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Growth kinetics and stability assessment of siphoviridae like *Campylobacter* phages

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ABSTRACT

Campylobacter infections pose a severe threat to the poultry ecosystem leading to high economic loss. The antibiotic treatment against these infections is not effective due to the emerging antibiotic resistance exhibited by the bacteria and no vaccines are currently available to prevent this infection. Phage therapy has been suggested as an alternative antimicrobial therapy. The phages were isolated, purified and the concentration was 22 ×10^{13} PFU (Plaque Forming Units) mL^{-1}. Over a period of 35 days, these phages were found to be stable when stored both in SM buffer and PBS at 4 - 7°C. The stability of phages was also analyzed at temperature and pH ranging from 50 to 90 °C and 2 to 12 respectively. The phage growth parameters such as the latent period, the burst period and the burst size were 30 min, 30 min and 4 PFU per infective center respectively. These phages possess a wide host range. From the AFM analysis, the head diameter and tail length were 140 nm and 248 nm respectively. From the results of genome finger printing analysis and pulse field gel electrophoresis, the genome size was found to be around 20 Kbp. The presence of different phage proteins in the molecular weight range of 15 – 85 KDa were observed by SDS-PAGE.

Key words: *Campylobacter*, phage characterization, production, stability analysis.

INTRODUCTION

Campylobacter jejuni is a gram-negative, slender, comma or spiral shaped rods [1]. *Campylobacters* have been frequently isolated from the poultry ecosystems and studies on this bacterium gained importance due to its pathogenic activity [2]. It causes campylobacteriosis, characterized with diarrhea and other related symptoms [3]. It also spreads rapidly.

The antibiotic therapy against campylobacteriosis is greatly affected because of the bacterial resistance to a wide range of antibiotics [4]. The phage therapy employed as an alternative approach against this pathogenic bacteria requires knowledge on the lytic phages specific against this pathogenic bacterium [5]. Here, we describe the isolation, large scale production, purification and further characterization of *Campylobacter* phages suggesting it to be to the biocontrol agent in the poultry ecosystems.

EXPERIMENTAL SECTION

Bacterial Strains and Culture Conditions

The bacterial strain used for the study was isolated from the fecal sample of a diarrhea affected chicken obtained from a poultry farm. 1 g of this sample was added to 10 ml of 0.1% peptone water and serially diluted up to 10^{-4}. The diluted samples were plated onto Muller Hinton agar and incubated at 42°C for 48 hours. The colonies were then subjected to biochemical analysis for the identification of the organism [6]. The bacterial strains used for host range analysis are tabulated in Table 2.

Isolation of Phages

The phages were isolated from different poultry farms. Chicken fecal samples were suspended (1:10) in SM buffer (NaCl – 100mM, $MgSO_4.7H_2O$ – 8mM, Tris-Cl – 50mM, pH – 7.5, 0.002% gelatin) and the bacteriophage was allowed to stabilize at 4°C overnight with gentle shaking. The supernatant was centrifuged at 13,000 g for 10 min and filtered through 0.22 micron pore size membrane filter. For the confirmation of phages, the soft agar overlay method of Frost (1999) was adopted. Different dilutions of bacteria and phage lysates were added and incubated at 42°C for 30 min to aid in the process of adsorption. The samples were then added to 5 mL of NZCYM agar (0.7%) which was maintained at 45°C in water bath. It was then overlaid onto NZCYM hard agar (1.2%) plates. The plates were incubated at 42°C for 48 h under micro-aerophilic conditions for isolation of plaques.

Concentration of Phages

The isolated plaques were precipitated using PEG 6000 [7, 8]. The plaques along with the soft agar were resuspended in distilled water and centrifuged at 10000 rpm for 30 min at 4°C. To the supernatant, 10% PEG was added and dissolved at room temperature. It was kept in ice for 1 h. Centrifugation was repeated again and the pellet was resuspended in 5 ml of saline (0.9% NaCl).

