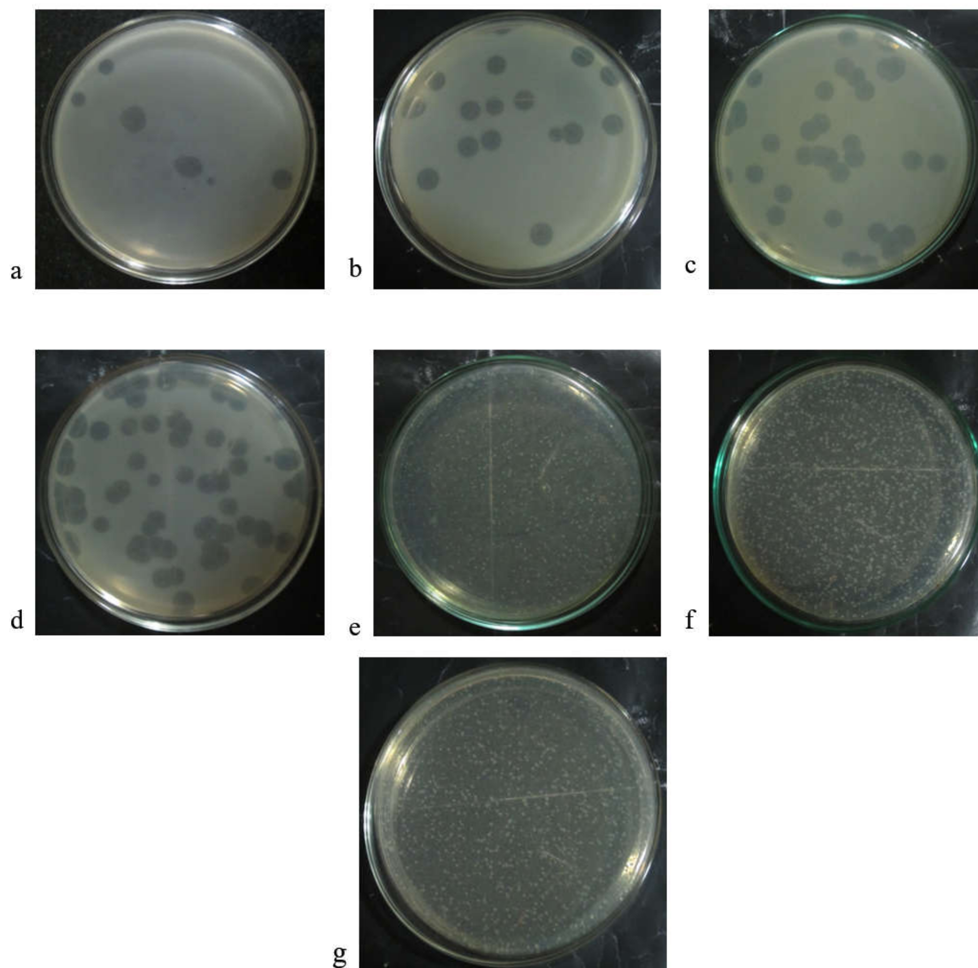

Image 5. One step growth curve, a: Lysate from 0 min, b: Lysate from 30 min, c: Lysate from 60 min, d: Lysate from 90 min, e: Lysate from 120 min, f: Lysate from 150 min, g: Lysate from 180 min

Water sample collection sites for isolation of *Salmonella* bacteriophage

Water sample sites selected for the isolation of *Salmonella* bacteriophages were contaminated river water sites. The possibility of presence of bacteriophages in such environment was greatest due to fecal and non-fecal contamination of sites and presence of host in larger numbers.

Phage enrichment, isolation and quantification

Clear plaques with large diameter were observed after incubation. A clear plaque indicates the presence of lytic phages and the diameter of plaques directly indicates increased rate of infectivity and the burst size. The quantification of phages was done by calculating total number of plaques present on agar plate. Each phage gives rise to one plaque. Thus number of phages [Plaque Forming Units (PFU)] present per mililiter of the enriched phage lysate calculated by counting number of plaques observed upon incubation.

One step growth curve

The burst size was determined using one step growth curve. Burst size is a point on one step growth curve graph with maximum relative titer. The burst size for the isolated phage was found out to be 712 x 10^5 PFU/ml.

Genome analysis

The molecular analysis of phages was done to determine genome size and restriction profile. The molecular weight of standard marker was 20000 bp, 10000 bp, 5000 bp, 3000 bp and 1000 bp. DNA molecular size of phage was approximately 33967 bp.

