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A broad-host range coliphage against a clinically isolated E. coli O157: isolation and characterization

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ABSTRACT

Escherichia coli O157 is one of the common and problematic pathogens, particularly in developing countries. To isolate a phage against this pathogen, a total of 32 water samples were enriched for phage and two of these samples were found to contain lytic phages that grow on and kill enterohaemorrhagic *E. coli* serotype O157. Following the primary evaluation, the most efficient phage was chosen for further characterization. The multiplicity of infection, morphology, killing efficiency and bacterial host range were determined under controlled conditions in the laboratory. Isolated phage was designated gBSN-MGB13. According to transmission electron microscopy, this lytic phage morphologically belonged to the myoviridae family. Based on phage efficiency test, a multiplicity of infection of 5 logs of gBSN-MGB13 resulted in 50% reduction in viable bacterial cell count after 20 min incubation in 37 °C without shaking. Since gBSN-MGB13 is a broad-host range phage and effective against several pathogenic species, *E. coli* O157 as well as *Proteus vulgaris* and *Pseudomonas aeruginosa*, its applications could be investigated in complex infections as combinatory therapy. This is an exciting aspect of phage therapy.

INTRODUCTION

Over the last years, despite the strict controls to prevent food born diseases; enterohaemorrhagic *Escherichia coli* (*E. coli*) *E. coli* (EHEC) serogroup O157 has emerged as a worldwide threat to public health following its first identification in an outbreak occurred by year 1982, when the illness was associated with the consumption of undercooked ground beef (Aslantas *et al.*, 2006; Dontorou *et al.*, 2003). According to the U.S. center for disease control and prevention (CDC), *E. coli* O157 is still an important cause of human illness in the United States and CDC reports multistate outbreaks of *E. coli* O157 regularly in which one of the last cases was in 2014 (Centers for Disease Control and Prevention, 2014). This food-borne pathogen is the reason of human illness varies from self-limited watery diarrhea and hemorrhagic colitis to critical manifestations such as the hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Boyce *et al.*, 1995). *E. coli* is usually found in intestinal tracts of human and animals. Cattle and sheep are known to be the most important primary reservoirs for this human pathogen. Direct contact with animals, contaminated drinking or swimming water, and person-to-person infections are important routes of transmission for sporadic human infections (Cornick and VuKhac, 2008; Gansheroff and O'Brien, 2000). CDC approximates that *E. coli* O157:H7 causes just about 73,400 illnesses and 60 deaths annually in the United States. Schroeder *et al.* (2002) pointed out that antibiotic resistant *E. coli* pose a growing threat. Phages are viruses that infect prokaryotes. Like all viruses, phages are obligate intracellular parasites, which have no intrinsic metabolism and need the metabolic machinery of the host cell to hold their reproduction. Bactericidal phages propose as a natural, nontoxic, feasible strategy for controlling several human pathogens.

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Phages control bacterial populations by infecting, killing and changing them by exerting evolutionary pressure on the host cell often specifically (Weber-Dabrowska *et al.*, 2000; Yoichi *et al.*, 2004). Although phages have originally been discovered since 1915, their potential application as an antibiotic alternative has been renowned only recently (Lorch, 1999).

Obviously, phages are an important part of the environmental ecosystem and are produced naturally at the universe (Karunasagar *et al.*, 2007; Lorch, 1999). Isolating *E. coli* O157 infective phages from natural resource and testing their use to control *E. coli* O157 in vitro, have been done by various research groups (Kudva *et al.*, 1999; O'Flynn *et al.*, 2004; Viazis *et al.*, 2011). The aim of our current study was isolation, identification and characterization of a lytic phage from environmental natural water recourse against *E. coli* O157. The isolated phage is broad-host range and can kill *Bacillus subtilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa*, as well. This lytic phage can potentially be used in controlling these four pathogens.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The enterohaemorrhagic *E. coli* O157 was used for the screening and assessment of phage titer. This strain was originally isolated in Iran and was obtained from reference laboratory of Bu Ali hospital. Also, bacterial species (Table 1) were used for determining the host range of phage. Bacterial cultures were grown in Luria-Bertani (LB) broth (1% peptone, 0.5% yeast extracts and 1% NaCl, pH 7.2). Agar plate (1.5% Agar) was used for growing *E. coli* O157. Cultivation was carried out by picking an isolated colony from streak LB agar plate, inoculating into LB broth allowing it to incubate at 37 °C, under aerobic conditions with agitation of 120 rpm.