Host Range Analysis

Phage host range was established by using the spot test method [9]. Ten different bacterial isolates were analysed for their sensitivity to the phage isolate 4 (Table 2). The plate inoculum consisted of 5 mL of soft agar was mixed with 100 µL of the overnight bacterial culture and equal volume of $CaCl_2$ (300 mM). The mixture was overlaid onto the surface of hard agar. 3 µL of each phage lysate were spotted onto the inoculated hard agar plates, and the plates were incubated at 42°C for 48 h. Bacterial sensitivity to the bacteriophages was established by the appearance of clear zone at the spot. A control plate was maintained using SM buffer which showed no zone of clearance.

Large Scale Production of Phages

Four batches of 250 mL Muller Hinton broths were inoculated with *C. jejuni* and incubated at 42°C overnight. The phage inoculum (0.1%) containing 10^7 PFU mL^{-1} was added to the cultures and incubated till the turbidity was changed. The phage lysate was then subjected to overlay as described above.

Stability Analysis

The isolated plaques were resuspended in SM buffer and PBS separately and then incubated at 4 - 7°C. During this period, samples were analyzed at regular intervals to detect the reduction in titer, if any.

Thermal Sensitivity Test

To analyze the temperature sensitivity of phages, 900 µL of sterile distilled water was preheated to temperature, ranging from 50 to 90°C. Then 100 µL of phage solution (1.05×10^3 PFU mL^{-1}) was added to the preheated water. It was then heated at different temperatures for 30 min and then kept at room temperature. Surviving phage titer was assayed by the double layer method [10].

pH Sensitivity Test

The phage (at the final concentration of 1.05×10^3PFU mL^{-1}) was incubated overnight at 25°C in phosphate buffered saline (135 mM NaCl, 1.3 mM KCl, 0.5 mM KH_2PO_4, 3.2 mM Na_2HPO_4, pH 7.4) at pH ranging from 2 to 12. The phage samples were then re-adjusted to pH 7 and the double layer method was performed to determine the phage titer [10].

Determination of Phage Adsorption Rate

Phage adsorption was studied using the modified method of Shao and Wang as described below: log phase growing *C. jejuni* culture was centrifuged and the cells were resuspended in fresh medium to a final concentration of 10^8 CFU mL^{-1}. Phage suspension was added at a multiplicity of infection (MOI) of 0.01, and the mixture was incubated at 42°C. Samples were collected every minute during a total period of 15 min. The samples were centrifuged at 14000 rpm for 20 min to sediment the phage adsorbed cells. The titers of unabsorbed free phages in the supernatant were determined as indicated above and the rate of adsorption was found [11].

Phage Growth Characteristics

10 mL of 24 h culture was harvested by centrifugation at 14,000 rpm for 20 min. The pellet was resuspended in 5 mL of fresh medium to obtain a final concentration of 10^8 CFU mL^{-1}. Phage suspension (MOI- 0.01) was added to this and the mixture was incubated at 42°C for 30 min. It was then centrifuged for 14,000 rpm for 20 min and the pellet was then resuspended in 10 ml fresh medium. Samples were taken every 5 min over a period of 3 h and were immediately titered [12].

Genome Fingerprinting Analysis
Phage DNA was isolated according to the procedure of Jonathan, 2003 [13]. Restriction digestion was carried out using the following enzymes: EcoRI, HindIII, XhoI, BamHI, HinfI, SacI and NheI. One µg of phage DNA (as quantified by UV spectrophotometry at 260 nm) was used in each digestion. The restriction digestion was performed in restriction endonuclease reaction buffer with 1 µg of DNA and 1 unit of respective enzyme. Digestion was carried out for 2 hours at 37°C. The restriction digestion pattern was observed on 0.7% agarose gel stained with ethidium bromide. The phage genome sizes were determined by using semi-log plotting method.

Pulse Field Gel Electrophoresis
For preparation of phage genomic DNA, 10 µl of phage suspension was diluted in 40 µl of TE buffer (10 mM Tris, 1 mM EDTA [pH 7.5]). This was mixed with an equal volume of 1.4% molten agarose (pulse field gel electrophoresis [PFGE] grade) in TE buffer and dispensed into plug molds. The plugs were allowed to set at room temperature and were then transferred to eppendorf tubes containing 5 ml of lysis buffer (100 mM EDTA, 10 mM Tris [pH 7.2], 1% Sarkosyl [wt/vol], 0.1 mg of proteinase K per ml). The plates were incubated at 55°C for 18 h with gentle shaking to lyse the phage capsids and digest the protein components. The lysis solution was discarded and proteinase K was inactivated by the addition of 5 ml of 1 mM phenylmethylsulfonyl fluoride in wash buffer (50 mM EDTA, 20 mM Tris [pH 7.2]) and incubated for 1 h at room temperature with gentle shaking. The plugs were then washed three times for 20 min each with successive changes of wash buffer at room temperature with gentle shaking. A 2-mm slice of each plug was then inserted into the wells of a 1.2% agarose gel. The gel was run using a Gene Navigator system in 0.5 TBE for 18 h at a range of 100 – 130V with a switch time of 30 to 60 s [14].