Image 6 a. Agarose gel electrophoresis

M: Standard Molecular weight marker lane with molecular weight 20000 bp, 10000 bp, 5000 bp, 3000 bp and 1000bp; Lane 2: *Salmonella* bacteriophage genome with molecular weight approximately 33967 bp

The restriction enzymes EcoRI and HindIII did not cleave Phage DNA. Restriction enzyme AluI yielded three fragments of sizes 20773 bp, 7008 bp and 5310 bp and MluI enzyme treated genome of bacteriophage yielded two different fragments of sizes 21938 bp and 11361 bp.

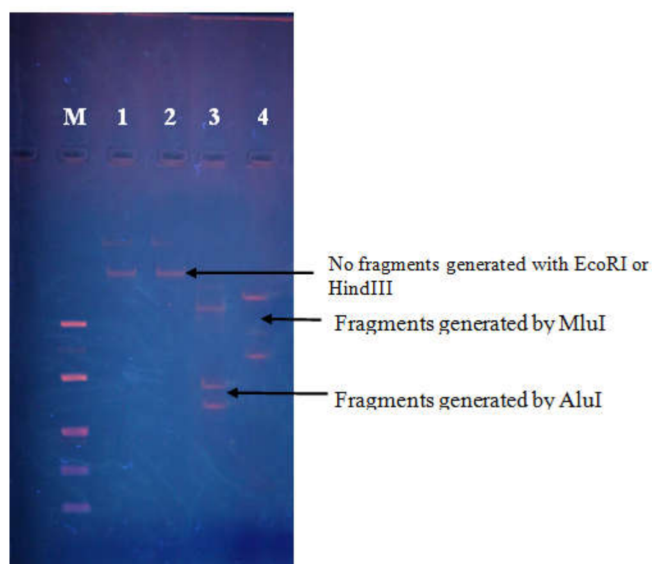

Image 6 b. Restriction digestion of *Salmonella* bacteriophage genome

M: Standard Molecular weight marker lane with molecular weight 20000 bp, 10000 bp, 5000 bp, 3000 bp and 1000bp
Lane 1: gDNA digested with EcoRI with molecular weights 33967 bp
Lane 2: gDNA digested with HindIII with molecular weights 33967 bp
Lane 3: gDNA digested with AluI with molecular weights 20773 bp, 7008 bp and 5310 bp
Lane 4: gDNA digested with MluI with molecular weights 21938 bp and 11361 bp

Proteome analysis

The proteome analysis suggested presence of 9 different proteins present in *Salmonella* bacteriophage. The standard molecular weights of marker were 200 kD, 150 kD, 94 kD, 66 kD, 40 kD, 18 kD and 3 kD. The molecular weights of Phage proteins were found out to be 168 kD, 118 kD, 86 kD, 37 kD, 29 kD, 24 kD, 17 kD, 12 kD, 10 kD and 7 kD.

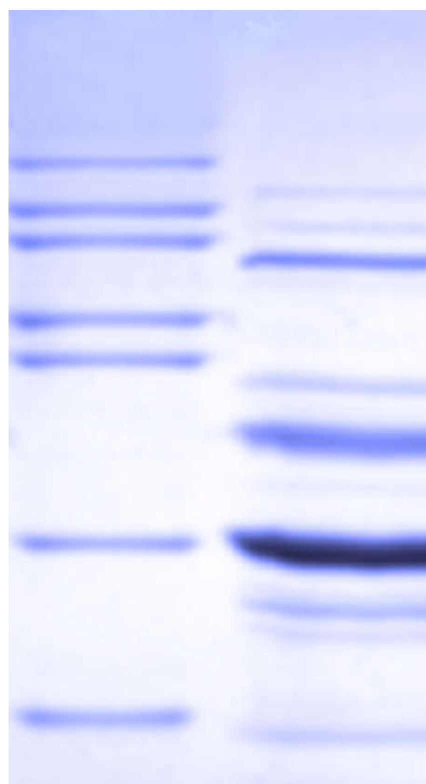

Image 7. Proteome analysis by SDS PAGE

M: Standard molecular weight marker lane with molecular weights 200 kD, 150 kD, 94 kD, 66 kD, 40 kD, 18 kD and 3 kD
Lane 1: Proteins extracted from *Salmonella* bacteriophage with molecular weights 168 kD, 118 kD, 86 kD, 37 kD, 29 kD, 24 kD, 17 kD, 12 kD, 10 kD and 7 kD

The present study focuses on isolation of *Salmonella* phages and their molecular analysis. In-depth research on similar lines will open ample opportunities to develop therapies against multi drug resistant bacteria and their application in the field of Healthcare and Medicine.

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