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Characterization and development of a phage cocktail for *Escherichia coli* causing gastrointestinal diseases

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Abstract

Escherichia coli (*E. coli*) is one of the most important causes of gastrointestinal diseases in developing countries. It considered the third cause of diarrhea in children. In this study, bacteriophages were isolated and detected from wastewater against isolates of *E. coli* by using plaque and spot test assay. Characterized three phages were differed on plaque morphology, particle morphology, host range and titration. According to phage characterization, the three phages were classified into two from Siphoviridae and one from Podoviridae. Combinations of the bacteriophages had broad host range with strong lytic activity against pathogenic *E. coli*. Phage cocktail revealed high stability at 45°C, pH 9 and 20% of salt concentration. According to the previous characters of a phage cocktail properties can broaden the spectrum of application of phage therapy. Formulated phages were prepared by mixing phages with royal gelly, dried skimmed milk and a mixture of sucrose and corn flour at ratio 1:1(V: W) at phage cocktail concentration $2.2x10^{11}$ plaque forming units (pfu) per mL. These phages formulation remained active for at least 48 days when stored at 4°C in a light protected container with titres of $4.0x10^{10}$, $3.6x10^{10}$ and $3.4x10^{10}$ pfu/mL for royal gelly, dried skimmed milk and a mixture of corn flour and sucrose respectively.

Key words: bacteriophage, *E. coli*, Siphoviridae, Podoviridae, phage cocktail, formulated phage

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1. Introduction

Phage therapy is the therapeutic use of bacteriophage to treat bacterial infections. The history of using of bacteriophage in therapy for human bacterial infections backs to 1919 when Felix d'Herelle the co-discoverer of the bacteriophage suggested the use of bacteriophage for the treatment of bacterial-induced diarrhea [1]. Phage-based therapies were sold by American pharmaceutical companies in the 1930s and also used by soldiers in the Second World War to fight off dysentery [1]. The practical application of antibiotics for the treatment of bacterial infections thwarted the use of phage therapy in the West [2]. In the Soviet Union phage therapy continues to be a common method of treatment where a number of companies, namely Microgen Inc., sell phage cocktails due to a shortage of antibiotics [3, 4]. Recently, the increasing rate of multi-drug-resistant bacteria has induced scientists to return phage therapy for bacterial infections [2]. Phages can be used for treatment of drug-resistant pathogens as monotherapy or in combination with other antibiotics, although it is unlikely that antibiotics will be replaced by phages in near future [5, 6]. The efficacy of phage therapy essentially depends on the phage concentration (i.e. the dose), timing, formulation and administration, with pharmacokinetics and pharmacodynamics characterized for each phage or phage cocktail [7, 8].

Lytic bacteriophages are considered better candidates for therapeutic application than their lysogenic counterparts, because they kill their hosts with high specificity and limited collateral damage to the natural microbiome [8]. There are several research trials for assessing the safety, effectiveness and pharmacodynamics of therapeutic phage cocktails for burn wounds infected by *Escherichia coli* [8].

E. coli is a member of the Enterobacteriaceae family, gram negative, rod-shaped, non-spore forming and facultative anaerobic. Some strains are pathogenic and cause gastrointestinal and urinary tract diseases [9]. *E. coli* strains

causing diarrheal disease are categorized into groups based on their virulence properties, mechanism of pathogenicity, clinical syndrome, and antigenic characterization [10].

The aim of this study was to isolate and identify a collection of lytic bacteriophages capable of infecting *E. coli* causing gastrointestinal diseases, forming a cocktail from three phages and formulate them with three different materials for potential application in treatment of *E. coli* infections.

2. Material and methods

2.1. Bacteria and culture media

Escherichia coli isolates were obtained from Microbiology Laboratory, Nassser Institute Hospital and Benha children's Hospital (Table 3) which previously isolated from human clinical samples, while *E. coli* Ww and *E. coli* Hw isolated from Benha University Hospital wastewater and confirmed morphologically and biochemically. Also, *Salmonella* isolates were obtained from Microbiology Laboratory of Benha University Hospital. All bacterial stock cultures obtained were stored at -80°C in Luria-Bertan (LB) broth containing 50% (v/v) glycerol. All frozen stock cultures were activated on LB agar plates before trail. Colony of *E. coli* was isolated from LB plate and inoculating in to LB broth at a 37°C until the OD_{600} reached 0.8. Luria-Bertani (LB) medium consisted of tryptone (10g), yeast extract (5g) and sodium chloride (10g) per 1000 ml distilled water (pH 7) [11]. For phage plaque formation, LB based solid medium containing 1.5 and 0.7% agar was used for the lower and upper layer, respectively [12].