A 2-mm-wide slice from each plug was cut with a scalpel or single-edge razor blade and transferred to a tube containing 1X restriction buffer solution. The plug slices were incubated in this restriction buffer at room temperature for 5 min. Then, the prerestriction mixture was removed, and 200 ml of the restriction enzyme mixture containing BamHI was added to one tube. The plug slices were incubated at room temperature (37°C) for 2 h. Prior to casting of the gel, the restriction mixture was removed from each tube and replaced with 200 ml of 0.5X TBE (10X TBE contains 0.89 M Tris borate and 0.02 M EDTA, pH 8.3). The plug slices were allowed to stand at room temperature for 5 min, after which they were loaded into the appropriate wells of a 1.2% agarose gel [15].

Analysis of phage proteins
The phage proteins were analysed by SDS-PAGE as described by Laemmli, 1970. 12% separating gel (Stock acrylamide - 12 mL, Tris HCl pH 8.8 - 7.5 mL, water - 10.05 mL, ammonium per sulfate - 0.2 mL, 10% SDS - 300 µL and *N, N, N', N'* - tetraethylethylene diamine - 20 µL) and 4% stacking gel (acrylamide – 2.7 mL, Tris HCl pH 6.8 - 2 mL, water - 15 mL, 5% ammonium per sulfate - 50 µL, 10% SDS - 200 µL and *N, N, N', N'* - tetraethylethylene diamine - 20 µL) were used. The protein samples were pretreated with SDS at 95°C for 15 min to denature the protein molecules. The samples were electrophoresed at 150 V for 3 h and stained with coomassie blue followed by silver staining.

Atomic Force Microscopy
Glass slide cleaned with 5% HNO_3 was taken. Phage sample was diluted 50 fold in 10 mM Tris-HCl of pH 8.8. 5 µ L of this sample was deposited on the glass slide and dried at room temperature in humid condition for 12 h. It was again dried at room temperature and gently rinsed with Milli-Q water [16]. It was then imaged in semi-contact mode, using NT-MDT Ntegra Auro Scanning SPM imager.

RESULTS AND DISCUSSION

Isolation of Bacteria
Ten different samples, one diarrheal and rest normal fecal samples were collected. One isolate from each sample were found to be *Campylobacter jejuni* based on biochemical screening.

Isolation and Concentration of Phages
Clear plaques on a well spread bacterial lawn were observed. The optimized ratio of bacteria to the different dilutions of phage lysate was 1:10. The phage isolate 4 from the diarrheal sample showed a concentration of 0.75×10^6 PFU mL^{-1}. This phage isolate was further purified by PEG precipitation which showed a concentration of 22×10^{13} PFU mL^{-1}. As the phage titer was higher in the diarrheal sample, it was taken for the further analysis.

Host Range Analysis
Out of 10 bacterial isolates, nine isolates showed sensitivity to the phage isolate 4 showing a wide host range (Table 2).

Large Scale Production and Purification of Phages

Turbidity change was observed after 24 h and the concentration was found to be 3.1×10^{23} PFU $mL^{-1}$for an inoculum size of 0.1 %. A 3.8 fold increase in phage yield was attained from 1 litre scale-up. This yield is higher compared to the yield of 11×10^{10} PFU mL^{-1} obtained from the large scale production of *E. coli* phages with an inoculum size of 0.5% and an incubation period of 19 h [17]. Using PEG 6000 the concentration of purified phages obtained was 9×10^{30} PFU mL^{-1}.