Table 1: Host range of phage gBSN-MGB13.

Bacterial isolate	PTCC*	Plaque formation
E. coli O157	Clinically isolated	+
Bacillus subtilis	1023	+
Proteus vulgaris	1312	+
Pseudomonas aeruginosa	1074	+
Salmonella typhi	1609	–
Staphylococcus aureus	1112	–
Escherichia coli	1329	–
Staphylococcus epidermophilus	1114	–
Shigella dysenteriae	1188	–
Vibrio cholera	1611	–
Shigella flexenari	1234	–
Streptococcus pyogenes	1447	–
Bacillus stearothermophilus	1353	–

*PTCC: Persian Type Culture Collection

Phage isolation, propagation, and titration

To isolate *E. coli* O157 specific phages, 32 water samples were collected from natural water resources of Iran in 2011 to 2012 (Table 2). Samples were collected in sterile 15 mL tubes and shipped to the laboratory in 24 h. Subsequently, the samples were centrifuged at 6000 rpm for 20 min, and the supernatants were filtrated through a 0.22 µm pore-size filter. The filtrate was incubated at 37°C with an exponential growth-phase culture of *E. coli* O157 (~10^8 CFU/mL).

Table 2: List of sampling locations of natural water samples were collected for phage enrichment and isolation.

Sample No.	Site- City	Lysis on *E. coli* O157
1	Talar river- Bahmanmir	-
2	Shazdeh river- Babolsar	-
3	Babol river- Babolsar	-
4	Fereidonkenar river- Fereidonkenar	-
5	Sorkh river- Sorkhroud	-
6	Vaghfi river- Mahmoudabad	-
7	Kheshtsar river- Kheshsar	-
8	Nour river- Nour	-
9	Royan river- Royan	-
10	Keshmeh- Nour	-
11	Vazivar- North of Iran	-
12	Kheir river- Nowshahr	+
13	Nowshahr river- Nowshahr	+
14	Chalous river- Chalous	-
15	Haj river- Hajroud	-
16	Beach- Sari	-
17	Beach- Kish Island	-
18	Beach- Persian Gulf	-
19	Kalarabad river- Kelarabad	-
20	Salmanshahr river- Salmanshahr	-
21	Abbasabad river- Abbasabad	-
22	Nashtarour river- Nashtaroud	-
23	Shiroud river- Shiroud	-
24	Chakroud river- Chakroud	-
25	Ramsar river- Ramsar	-
26	Kileh river- Tonekabon	-
27	Haraz river- Amol	-
28	Tajan river- Sari	-
29	Siahroud river- Ghaemshahr	-
30	Neka river- Neka	-
31	Behshahr river- Behshahr	-
32	A well- Ghaemshahr	-

After overnight shaking (120 rpm), debris and bacteria were removed by centrifugation at 6000 rpm for 20 min. Following this enrichment procedure, the supernatants were filtered through 0.22 µm pore-size filter and analyzed by plaque assay on *E. coli* O157 using double agar layered method of Adams (Van Helvoort, 1992). The single plaques formed on the plates were stabbed with a needle and eluted with a small volume of phage buffer. Each phage suspension was serially propagated twice on the same strain. The most efficient phage with lytic (clear) plaques was selected for further study in our work and designated gBSN-MGB13. The corresponding location for selected phage was a river in Nowshahr city of Iran. Isolated phage was incubated with an exponential growth-phase culture of *E. coli* O157 for 20 min without agitating, and then with shaking (120rpm) until visible lysis was begun. The phage titer of 10^{12} PFU/mL was obtained from this step. The lysate was centrifuged at 6000 rpm for 20 min. NaCl and PEG8000 were added to the supernatant to reach the final concentration of 0.5 M and 20% (v/v), respectively and phage particles were precipitated at 4°C overnight. The phage pellet was dissolved in SM buffer (0.05% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 0.005 M Tris-HCl, pH 7.5) (Van Helvoort, 1992). In addition, phage activity in the supernatant was

evaluated by a spot assay that entailed placing 5 µl of the supernatant on LB agar seeded with *E. coli* O157. The plates were checked for plaque formation after overnight incubation at 37°C (Sheng *et al.*, 2006).