2.2. Isolation of bacteriophages

Coliphages were isolated from wastewater (collected from the Benha University Hospital). Phages were detected in wastewater by spot test according to Adams [13] using *E. coli* as a host. The obtained bacteriophage plaques were purified by repeated single plaque isolation to ensure that each contained only one type of bacteriophage. To produce bacteriophage stocks in sufficient quantities for the experiment, the protocol used for enrichment of phages including overnight growth of phages with their host bacteria then centrifugation and purification with 0.45µm sterile membrane filter. This method was used for all three bacteriophages.

2.3. Morphology of bacteriophages

The morphology of each of the three bacteriophages was examined by transmission electron microscopy. Suspensions of concentrated phages were spotted onto a carbon-coated copper grid and stained with 2% phosphotungstic acid (pH 7.0). The grid was dried for 5 min and then observed at 80 kV using transmission electron microscope. The bacteriophages were classified according to Ackermann [14].

2.4. Phages and phage cocktail host range determination

Aliquots of phage cocktail were prepared using equal volumes of a 1:1:1 mixture of three single lytic phages. The three single phages and phage cocktail were tested against a panel of clinical isolates (*E. coli* and *Salmonella*) using spot-test procedure [15]. Briefly, 3 ml top-0.7% LB agar was added to 200 µl overnight culture of each clinical isolate and poured over LB Agar. The agar was allowed to solidify, after which 5 µl of each bacteriophage and phage cocktail suspensions were spotted on the bacterial lawn of each different isolate. The drop was allowed to dry, and the plates were incubated over night at 37°C. Based on the degree of lysis and clarity on the bacterial lawn, the spots were differentiated into four categories: strong, moderate, weak, or no lysis.

2.5. Physical properties of phage cocktail

The effects of temperature, pH, salinity and dilution on the lytic activity of a phage cocktail (2.2×10^{11} pfu/mL) against *E. coli* were checked. For evaluation of phage cocktail stability under various temperature conditions, phage cocktail suspensions were prepared in 1.5 ml Eppendorf tubes then, tubes were incubated at 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70°C for 1 h. For their susceptibility to different pH conditions, the phage cocktail suspensions were incubated at 37°C for 24 h at different pH degrees which were obtained using HCl and NaOH in a range of 3 to 10. Phage cocktail susceptibility to three sodium chloride concentrations (10, 15 and 20%) was also determined. For evaluation of phage cocktail activity under different dilutions, the phage cocktail suspensions were diluted in LB broth through various serial dilutions from10^{-1} to10^{-12} and finally the lytic activity of a phage cocktail was checked against *E. coli* using spot test.

2.6. Formulation of phage cocktail

The phage cocktail was mixed with three different carriers to form three formulas. The first was by using royal gelly and phage cocktail with ratio 1:1(w/v). The second by using dried skimmed milk powder and phage cocktail with ratio 1:1(w/v). The third by using mixture of corn flour and sucrose and phage cocktail with ratio 1:1:1(w/w/v). Every 1ml of phage cocktail (2.2×10^{11}pfu/mL) was added to 1g of its carrier. Phage cocktail formulas were stored at 4°C in a light protected container. The three formulas were checked for phage cocktail viability twice weekly up to 48 days using double agar layer (DAL) method.

3. Results

3.1. Isolation of bacteriophages

Three lytic bacteriophages, designated as øECP2, øECP5 and øECP7 were isolated from Benha Hospital wastewater. These phages formed clear lytic plaques with different shapes of approximately 1.0 to 3.0 mm in diameter on *E. coli* lawns (Table 1). Plaques produced by the phage (øECP5) were surrounded by growing opaque halo zones. Phage øECP2 produced smaller plaques of 1 mm in diameter while phage øECP7 produced plaques of about 2 mm in diameter (Fig. 1).

