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Abstract

A number of 8 phages lytic of *V. cholerae* O1- biotype-El-Tor, serotype Inaba were selected for the formulation of a phage cocktail. A phage cocktail composed of 8 phages was prepared and showed 100% inhibition of *V. cholerae* isolates comparing with a percentage ranged from 0-75% for every single phage in growth inhibition assay. The potential activity of phage cocktail of prophylactic therapy for infant rabbits model of cholera was evaluated through phage retention time and length of phage prophylaxis studies. Results have been showed that phage cocktail was potent in the prevention of development of cholera in infant rabbits dosed with 5×10^9 PFU of phage cocktail in 3, 12, and 24 hours prior to infection with *V. cholerae*. The infant rabbits administered with the prophylactic dose of the phage cocktail showed no signs of profuse diarrhea and lost < 10% of body weight after 20 hours of infection with *V. cholerae* comparing with the infant rabbits non-treated with prophylactic phage dose which lost 10-15% of the bodyweight within 10-14 hours after infection with *V. cholerae*. It is concluded that the results of the animal models confirmed the ability of phage therapy for cases of cholera and it is a highly promising alternative antimicrobial therapy for such cases.

Keywords

Vibrio cholerae O1, Phage therapy, phage cocktail, infant rabbits

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Cover Page Footnote

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1. Introduction

Cholera is an acute watery diarrhea that results from infection of small intestine with *Vibrio cholerae* that produce cholera toxin (CT) and resulted in profuse watery diarrhea, loss of electrolytes and rapid dehydration. This could result in hypovolemic shock and metabolic acidosis [1]. Mortality rate of cholera could be as high as 70% in the cases of late rehydration of the patients. It is estimated that global burden of cholera is 1.4–4.3 million cases with deaths ranging from 21,000 to 143,000 yearly [2]. 34 countries documented 1,227,391 cases with 5654 deaths only in 2017 [3].

Several antibiotics have been used as medication of cholera such as tetracycline, azithromycin and fluoroquinolones [4]. However, in the last decade, a failure in treatment of cholera cases was repeatedly documented due to emergence and spread of drug-resistant *Vibrio cholerae* [5]. The phenomenon of multi-drug resistance (MDR) and extensive drug resistance in *V. cholerae* is attributed to many genetic mechanisms such as plasmids, transposons and insertion sequences (IS) [6,7].

Phage therapy is the most useful alternative therapy for treatment of drug-resistant *Vibrio cholerae*. Recently, many studies have focused on phage therapy for treatment of cholera in the animal models [8–11].

The targeted prophylactic therapy for cholera is recommended for closed institutional settings like prisons and orphanages, where exposure to certain pathogen is likely to occur over short period of time. This targeted prophylactic therapy must be given to all people, I mean patients, at the same time immediately after the first case of infection is recognized [12]. The prophylactic therapy of phages may be superior over the use of antibiotics as it results in no side effects and unrelated to development of drug-resistance on targeted bacteria as antibiotics do [13].

This study aims to formulate a phage cocktail active in treatment of cholera via selection of potent lytic phages for isolation *Vibrio cholerae* O1, and to evaluate their *in vitro* and *in vivo* activity against isolates of *V. cholerae*.

2. Materials and methods

2.1. Bacteria

A number of 26 isolates of *V. cholera* O1- biotype- EI- Tor, serotype Inaba were supplied from central

general public health laboratory in Baghdad. Identification, serotyping and biotyping of *V. cholerae* isolates were carried out by using cultural, biochemical, serological and molecular methods in the central public health laboratory in Baghdad. The culture media for isolation and selection of *V. cholerae* included the TCBS agar (Himedia, India), alkaline peptone water (Himedia, India) and MacConkey agar (Himedia, India). The serological tests included rapid immunochromatographic detection (IndianMart, India), antigen rapid test cassette (Novatest, Italy) and Combo rapid test for *V. cholerae* O1/O139 antigen (Liming Bio, China). The molecular diagnosis was by real time PCR by the *V. cholerae* TaqMan probe/primer and control set (Norgen Biotek, Canada).

2.2. Phages

A number of 8 virulent lytic phages against *V. cholerae* O1- biotype- EI-Tor, serotype Inaba isolated from sewage water were selected for preparation of phage cocktail and evaluation of their activity in prophylactic therapy of infant rabbits model of cholera. Those phages were PCV6, PCV8, PCV9, PCV15, PCV19, PCV20, PCV23 and PCV25 (unpublished data).