Determination of the host range of phage gBSN-MGB13

Isolated phage was tested for its ability to form plaque on a range of gram-positive and gram-negative bacterial species (Table 1). The sensitivity of each bacterial species to *E. coli* O157-infective phage was determined using the spot assay, as described above. The presence of a lytic zone was considered evidence of phage susceptibility, no cell lysis was considered a negative response indicating bacterial resistance to phage (Sheng *et al.*, 2006).

Determination of phage optimal MOI

The optimal multiplicity of infection (MOI) for *E. coli* O157 was investigated by mixing and incubating bacteria and lytic phage at ratios ranging from 10^{-1} to 10^{5} PFU/CFU in tubes containing 10^{3} CFU/mL of *E. coli* O157. Mixtures were incubated for 20 min at 37 °C. Phage-free cultures (containing only bacteria) and cell-free cultures (containing the only phage) were used as controls. Subsequently, the number of viable cells in mixtures was determined by plating on LB agar and incubating at 37 °C overnight.

Killing efficiency of phage gBSN-MGB13

Bacterial cells with 10^{3} CFU were added with an equal volume of phage lysate (10^{3} PFU/mL) at a multiciplity of infection (MOI) of 1. Phage-free *E. coli* culture was used as control sample in experiments. The mixtures were incubated at 37 °C for 9 h. The optical density of test and control samples was recorded at 600 nm and was set zero at the time of infection. Spectrophotometric monitoring of bacterial growth in phage-free sample and the infected sample was carried out through viral lysis every 60 min.

Electron microscopy

Pure, high-titer phage stocks were used. The sample was deposited on copper grids and allowed to adsorb for about one minute. Uranyl acetate (2%) was used to stain the phage negatively for one minute. After the excess staining solution had been drained with filter paper, the grids were examined with an electron microscope (Philips, UK).

Genome and proteome analysis of gBSN-MGB13

Phage genome was extracted and purified from phage lysate using the phenol-chloroform method. The type of phage genome was examined by treatment with DNase I and RNase A. for restriction analysis, two enzymes were used (*Eco*RI and *Hin*dIII) which are added to phage nucleic acid using methods as recommended by the supplier. Treated nucleic acids were analyzed using 1% agarose gel. The phage proteins were analyzed using SDS-PAGE according to the Laemmli method (Laemmli, 1970). Purified phage solution was precipitated with four volumes of ice-cold acetone. After centrifugation (16,000×g 20 min, 4 °C), the pellet was air-dried and re-suspended in the buffer. Briefly, 50 µL of sample was added to 10 µL of loading buffer and boiled for 10 min. The sample was then loaded on 12.5% SDS-PAGE slab gel and electrophoresed with Tris-glycine buffer.

RESULTS AND DISCUSSION

Isolation and host range determination of phage gBSN-MGB13

Screening tests for detection of lytic phages were performed with *E. coli* O157. Two samples from 32 collected water resources were active against the bacteria in the plaque assay. Each sample contained several phages. Through picking, re-enriching and examining of clear plaques by plaque and drop assay, we found a lytic phage with remarkable lytic activity. Among the phage isolates, gBSN-MGB13 was more effective than the others at lysing *E. coli* O157. gBSN-MGB13 formed large clear plaques on the bacterial lawn (Fig. 1). To determine phage host range, gBSN-MGB13 was spot tested against 13 bacterial samples including gram positive and gram negative bacteria (Table 1). The results indicated that isolated phage has a lytic effect on 3 of 13 representative bacterial species as well as *E. coli* O157. gBSN-MGB13 produced plaques on *Bacillus subtilis* (*B. subtilis*), *Proteus vulgaris* (*P. vulgaris*) and *Pseudomonas aeroginosa* (*P. aeroginosa*). Therefore, it is concluded that this coliphage is polyvalent and not genus specific.

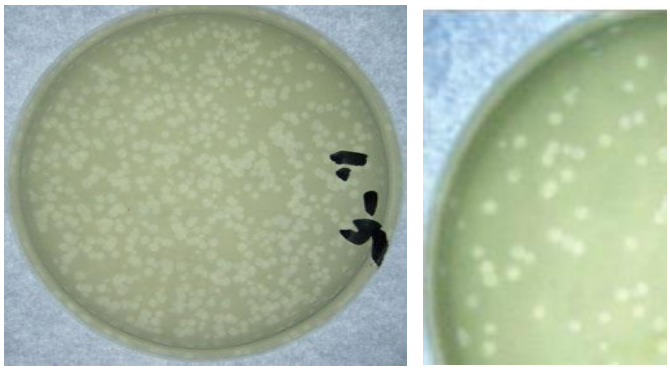

Fig 1: The plaque of gBSN-MGB13 using the double agar layered method on *E. coli* O157 lawn.