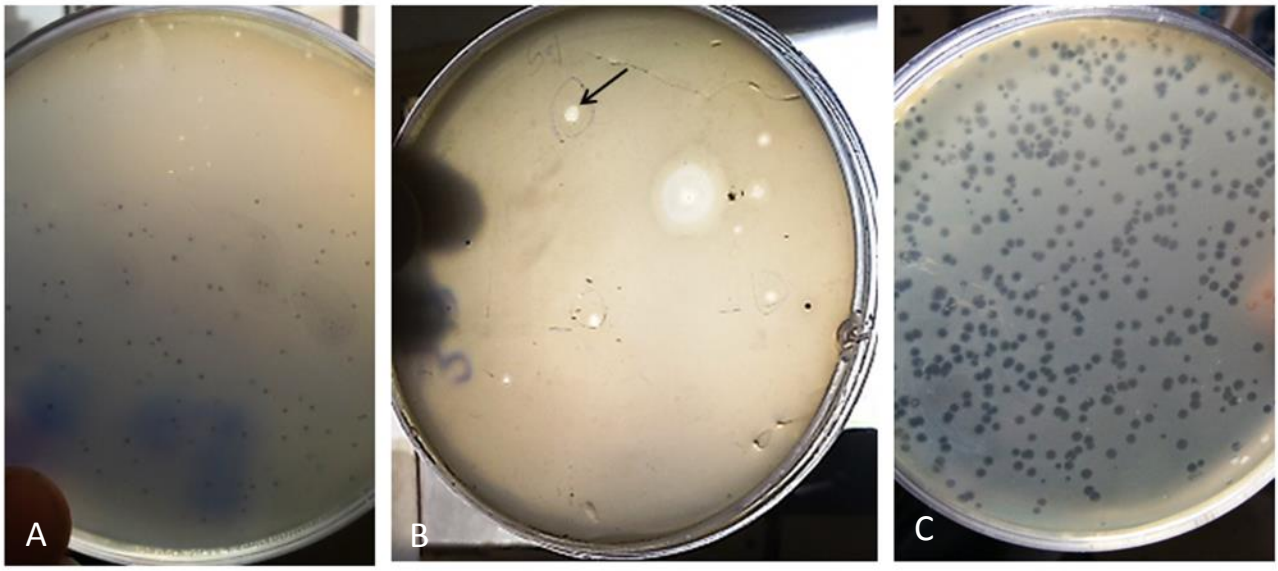

Fig (1): Plaques formed by phages on their host isolates of *E. coli* after an overnight incubation at 37°C A: øECP2, B: øECP5 and C: øECP7.

Table1. Plaques morphology of three coliphages.

Phage isolate	Plaques morphology		
	Diameter (mm)	Shape	Appearance
øECP2	1.0	Circular, regular	Clear, without center and halo
øECP5	3 .0	Circular, irregular	Clear, with center and halo
øECP7	2 .0	Circular, regular	Clear, without center and halo

3.2. Morphological characteristics

The morphological characters and dimensions of the three bacteriophages are presented in Fig. (2) and Table 2. The bacteriophages (øECP2 and øECP5) appeared to be composed of long non-contractile tail with an isometric head. These features allowed us to classify them as members of the family Siphoviridae. The bacteriophage (øECP7) showed characteristic features of the family Podoviridae.

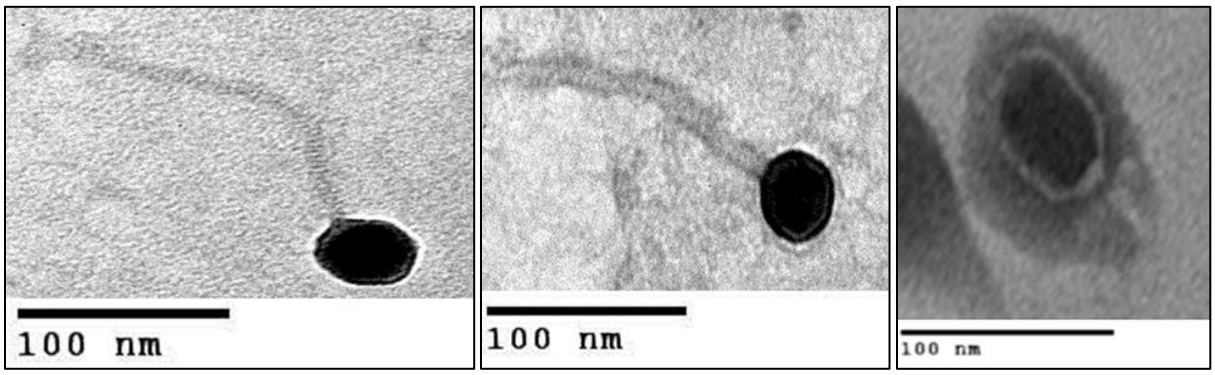

Fig (2): Electron Micrographs of three isolated phages øECP2, øECP5 and øECP7, respectively from left to right. The size bar corresponds to 100 nm.