2.3. Phage titration

A single colony of *V. cholerae* isolate was cultured in Lauria Bertani broth till development of optical density (O.D.) (Fisher Scientific, USA) to 0.5. The Phage titration was calculated by top agarose method. Briefly, an aliquot of top agarose (3 ml) was dispensed on plane tubes and equilibrated at 45 °C to prevent solidification. A volume of 10 µl of phage preparation and 100 µl of host *V. cholerae* culture (mid-log phase) were added to tubes. Serial dilutions were made for each by using the same equilibrated top agarose tubes. Tubes were cooled and 1 ml of mixture from each tube was poured on the surface of Lauria Bertani agar. The plates then cooled and incubated at 37 °C for 18 h. Phage titration was calculated as plaque forming units (PFU) per milliliter of phage preparation [14].

2.4. Preparation of phage cocktail

Each single phage in a phage cocktail was prepared in the desired concentration in SM buffer

(Sigma–Aldrich, USA). Equal volumes of each phage preparation were mixed to give the phage cocktail with equal concentration for each single phage in the cocktail.

2.5. Growth inhibition assay

Each isolate of *V. cholerae* was cultured in Lauria Bertani broth and incubated in a shaker incubator at 37 °C under 150 rpm/min for 24 h. A volume 50 µl of Lauria Bertani broth (2X) was added into each well of a microtiter plate (flat-bottom 96 micro-titer plate). A concentration of bacterial culture (2×10^8 CFU/ml) was prepared through serial dilution, culture of each dilution, incubation of plates for 24 h and calculation through the equation:

$$\text{CFU/ml} = \left(\frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Volume of inoculum cultured}} \right)$$

A volume 50 µl of bacterial culture was added into each well, followed by addition of 100 µl of phage preparation. A volume of 50 µl of filter sterilized 0.1% TTC (Zellibio, Germany) was dispensed into each well. The microtiter plate was incubated at 37 °C for 24 h and measured at OD 540 nm by microtiter plate reader (BioTeck, USA). The %inhibition of *V. cholerae* isolates by single and cocktail phages was calculated according to the equation [15]:

$$\%inhibition = 100 \left(\frac{\text{The absorbance of controls} - \text{The absorbance of treated wells}}{\text{The absorbance of controls}} \right)$$

2.6. Animals

Three days old of New Zealand White infant rabbits (Biotechnology Research Center Animal House, Nahrin University) of both sexes were used for the animal models of cholera.

2.7. The ethical treatment of the animals

The animal studies were done according to the recommendations of Committee of the Ethics of Laboratory Experiments of the animals in Ministry of Higher Education and Scientific Research in Iraq.

2.8. Phage retention time

A number of 9 infant rabbits were orogastrically administered with 3×10^8 PFU of phage cocktail diluted in the sodium bicarbonate (2.5%). At intervals of 3, 12 and 24 h, a number of 3 infant rabbits were sacrificed and dissected. The intestine of each sacrificed infant rabbit was homogenized in Lauria Bertani broth supplemented with 20% glycerol and PFU for each homogenized intestine was calculated by top agarose method [16].

2.9. Length of phage prophylaxis

Length of prophylaxis obtained from administration of phage cocktail was tested. A number of 9 infant rabbits were orally administered with 5×10^9 PFU of phage cocktail diluted in oral solution of sodium bicarbonate (2.5%). Three hours prior to establishment of *V. cholerae*, infant rabbits were injected intra-peritoneally with-ranitidine-hydrochloride (Sigma–Aldrich, USA) (2 µg per gram body weight) to

reduce acidity of the stomach. Each animal was dosed orally with 5×10^8 CFU of bacterial pathogen diluted oral solution of sodium bicarbonate (2.5%) in 3, 12 and 24 h after administration of phage cocktail. Control group of 3 infant rabbits were not dosed with *V. cholerae* to be compared with other groups. Weight of infant rabbits was recorded in the beginning and in the end of the experiment to calculate the percent body weight as below:

$$\text{Body weight}\% = 100 \left(\frac{\text{body weight at the end of the experiment}}{\text{body weight in the beginning of the experiment}} \right)$$

The animals were sacrificed from 10 to-20 h according to percent body weight. Infant rabbits losing 10 to-15% of their body weight were sacrificed from 12 to-14 h, whereas those losing <10% of body weight were sacrificed 20 h post infection.