Plaque size and morphology of phage gBSN-MGB13

E. coli O157 lytic phage grew into clear plaques at 37 °C, with a plaque size of 3 mm in diameter (Fig. 1). An electron micrograph of isolated phage showed that it has a rather long tail (not long as siphoviridae and not short as podoviridae) and an isometric head (Fig. 2). gBSN-MGB13 morphologically belongs to the myoviridae family. Its length is approximately 130 nm, and the head is about 50 nm in diameter. The plaque size of gBSN-MGB13 (3 mm) is relatively large comparing to previously isolated phages against *E. coli* O157 such as KH1 (<1 mm) and SH1 (2 mm) (Sheng *et al.*, 2006). There is no study investigating the relation between phage plaque size and its efficacy.

Consequently it cannot be concluded that gBSN-MGB13 lyric effect against bacteria is superior to other isolated phages with the smaller size.

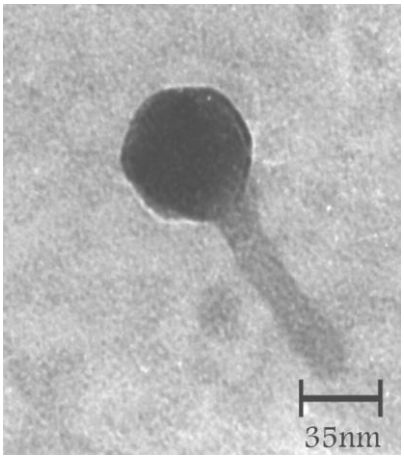

Fig 2: Electron micrographs of gBSN-MGB13 negatively stained with 2% uranyl acetate.

Phage killing efficacy analysis

To further characterize the isolated phage, different MOIs for the elimination of *E. coli* O157 were investigated. At MOIs ranging from 10^{-1} to 10^{5} PFU/CFU, a reduction in bacterial titer was detected. At MOI of 10^{5} PFU/CFU, 50% bacterial growth inhibition relative to control samples without phage was observed in 20 minutes after infection. Phage gBSN-MGB13 showed a strong activity against the bacterium. The time of action was really short and remarkable. According to other studies, in *E. coli* O157 culture-positive samples, phage treatments reduced the number of bacteria compared to untreated controls but did not eliminate the bacteria completely (O'Flynn *et al.*, 2004; Tanji *et al.*, 2005).

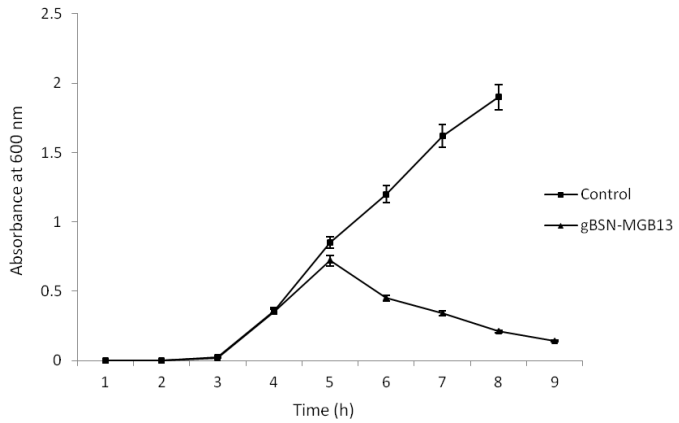

Fig 3: Killing efficiency of phage gBSN-MGB13. *E. coli* O157 cells were infected at the early log phase with phage lysate. Killing efficiency of phage-infected culture (♦) and control culture (■) were monitored spectrophotometrically.

Moreover, to determine the efficacy of phage on *E. coli*, the survival of bacteria was measured spectrophotometrically at MOI of 1 PFU/CFU (Fig. 3). Following four hours of the infection of bacterial cells with lytic phage, OD measurements of the control and test samples begin to diverge dramatically. At the 5th hour, the curve of test sample dropped quickly indicating fast dying of bacteria regarding phage-free bacterial growth curve which showed a rising pattern. Based on OD-time curve in Fig. 3, the OD values of infected culture declined rapidly but did not reach to zero after nine hours of infection.

