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In vitro study on using bacteriophages in the treatment of pathogenic *Escherichia coli* in Iraq

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Abstract

Background Bacteriophages, or phages, are virus-like agents that infect bacteria. Lytic phages can be used as biological antimicrobials and can kill bacteria.

Objective To formulate therapeutic phage cocktails able to overcome multiple-drug resistant *Escherichia coli* in Iraq.

Methods *Escherichia coli* were isolated from Iraqi hospitals and were characterized in terms of site of isolation, patient's age, sex, and disease. Antibiotics sensitivity test was used to evaluate antibiotics effectiveness. Accordingly, isolated bacteria were grouped in terms of resistance to antibiotics, infection type, and infection site. Wild phages specific to *Escherichia coli* were isolated from different areas. The isolated phages were optimized and their biokinetics were measured.

Results A total of 10 samples of *Escherichia coli*, 7 samples of them revealed specific phages. The formulated phage cocktail to *Escherichia coli* was shown to remarkably minimize the bacterial resistance to individual phages.

Conclusion Bacteriophage cocktails are useful to tackle the problem of MDR bacteria.

Keywords Multi-drug resistance, bacteriophage, phage therapy, *Escherichia coli.*

List of abbreviation: MDR = multiple drug-resistant, *E. coli* = *Escherichia coli*, BT = burst time, BS = burst size, IP = infective percentage

Introduction

Nowadays, disease-causing microbes that have become resistant to drug therapy are considered as an increasing public health problem and is one of the great challenges for modern healthcare [(1)].

Bacteriophages, or phages, are viruses whose hosts are bacterial cells. Like all viruses, phages are metabolically inert in their extra-cellular form, reproducing only after infecting suitable host bacteria. Discovered over 80 years ago, they have played a key role in the development of modern biotechnology. Their initial isolation appeared to offer the first therapeutic for the control of infectious disease [(2)].

The discovery of antibiotics in the 1940s eclipsed bacteriophage-based therapies. However, the increasing rate of emergence of multiple drug-resistant (MDR) bacteria led scientists and health bodes in the world to re-evaluate phages as the basis of new therapeutic strategies.Phage therapy is the use of phages as antimicrobial agents for the control of pathogenic and other problem bacteria [(3)].

The main problem of phage therapy is the rapid development of bacterial resistance which is much faster than that against chemical antimicrobials. However, the strength point in

phage therapy is that phages are endless source of new anti-pathogen phages[4].

Therefore, it has been conceived that formulating a cocktail of specific lytic bacteriophages against certain bacteria would mask the rapid development of bacterial resistance against attacking phages. Moreover, bacteriophage cocktail can be used very efficiently in combination withantibiotics. Since mechanisms of bacterial resistance against antibiotics are different from that against phages, combinational phage-antibiotics therapy can be the last resort of holding up the inevitable point when human beings reach the post-antibiotic era which is expected to reach 15 to 25 years from now[5].

The objectives of this study was to formulate therapeutic phage cocktails able to overcome multiple-drug resistant (MDR) *Escherichia coli* in Iraq.

Methods

Bacterial sampling:

Isolates of *Escherichia coli*(*E. coli*) were collectedfrom 10 samples(4 urine, 3 blood, 2 ear swab and one knee fluid).These samples were obtained from Al-Kadhimyia Teaching Hospital, Central Teaching Hospital for Children, and Al-Kindi Hospital in Baghdadduring the period from December 2012 to January 2013.

Bacteriophage sampling:

Different crude samples for phage isolation obtained from sheep faeces, cattle faeces, cattle manure, cattle farms, farms soil, sewage, mastitis discharge of human and cattle, burns, wounds, and hospital wards were collected from different regions in Baghdad

Isolation, preparation, and propagation of bacterial samples:

The bacterial samples were cultured by spreading on the nutrient agar;MacConkey agar and blood agar plates. Plates were incubated overnight at 37^{o}C. Cultures were then examined,refreshed, subcultured, and finally stored in 20% glycerol as backup samples.

Antibiotic susceptibility tests:

Antibiotic susceptibility tests were carried out on isolated and identified colonies of bacterial isolates using commercially prepared antibiotic sensitivity discs[6]. Antibiotics used were:amikacin (10 µg), ampicillin (10 µg), gentamcin (10 µg), vancomycin (30 µg), ciprofloxacin (10 µg), cefotaxime (10 µg), methicillin(30 µg), tetracycline (30 µg), ceftazdime (30 µg), rifampicin (5 µg), amoxicillin (25 µg), nitrofurantion (100 µg), cloxacillin (10 µg), clindamycin (2 µg), chloramphenicol(10 µg) and imipenem (10 µg).