Stability Analysis

The reduction in phage titer from 0.9×10^{29} PFU mL^{-1} at regular intervals of time is shown in table 3 .The initial titer of phages before temperature and pH treatment was 1.05×10^{3} PFU mL^{-1}. Phages were found to be comparatively stable at temperatures like 50°C, 60°C and 70°C with 3.5 fold reduction in titer (Fig.1.). The phage titer was considerably reduced by 12 folds when treated at 80 and 90°C revealing its temperature dependence. At temperatures other than the optimal level, only fewer phage genetic material penetrate into bacterial host cells and therefore, fewer of them can be involved in the multiplication phase [18]. This might be the cause of considerable reduction of phage titer at higher temperatures. The thermal stability of the viruses is influenced by many factors. For instance, in mouse retrovirus it was reported that depletion of cholesterol level in the viral lipid shell and host cellular plasma membrane results in increased thermal stability [19]. The phage titer was reduced by 15 folds at lower pH 2 and 3 and the percentage reduction varied from 11% to 75% for treatment at higher pH as shown in Fig. 2. Neutral pH was found to be favourable for its lytic activity since only 1.1 fold reduction was observed. Poultry processing steps involve heat treatment at 40 to 70°C and acid treatment resulting in low pH condition [20]. The results indicate that the activity of phages may not be hindered by these conditions. Moreover, the inactivation of phages due to the low pH in the bird's gizzard is nullified by the protective functions of the food constituents. This benefit could be realized when the phage is administered along with food. It has been further reported that the administration of phage along with an antacid, neutralize the low pH effect on phages and improves its efficacy [21, 22, 23].

Determination of Phage Adsorption Rate

In our study, the time taken by the viral particles to reach maximum adsorption was around 28 min. The result obtained was in accordance with the Siphoviridae family of phages active against *Vibrio harveyi.* [10]. However, adsorption rates vary between different families of phages. For instance, adsorption studies carried out in T7 group of phages active against *Pseudomonas fluorescens* showed a rapid adsorption rate for 5 min followed by a slower rate for the next 5 min [24]. The adsorption rate representing the affinity of phages towards the host cell is greatly influenced by factors like pH and presence of ions like Mg^{2+} and Ca^{2+}. The influence of temperature on adsorption has also been reported [25]. The Mg^{2+} and Ca^{2+} ions stabilize the coiled DNA inside the phage capsid which greatly improves the adsorption rate and also controls the penetration efficiency of phage DNA into the bacterial cells [25]. Mg^{2+} ions, even at concentrations below 1 mM can prevent the inactivating effect of Tris HCl, generally used as buffering medium, in phage adsorption [26]. Maximum stability was attained in 2.5 mM solutions of these salts [27]. Here, the adsorption is highly favoured by suitable Mg^{2+} ion concentration. The viral particle generally makes a random movement to find its host. If the mixture is highly diluted, the phages find it very difficult to infect the host. However, the adsorption efficiency observed in the present study may not get reflected in vivo since the viscosity due to the mucous in the intestine influences the adsorption rate [28]. Hence, further studies are warranted to understand the adsorption characteristics of this phage.

Phage Growth Characteristics

The growth kinetic studies of *Campylobacter* phages, revealed that the latent period and the burst period were 30 min, each. The burst size representing the maximum number of phages released was 4 PFU per infective center as shown in Fig. 3. During latent period, there will be no release of phages. Latent period depends on the nature of the phage, physiological state of the host as well as the composition of the growth medium. For instance, tryptophan, a key ingredient in the growth medium interacts with the tail fibres of the phage, reversibly, enabling it be activated and adsorbed onto the host. It is then followed by a period of rise during which the lysis of host occurs and thereby virions are released. Latent period also depends on the quality and the quantity of the host. A phage will evolve a shorter latent period when either host density is high or host quality is good [29]. The burst size obtained was comparable to the burst size of *Campylobacter* phages which has been previously reported [30]. Burst size to the maximum of 24 PFU per infective center for *Campylobacter* phages was also reported [31]. The burst size reported for other groups of phages was comparatively higher indicating that *Campylobacter* host cell lysis can happen even at the release of very low number of phages. This is due to the tendency of some phages to lyse the host even when they are not completely full [32].