It is assumed that after infection, phage-bacteria culture reaches to a balanced state in which phages are not able to completely eliminate infected bacteria possibly due to mutations. Therefore, the initial rate of action decreases gradually hours after infection.

Genome and proteome analysis of gBSN-MGB13

The nucleic acid of the phage was extracted and subjected to enzymatic digestion analysis. The genomic nucleic acid of the phage was found to be a double stranded DNA following successful digestion with DNase I but not with RNase A. Treatment of the genome of gBSN-MGB13 with restriction enzymes *Eco*RI and *Hin*dIII were shown no bands on agarose gel electrophoresis (data not shown). This may probably due to the phage genome have no restriction sites for these enzymes, or the restriction sites have changed (by chemical modification) that *Eco*RI and *Hin*dIII cannot restrict the phage genome.

According to SDS-PAGE result, the protein composition of gBSN-MGB13 includes at least 8 distinct bands. The most predominant band could be accounted for major capsid protein which has a size of approximately 45 kDa (Fig. 4).

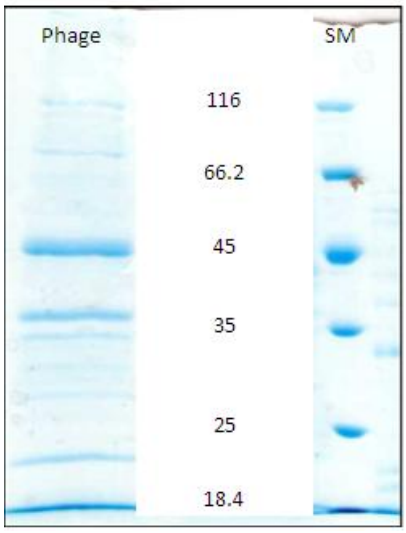

Fig 4: SDS-polyacrylamide gel electrophoresis analysis of phage gBSN-MGB13. "Phage" is the phage proteins and "SM" is the protein size marker.

CONCLUSION

Treatment of O157 infection with antibiotics due to emerging resistant bacteria and increased expression of Shiga toxin genes is contraindicated (Igarashi *et al.*, 1999; Proulx *et al.*, 1992; Yoh and Honda, 1997). The risk of releasing Shiga toxin, thereby developing HUS (hemolytic uremic syndrome) is an

undesirable side effect of antibiotics. Therefore, considering the above drawbacks and the relatively easy access to the natural water resources, phage therapy might be considered as an approach to apply in prevention and treatment of infectious diseases (Nakai and Park, 2002). Lytic phage therapy is one of the therapeutic alternatives for the control of *E. coli* O157 (Stevens *et al.*, 2002).

Some studies have been carried out to assess the abilities of the phage to fight *E. coli* O157 (Bach *et al.*, 2003; Kudva *et al.*, 1999; Tanji *et al.*, 2005).

In this regard, gBSN-MGB13 was isolated from water resources against *E. coli* O157 and was screened by spot testing against 13 different strains. Three other strains were sensitive to phage and gBSN-MGB13 formed clear plaques and was capable of lysing them (Table 1). Therefore, this potent lytic phage is not specific to *E. coli* O157. Since most bacterial infections do not have a clinical presentation that is specific enough to differentiate them from other acute bacterial infections and because broad-host range phages like gBSN-MGB13 are effective against several pathogenic species (in this case, *E. coli* O157 as well as *Proteus vulgaris* and *Pseudomonas aeruginosa*), their applications could be investigated in complex infections as combinatory therapy. This is an exciting aspect of phage therapy, particularly regarding that no adverse events related to phage application were reported following *E. coli* phage administration even by healthy adult volunteers received phage in their drinking water in other studies (Bruttin and Brussow, 2005).

There are some efforts to modify phages to more efficient forms and to address probable ecosystem considerations (Moradpour and Ghasemian, 2011; Moradpour *et al.*, 2009). To improve the features of current phages and to modify them to meet the medical and regulatory requirements, it is essential to isolate new phages and explore detailed features of these isolates. This will motivate phage science toward developing new reliable biopharmaceuticals.

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