Table 2. Morphological properties and prospected families of three coliphages.

Phage	Head diameter (nm) and shape	Tail length (nm) and character	Phage titre (pfu/mL)	Prospected family
øECP2	47 Isometric	174 Non-contractile	$3x10^{10}$	Siphoviridae
øECP5	48 Isometric	160 Non-contractile	$3.5x10^{6}$	Siphoviridae
øECP7	68 x 45.5 Elongated icosahedral	Short non-contractile	$2.2x10^{7}$	Podoviridae

3.3. Phages and phage cocktail host range

To gain insight into the host range of the selected coliphages and a phage cocktail; the susceptibilities of the eight isolates of *E. coli* and four *Salmonella* isolates were tested using spot test as showed in Table 3. There was a degree of variability between the selected single phages and a phage cocktail for their lytic potential on pathogenic *E. coli*. Phage øECP2 produced a weak lytic activity against *E. coli* isolates and there was no lysis against *Salmonella* isolates. While øECP5 showed a strong lytic activity against *E. coli* U isolate only. All tested *E. coli* isolates were susceptible to a phages cocktail with a strong lysis except *E. coli* S2 isolate revealed a weak lysis. In contrast, *Salmonella* S.1 isolate gave weak lysis with a phage cocktail.

Table 3. Host range analysis of the single phages and a phage cocktail.

Isolates		Source	Spot test*			
			Phage cocktail	øECP2	øECP5	øECP7
E. coli isolates	*E. coli* S1	Benha children's Hospital	+++	+	++	+
	E. coli Af	Nasser Institute Hospital	+++	+	++	++
	E. coli W	Nasser Institute Hospital	+++	+	++	+
	E. coli A	Nasser Institute Hospital	+++	+	+	+
	E. coli U	Nasser Institute Hospital	+++	+	+++	++
	E. coli S2	Benha children's Hospital	+	-	-	+
	E. coli Ww	This study	+++	+	+	-
	E. coli Hw	This study	+++	+	+	+
Salmonella isolates	S.1	Benha University Hospital	+	-	+	-
	S.2	Benha University Hospital	-	-	-	-
	S.3	Benha University Hospital	-	-	-	-
	S.4	Benha University Hospital	-	-	-	-

*Based on the degree of lysis on the bacterial lawn: strong lysis (+++), moderate lysis (++), weak lysis (+) and no lysis (-)

3.4. Physical properties of a phage cocktail

The result showed that a phage cocktail could lyse bacterial host at temperature from 25 to 45°C, while it failed at 50 to70°C. A phage cocktail was able to lyse its host at pH ranging from 4 to 9. It also was able to lyse its host at all salt concentrations used as shown in Table 4; a phage cocktail had no lytic activity at higher dilutions ($10^{-11}$and 10^{-12}).

Table 4. Effect of pH, temperature, salinity and dilution on a phage cocktail lytic activity.

Factor	Condition	Result
Temperature °C	25	+ve
	30	+ve
	35	+ve
	40	+ve
	45	+ve
	50	-ve
	55	-ve
	60	-ve
	65	-ve
	70	-ve
pH	3	-ve
	4	+ve
	5	+ve
	6	+ve
	7	+ve
	8	+ve
	9	+ve
	10	-ve
Salt concentration (%)	10	+ve
	15	+ve
	20	+ve
Dilution	10^{-1}	+ve
	10^{-2}	+ve
	10^{-3}	+ve
	10^{-4}	+ve
	10^{-5}	+ve
	10^{-6}	+ve
	10^{-7}	+ve
	10^{-8}	+ve
	10^{-9}	+ve
	10^{-10}	+ve
	10^{-11}	-ve
	10^{-12}	-ve

+ve represents lysis and –ve represents no lysis

3.5. Formulated phage cocktail stability

A phage cocktail stability for the three formulas was determined by plaque assay; a phage cocktail titre dropped from $2.2x10^{11}$ pfu/mL at day 1 to $4.0\ x10^{10}$ pfu/mL in royal gelly, $3.6\ x10^{10}$ pfu/mL in dried skimmed milk and $3.4x10^{10}$ pfu/mL in corn flour and sucrose mixture after 48 days of storage. Results indicated that royal gelly was the best phage cocktail carrier (Fig. 3).