Phage growth rates are a function of both the length of phage generation and the phage burst size. The phage generation time observed in this study was 58 min. Generation time is a function of both phage latent period and the rate of phage adsorption. This is given by the equation,

Phage generation time, $t_G = t_A + E + L - E$

where t_A - adsorption time, E - eclipse period and L - latent period. The phage eclipse period is the time during which infection is occurring but no mature phage progeny is found within an infected cell. Both burst size and the phage generation time are controlled by the phage latent period, with greater burst sizes associated with longer latent periods but shorter generation times associated with shorter latent periods [29].

Smaller burst size may also be due to the larger size of the phages and the smaller size of the host cell. The size of the host cell is important as it modulates the availability of receptors and its protein synthesizing machinery for the binding and growth of phages respectively [33].

The data tabulated in table 1 show the relationship between the burst size and the size of the host cell [10, 33,34,35,36,37,38].

The *Campylobacter* phages generally belong to either the family of Myoviridae or Siphoviridae [39]. Here, the lower burst size, shorter latent period and pH tolerance resembles the characteristics of Siphoviridae phages.

Genome Fingerprinting Analysis

The phage DNA was resistant to digestion with EcoRI, HindIII, HinfI, SacI, NheI and XhoI. However, restriction digests were obtained for BamHI. The genome size was found to be around 21,800 bp (Fig. 4.). The genome size of siphoviruses were reported to be in the range between 22 and 121 kb [40] and the genome size of this phage is in agreement with these reports.

Pulse Field Gel Electrophoresis

PFGE was used to estimate the overall genome size and banding patterns following restriction endonuclease digestion of the genome with BamHI. The restricted fragments revealed the genome size to be approximately 20 kb, thus coinciding with the fingerprinting results (Fig. 5.).

Protein profiling

From the SDS-PAGE profile, the phage was found to contain proteins in a molecular weight range of 15 to 85 KDa (Fig. 6.). The proteins with different molecular weights observed in the gel were suggested to be various types of whole phage proteins as shown in table 4 [41, 42, 43,44].

AFM Imaging

From the AFM analysis, head diameter and tail length were 140 nm and 248 nm respectively (Fig. 7.). Based on the morphology and the growth characteristics studied, this phage is suggested to be Siphoviridae phage since they lack contractile sheath [45]. Elford and Andrews (1932) [46] reported that the size of the phages inversely correlates the size of the plaques on agar. The smaller plaque size which might be due to the larger size of these phages observed in the present study (Fig. 8.) corroborates their findings.