Bacteriophages isolation:

Wild phages specific for *E. coli* can be isolated from different areas e.g. sewage. Phage-amplification assays were used by mixing culture broth of the *E. coli*with mixture of specimens in which specific phages were suspected. The mixture of E. *coli*broth and specimens were incubated at 37^{o}Covernight. Next day, if any phage was there, a phage amplification reaction was taken place giving rise to high titer of specific phages. Bacterial lawns were prepared to apply the phage-lawn assay as a screening test for detecting the presence of any specific lytic phages. Phages detected in bacterial lawns were picked up and isolated using the chloroform-shaking method. Isolated phages were kept in lambda buffer.Phage-based plaque assay were done to confirm the detected phages as well as to characterize phages in terms of plaque size, shape, opacity, edge, and depth, this assay was used to maximize the titer of the isolated phages.

Phages optimization and measurement of biokinetics of phages:

The plaque assay for phages, an optimization process, was used to apply phage passage in vitro for obtaining the best of the best plaque forming phages, i.e. pick up the best plaques in terms of size, clarity, depth.The biokinetics of the optimized lytic phages were measured using the novel patented single master tube biokineticassay. The biokinetic measurements are bursttime (BT), burst size (BS) and infective percentage (IP). Gradually build up a mixture of optimized (vertically bred) lytic phages specific

to a single target bacterial species, this mixture named as phage cocktail.Build up and increase the number of effective lytic phages in phage cocktails for *E.coli*in a way it was highly preferred that target bacteria is covered by more than one phage in order to minimize the development of resistance against attacking phages.

Results

Characteristics of *E. coli* samples:

A total of 10 samples of *E. coli*, 7 samples of them were enrolled in the present study because of revealing specific phages. The specimens, from which *E. coli* were isolated, were as follows: blood 2/7 (28.6%), knee fluid 1/7 (14.3%), urine 3/7 (42.9%) and ear swab 1/7 (14.3%).The patients' age ranged between one day to 80 years. This means that different age groups could be suitable source for *E. coli* isolates and good source for corresponding specific lytic phages. Sex ratio was females 4/7 (57.1%) and males 3/7 (42.9%).

Antibiotic susceptibility tests:

The results showed that different isolates of *E. coli* had different antibiotic profiles and these isolates were resistant to many important antibiotics; for example, all of the 7 isolates of *E. coli* were resistant to cefotaxime, rifampicin, amoxcillin and nitrofurantion. Moreover, the isolates were resistant to many antibiotics that have frequently been used in Iraq and as follows 1/7 (14.2%) amikacin, 6/7 (85.7%) ampicillin, 1/7 (14.2%) gentamicin, 2/7 (28.6%) imipenem, 2/7 (28.6%) ciprofloxacin, 7/7 (100%) cefotaxime, 6/7 (85.7%) tetracycline, 3/7(28.6%) ceftazdime, 6/7 (85.7%) ceftriaxone, 7/7 (100%) rifampicin, 7/7 (100%) amoxicillin, 7/7 (100%) nitrofurantion, 6/7 (85.6%) clindamycin and 4/7 (57.1%) chloramphenicol (Table 1).

Table 1. Antibiotic susceptibility test to phage-matched *Escherichia coli* bacteria.

Antibiotic	E1	E2	E3	E4	E5	E6	E7
Amikacin (AK)	*S	S	**R	S	***I	S	S
Ampicillin (AM)	R	R	R	R	R	R	I
Gentamicin (CN)	S	I	S	R	S	S	S
Imipenem (IPM)	S	R	I	S	S	R	S
Ciprofloxacin (CIP)	S	S	R	S	R	S	S
Cefotaxime (CTX)	R	R	R	R	R	R	R
Tetracyclin (TE)	R	R	R	R	R	R	S
Ceftazdime (CAZ)	S	S	S	R	R	S	R
Ceftriaxone (CRO)	R	R	R	S	R	R	R
Refampycin (RA)	R	R	R	R	R	R	R
Amoxcillin (AX)	R	R	R	R	R	R	R
Nitrofurantion (F)	R	R	R	R	R	R	R
Clindamycin (DA)	R	R	R	S	R	R	R
Chloramphenicol(C)	I	R	R	S	R	S	R

*S: Sensitive, **R: Resistant, ***I: Intermediate.

Characteristics of the isolated and optimized phages:

The nature of source specimen and plaque assay-based characteristics of the specific and lytic phages isolated and optimized to the 7isolates of *E. coli*. The characteristics of these phages are shown in terms of the type of phage specimen, size of plaques, shape of plaques, clarity of plaques, and plaques' margin cut as seen in table 2.The primary phages to *E. coli* were isolated as follows: the EP1-EP7 phages were isolated for E1-E7isolates, respectively. When these phages were optimized, each bacterial isolate was recognized by more than

the one primary phage; the newly recognizing phages were called secondary phages and as follows: the secondary phages to E1 were EP3, EP5 and EP7; the secondary phages to E2 were EP3 and EP6; the secondary phages to E3 were EP1 and EP6; the secondary phages to E4 were EP2 and EP7; the secondary phages to E5 were EP1, EP3 and EP4; the secondary phages to E6 were EP5 and EP7;the secondary phages to E7 were EP5 and EP6 (Table 3).