The intestines of dissected animals were homogenized in Lauria Bertani broth containing 20% glycerol, serially diluted and plated on Lauria Bertani agar - containing 100 µg/ml streptomycin to calculate CFU per intestine. For extraction of phages from homogenates of intestine, a portion of homogenate was treated with chloroform and centrifuged as described previously. The PFU per each intestine was determined by top agarose method [16].

3. Results

3.1. Phage cocktail

The 8 phages were formulated in a cocktail and tested versus each of the following single phage of PCV6, PCV8, PCV9, PCV15, PCV19, PCV20, PCV23 and PCV25 in growth inhibition assay. Results of growth inhibition assay for activity of each one of the 8 single phages and phage cocktail against the 26 isolates of *V. cholerae* O1 have showed that %inhibition of single phages ranged from 0 to 75%, whereas those for phage cocktail was 100%. Fig. 1 showed the growth inhibition assay for PVC15 and phage cocktail against

13 isolates of *V. cholerae* O1- biotype- EI- Tor, serotype Inaba.

3.2. Phage retention time

The study of phage retention time in the intestine of the infant rabbits showed drop in number of phages in a cocktail administered orally from 3×10^8 PFU to an average of 64.33 million PFU, 23 million PFU and 9.66 million PFU after 3, 12 and 24 h, respectively (Fig. 2).

3.3. Length of phage prophylaxis

The study of phage prophylaxis showed that the oral administration of *V. cholerae* 3 h after prophylaxis by phage cocktail resulted in 100% eradication of bacteria from infant rabbits intestine, whereas oral administration of *V. cholerae* 12 and 24 h after prophylaxis with phage cocktail resulted in same result with an exception of survival for only as few as an average of 233.33 and 533.33 CFU/intestine, respectively (Fig. 3).

Plaque forming units (PFU) were calculated in homogenates of sacrificed infant rabbits infected with *V. cholerae* in 3, 12 and 24 h after prophylaxis with phage cocktail. Results also showed the presence of large numbers of phage particles in homogenates of intestines owing to continuous lysis of *V. cholerae* in the presence of phage cocktail. The PFU were recovered

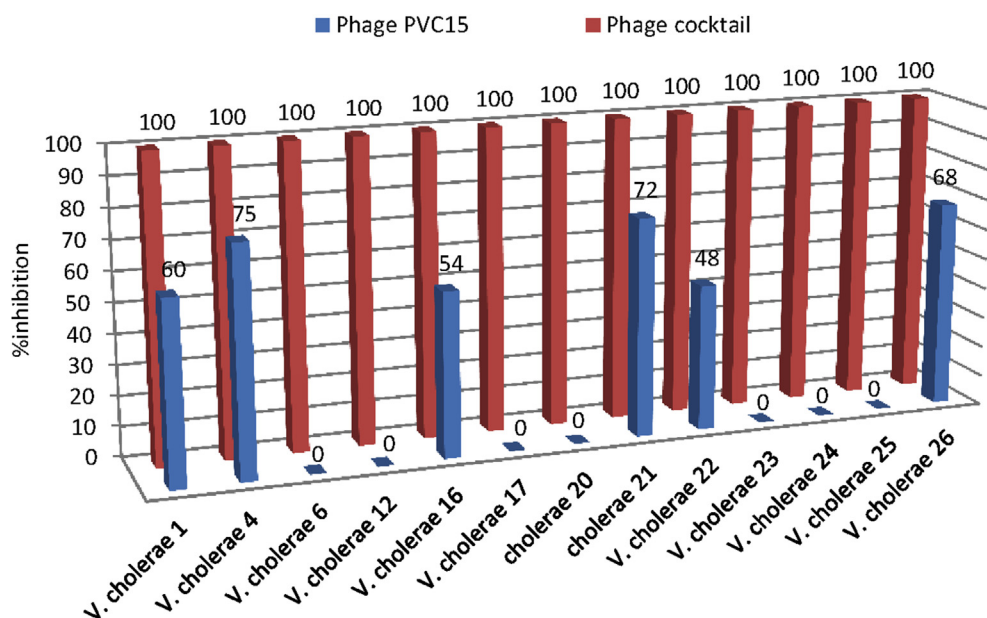


Fig. 1. The %inhibition for the phage PVC15 versus the 8 phages cocktail on 13 *V. cholerae* isolates by growth inhibition assay.