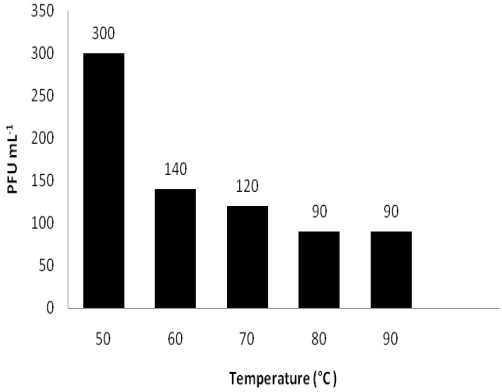

Fig. 1. Temperature sensitivity of phage isolate 4

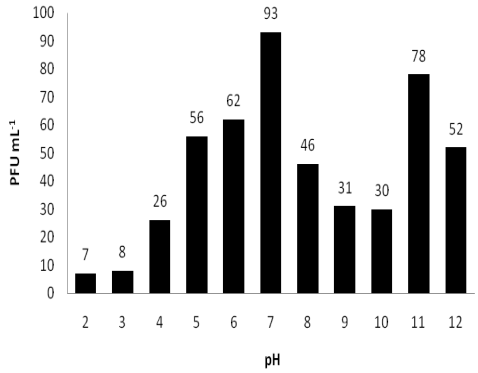

Fig. 2. pH sensitivity of phage isolate 4

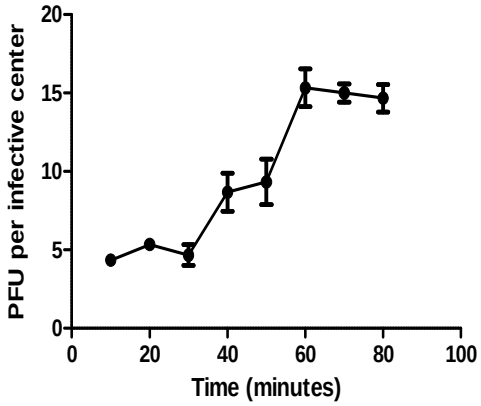

Fig. 3. One step growth curve of phage isolate 4

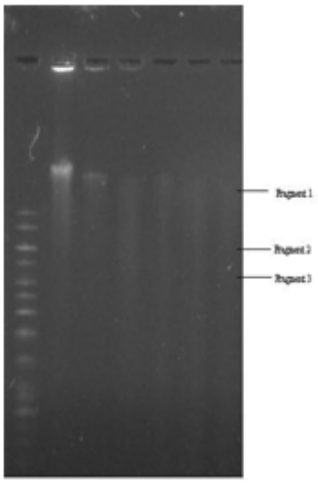

Fig. 4. Restriction analysis of phage DNA

Lane 1: 1 Kb DNA ladder; Lane 2: Lambda DNA uncut; Lane 3: XhoI restricted Lambda DNA; Lane 4: BamHI restricted Lambda DNA; Lane 5: XhoI subjected Phage DNA and Lane 6: BamHI restricted Phage DNA

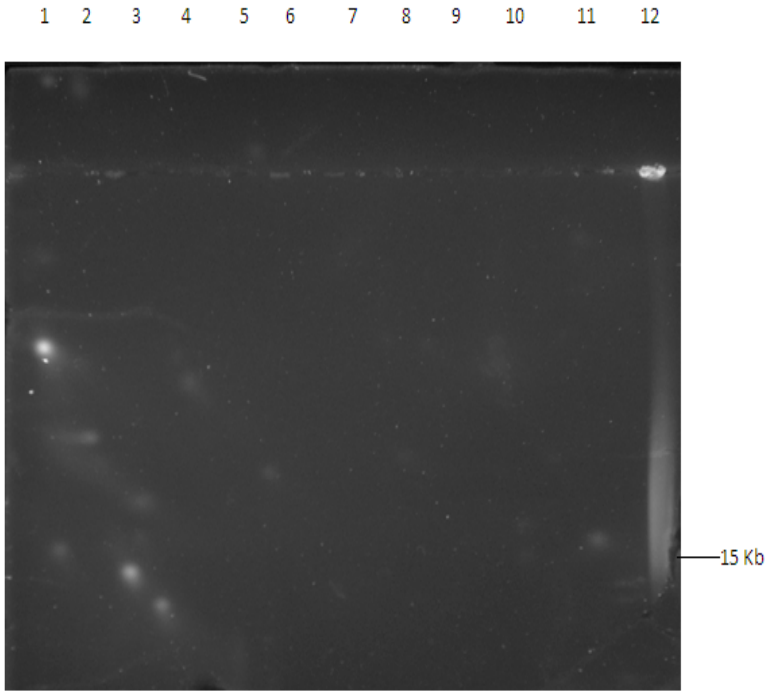

Fig. 5. PFGE analysis

Lane 1 - 10: Uncut DNA of phage isolates 1 – 10; Lane 11: BamHI restricted DNA of Phage isolate 4; Lane 12: PFGE mid range marker (291 to 15 Kb)