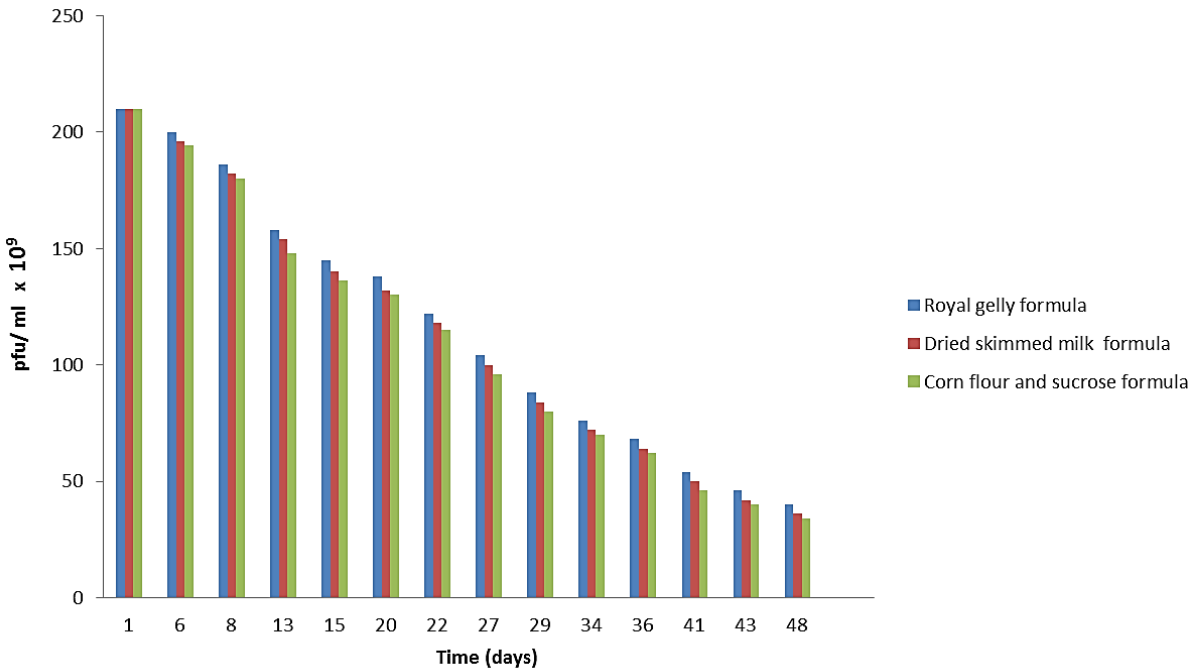

Fig (3): Histogram showing storage stability of phage cocktail formulas.

4. Discussion

Preparations of bacteriophage for therapeutic purposes require careful design through a multistep research process of bacteriophage characterization, purification and cocktailing. This process includes *in vitro* studies, such as those presented here and *in vivo* studies like studies by Mendes et al. [16]. Ideally, the characterization of bacteriophages for therapeutic purpose should be as thorough and complete as possible. However, in certain cases, it may be more practical to minimize this process and to focus the characterization on particular traits that are the most desirable for a specific application. Combining different bacteriophages in the same preparation (mixtures of two or more bacteriophages within a given formulation) results in a broader spectrum of antibacterial activity and lytic efficacy may allow targeting of bacteria under different conditions or in different environments [17].

The information from the morphological analyses revealed that the bacteriophages used here were two belonging to the family Siphoviridae and one belonging to the family Podoviridae. Host range is an important criterion for selection of bacteriophages for therapeutic use; host range should be as broad as possible and including clinically prevalent bacterial species [18]. In this study, the phage cocktail exhibited a broad spectrum with strong lytic activity against *E. coli* isolates and narrower host range against *Salmonella* isolates [19]. The overall morphology characterization and host range results suggested that these bacteriophages are very good candidates for therapeutic use. However, care must be taken for generalizing these results. Whilst, the use of high bacteriophage titres (2.2 $x10^{11}$ pfu/mL per spot) for host-range analysis is routine when considering bacteriophages for therapeutic use [20].