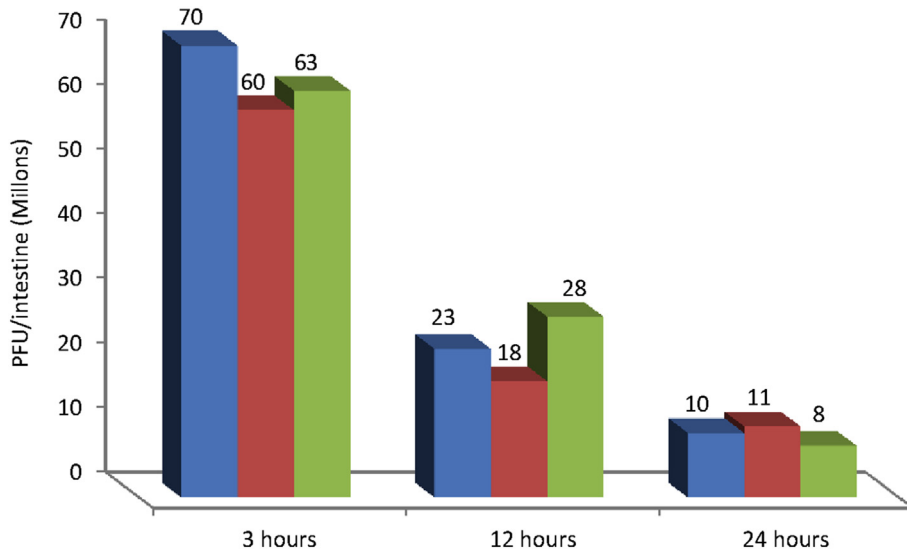


Fig. 2. The account of phages in infant rabbits intestine homogenates after 3, 12 and 24 h of oral administration of 3×10^8 PFU. Each column represents one infant rabbit.

from homogenates of intestines in an average of 7.66×10^9 , 5.16×10^9 and 3.26×10^9 after 3, 12 and 24 h of prophylaxis of infant rabbits with 5×10^9 PFU of phage cocktail (Fig. 4).

4. Discussion

The 8 lytic phages for *V. cholerae* had narrow-host spectrum and showed 0–75% activity against isolates of *V. cholerae* in growth inhibition assay. No single

phage showed broad-spectrum activity against all *V. cholerae* isolates and the most potent lytic phages was PVC15 that showed lytic activity for only 65% of isolates. The 8 phages cocktail showed 100%inhibition to all *V. cholerae* isolates through growth inhibition assay, whereas phage PVC15 showed %inhibition ranged from 0 to 75% against tested *V. cholerae* isolates. Host-range of phages is determined owing to phage adsorption to certain receptors on bacterial cell surface, especially Lipopolysaccharides (LPS) [17].

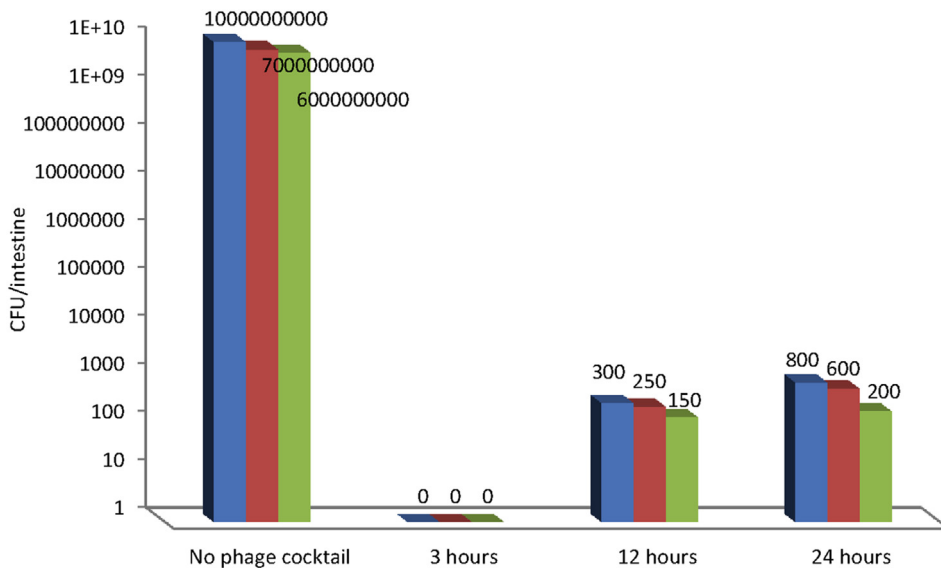


Fig. 3. The account of *V. cholerae* in infant rabbits intestine homogenates after 3, 12 and 24 h of oral administration of 3×10^8 PFU with the positive control not administered with phage cocktail. Each column represents one infant rabbit.