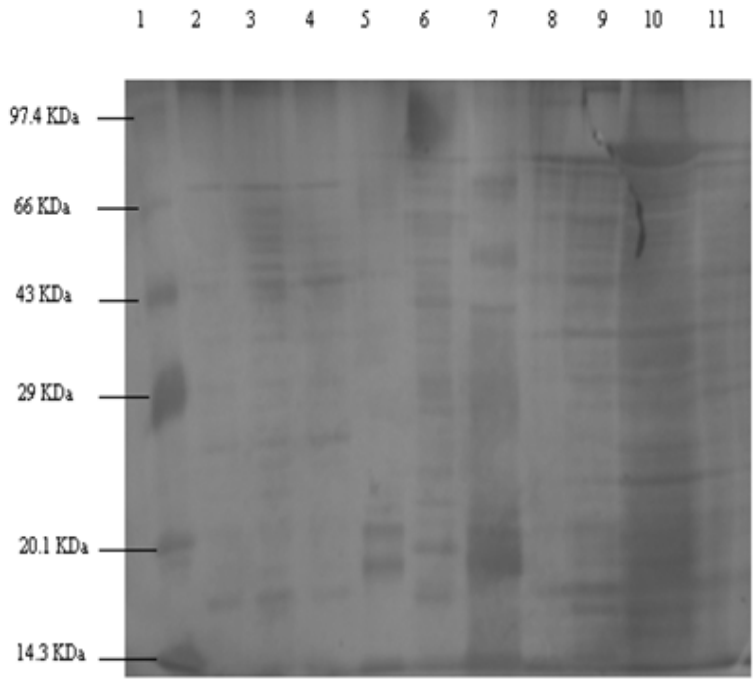

Fig.6. Protein profiling

Lane 1: Medium size marker; Lane 2: Phage isolate; Lane 3: Phage isolate 6; Lane 4: Phage isolate 7; Lane 5: Phage isolate 8; Lane 6: Phage isolate 9; Lane 7: Phage isolate 10; Lane 8: Phage isolate 2; Lane 9: Phage isolate 1; Lane 10: Phage isolate 4; Lane 11: Phage isolate 3

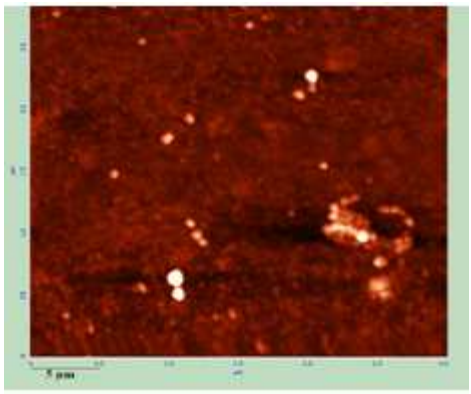

Fig. 7. AFM image of phage isolate 4

Fig. 8. Plaques on agar

Table 1: Relationship between burst size and host cell size

S.No.	Bacterial strains	Cell size	Burst size (PFU per infective center)
1	*Lactobacillus bulgaricus*	2 - 9 mm	130[a]
2	*Pseudomonas aeruginosa*	0.5 - 1.0 µm	10 – 200[b]
3	*Vibrio harveyii*	2 - 5 mm	78[c]
4	*Streptococcus*	0.5 - 1.2 µm	30 – 70[d]
5	*Lactococcus lactis*	0.75 – 0.95 µm	24[e]
6	*Escherichia coli*	0.5 µm	12 – 15[f]
7	*Leuconostoc fallax*	0.8 - 1.8 µm	80[g]
8	*Campylobacter jejuni*	0.2 - 0.8 µm	9 – 24[h]

The references for the above data are as follows: a – [34]; b – [6]; c – [10]; d – [36]; e – [37]; f – [33]; g – [38] and h – [31].

Table 2: Host range analysis

S. No.	Bacterial isolates	Lytic activity of Phage Isolate 4
1	Cjcf1	+
2	Cjcf2	+
3	Cjcf3	+
4	Cjcf4	+
5	Cjcf5	+
6	Cjcf6	+
7	Cjcf7	+
8	Cjcf8	+
9	Cjcf9	-
10	Cjcf10	+

+ -lytic activity ; - -no lytic activity
Cjcf- Campylobacter jejuni chicken fecal sample

Table 3: **Stability analysis**

S.No.	Time interval (day)	Phage titer - SM buffer (PFU mL^{-1})	Phage titer - PBS (PFU mL^{-1})
1	15	1.21×10^{22}	1.54×10^{22}
2	25	1.20×10^{21}	2.78×10^{21}
3	35	2.60×10^{19}	0.70×10^{19}

TABLE 4: Phage proteins observed from SDS-PAGE

S.No.	Observed molecular weights (KDa)	Phage proteins
1	110	Major tail fiber protein[a]
2	24 - 50	Minor tail fiber protein[a]
3	17, 19	Major tail tube protein[a]
4	36	Minor tail tube protein[a]
5	46, 56	Capsid proteins[b]
6	16	Lysozyme [c]
7	85	F protein[d]
8	48	A protein[d]
9	18	D protein[d]

The references for the above data are as follows: a – [41]; b – [42]; c – [43] and d – [44].

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