Temperature is one of the most important environmental factors that strongly affect many aspects of the biological systems. Influence of temperature upon the biological system is very vivid and it has been observed that evolution of phenotypic traits, species distributions, and extinctions in many cases can be traced to changes in temperature regimes [21]. Present study results are in confirmation with the above findings as during the experiment it was observed that yield of phage cocktail was highly temperature dependent. A phage cocktail was unable to develop and perform lysis on *E. coli* at temperature above 45°C, while on temperature between 25°C and 40°C, the activity was carried out, this study showed that at thermophilic temperature 45°C, the phage cocktail developed and performed lysis on its host bacteria and support the results of Pollard and Woodyatt [22] who reported that bacteriophage developed at 41.2°C. While, temperature regimes from 50°C, to 70°C proved as limiting factor and caused the actual inactivation of the bacteriophages. Study results regarding the inactivation are in confirmation with those observed by Basdew and Laing [23] who reported that increase in temperature decreases virus survival and activity. In the same way, findings by Pope et al. [24] that indicate an increase in bacteriophage yield till 30°C and 39°C corroborates the present study results which revealed that 37°C was ideal temperature for bacteriolytic activity of the phage cocktail against *E. coli.*

A phage cocktail is stable at pHs between 4 to 9 and pH finding of the study confirmed Langlet et al. [25] results

which indicated that virus exhibited stability at wide range of pH regimes.

A phage cocktail was able to lyse *E. coli* isolates after incubation in a high saline environment. The cocktail is a mixture of different three phages belonging to two different families and different characters, so the phage cocktail has the ability to adapt with conditions more hard than individual phages. Many phages were able to adapt with environments with high salinity. Several bacteriophages were isolated from marine water of different salinities. Wichels et al. [26] studied 22 phages which they found in water near Helgoland in the North Sea, all of them had tails and icosahedral heads of 50.2 to 99.3 nm, and they were classified into three different families: 11 phages to Myoviridae, 7 to Siphoviridae, and 4 to Podoviridae. No similarity in DNA structure was shown among phages belonging to different families present in this area. There are many conditions which may overlap with the phage and reduce the net concentration of the phage environment West and Kelly [27] reported that mixing of 0.1ml of 10% NaCl with an equal amount of the broth culture of the propagating strain of *Staphylococcus*, followed by immediate addition of 3 ml soft agar, quickly diluted the concentration of NaCl to about 0.8 %. The quantity of NaCl was further decreased on plating the soft agar; they had shown that the free phages differ in their NaCl tolerance. Their results discussed the positive results in high salt concentrations.

The mechanisms of bacteriophage lysis have been studied for the bacteriophage control of bacterial virulence in animals and human [28]. It has been found that lysis of the bacterial host is the final event in the infection cycle of a lytic bacteriophage [29]. The phage cocktail lysed from 10^{-1} to 10^{-10} dilutions, but at the highest dilutions 10^{-11} and10^{-12} a phage cocktail had no lysis activity. It means that at highest dilutions, coliphages failed to lyse. Lysis can be produced by phage dilution up to a certain point; as Worley-Morse et al. [30] reported that bacteriophage concentration is very important for lysis activity.

The three formulas were tested over about seven weeks. The results were expected because the capsulation material protects phages from environmental conditions. The royal gelly formula was the best used formula, it gave more protection to a phage cocktail, and however the crude royal gelly was not able to lyse bacteria. Studies on phage encapsulation have used a variety of hydrophilic and hydrophobic polymers including agarose [31], alginate [32, 33], chitosan [34, 35], and pectin [36]. Developing formulations that incorporate bacteriophage for therapeutic applications requires an appreciation of the chemical and physical stresses phage may encounter both during processing as well as during storage once formulated. Phage inactivation and long-term reduction in phage titre upon storage is highly undesirable. A polymer or lipid may be used to coat an existing structure containing the phage. Murthy and Engelhardt [37] sprayed phage on dried skimmed milk and then encapsulated them in a lipid coating and there are many techniques and processes that may be used for stabilizing, immobilizing and encapsulating phage [38]. In the current study, development of a phage cocktail was proposed based on many criteria: strong lytic activity; broad host range; and high stability. Also, formulated phage cocktail remained active for at least 48 days when stored at suitable conditions.

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