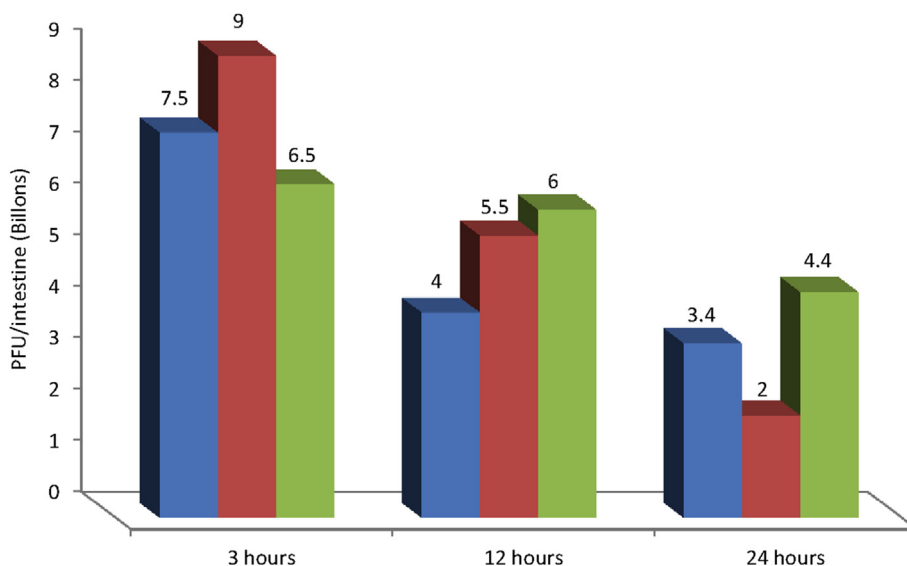


Fig. 4. The account of phages in infant rabbits intestine homogenates after 3, 12 and 24 h of oral administration of 3×10^8 PFU. Each column represents one infant rabbit.

The adsorbed phages to S (smooth)-type LPS exerts very narrow-host range owing to high variation of O-antigen even within the same bacterial species, whereas phage adsorbed to R (rough)-type LPS exerts broader-host range due to conservative composition of the core of LPS in different genera and species of the Gram negative bacteria species and genera of Gram-negative bacteria [18]. Some phages adsorbed both S-type and R-type of LPS [17]. The 8 phages selected for the formulation of phage cocktail had very narrow-host range that mostly attributed to their adherence to S-type LPS on *V. cholerae*.

Phages have the ability to adsorb to different receptors on bacterial cell surface as LPS, LPS-protein complex, Outer membrane proteins (OMPs), selective transport protein (s) and enzymes localized on outer membrane [17–20]. The broad-host range of the 8 phages cocktail may be attributed to combinational properties of phages included in the cocktail to adsorb to various receptors on cell surface of *V. cholerae* that guaranteed availability of one phage or more than one phage, in a cocktail, capable of adsorption on surface of target bacterium, whereas each single phage may be failed to adsorb to certain bacteria owing to incapability for adsorption to any receptor on bacterial surface [21–26].

The infant rabbit model of cholera confirmed the ability of phages in prophylactic prevention of development of cholera in animals administered high concentration of *V. cholerae*. The animals administered

with prophylactic dose of phage cocktail lost <10% of body weight after 20 h of administration of high infectious dose of *V. cholerae*, whereas animals non-administered with the phage cocktail dose developed profuse diarrhea and lost 10–15% of body weight within 10–14 h after taking the infectious dose of *V. cholerae*. The prophylactic therapy of phages in cholera is very important and superior over the use of antibiotics [12,13].

5. Conclusions

The successfulness of phage cocktail developed in this study and other *in vitro* and *in vivo* previous studies in treatment of cholera and other bacterial infections caused by drug-resistant bacteria is highly promising in the development of pharmaceutical phage cocktails at commercial level to be available for treatment of cholera and other bacterial infection in medical institutions.

6. Recommendations

More global attention must be paid towards development of new phage cocktail specific for treatment of various non-responsive drug-resistant bacterial infections. It is highly recommended for all health care institutions worldwide to incorporate units of phage therapy in their routine work to be an available alternative therapeutic method against drug-resistant

bacterial infection encountered daily in each medical care institution.

Declaration of competing interest

None.

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