Table 2. The source specimen and plaque assay characteristics of the isolated phages to *E. coli*.

Bacteriophage name	Crude specimen of the phage	Plaques Size (mm)	Margin cut	Plaques clarity	Plaques shape
EP1	Chicken litter	1	Irregular	Semi –clear	Oval
EP2	Sewage	7	Regular	Clear	Circular
EP3	Sewage	4	Irregular	Clear	Oval
EP4	Sheep stool	5	Regular	Semi-clear	Circular
EP5	Sewage	3	Regular	Clear	Circular
EP6	Sewage	2	Regular	Semi-turbid	Oval
EP7	Sheep stool	0.8	Irregular	Turbid	Circular

Table 3. Plaque assay characteristics of the secondary phages to *Escherichia coli*.

Bacterial isolate	Secondary phage	Plaques Size (mm)	Margin cut	Plaques clarity	Plaques shape
E1	EP3	0.3	Irregular	Semi-turbid	Oval
	EP5	0.2	Irregular	Semi-turbid	oval
	EP7	0.5	Irregular	Turbid	oval
E2	EP3	2	Regular	Semi-clear	Circular
	EP6	0.9	Irregular	Turbid	Oval
E3	EP1	0.7	Irregular	Turbid	Oval
	EP6	0.6	Irregular	Turbid	Oval
E4	EP2	5	Irregular	Semi-turbid	Circular
	EP7	0.6	Irregular	Turbid	Circular
E5	EP1	0.5	Irregular	Semi-turbid	Oval
	EP3	3	Regular	Semi-clear	Circular
	EP4	2	Irregular	Semi-turbid	Oval
E6	EP5	1	Irregular	Turbid	Oval
	EP7	0.3	Irregular	Turbid	Oval
E7	EP5	1	Irregular	Semi-turbid	Oval
	EP6	0.6	Irregular	Turbid	Oval

The titers of the specific and lytic phages isolated and optimized to the studied bacterial isolates were measured by using top layer plaque assay; the highest titers of the isolated and optimized phages shown in table 4.

As demonstrated in fig. 1, the optimized highly lytic phages are shown to be able to lyse completely target bacteria in whatever manner of phages application.

Biokinetics of the isolated and optimized bacteriophages:

The mean BS of isolated phages to *E. coli* ranged between 103.4± 0.06 to 326± 10.92 phage progeny. The mean IP ranged between 64.6±

1.18 to 96.2± 0.18 %.The minimum BT was 30 min, while the maximum one was 35 min (Fig. 2).

Table 4. The titer of the specific and lytic phages to *Escherichia coli*.

Bacteriophage name	Titer (PFU/ml)
EP1	1.3×10^7
EP2	7×10^9
EP3	6×10^{11}
EP4	9×10^7
EP5	2.2×10^7
EP6	5×10^8
EP7	8×10^7

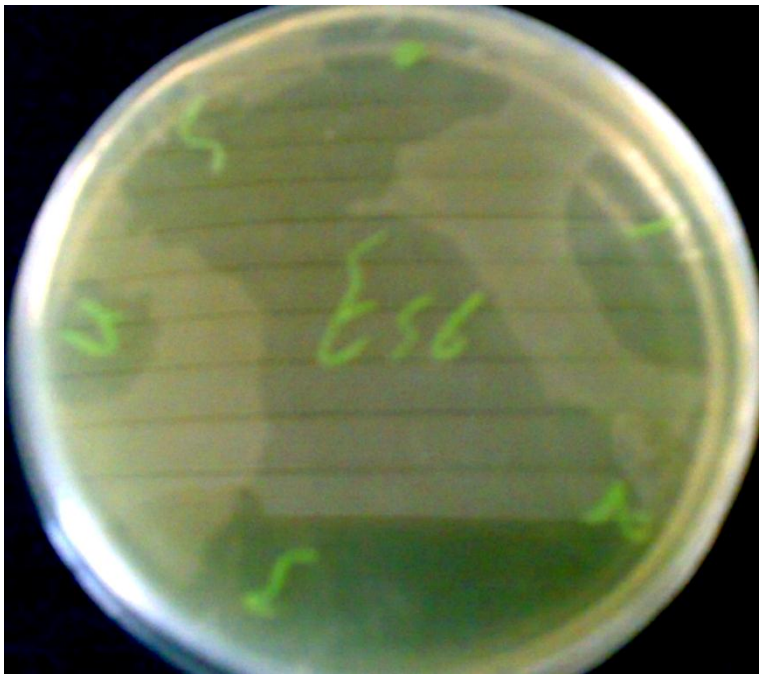

Fig. 1. Phage spot lysis plaque assay of bacteriopahge to *Echerichia coli*.

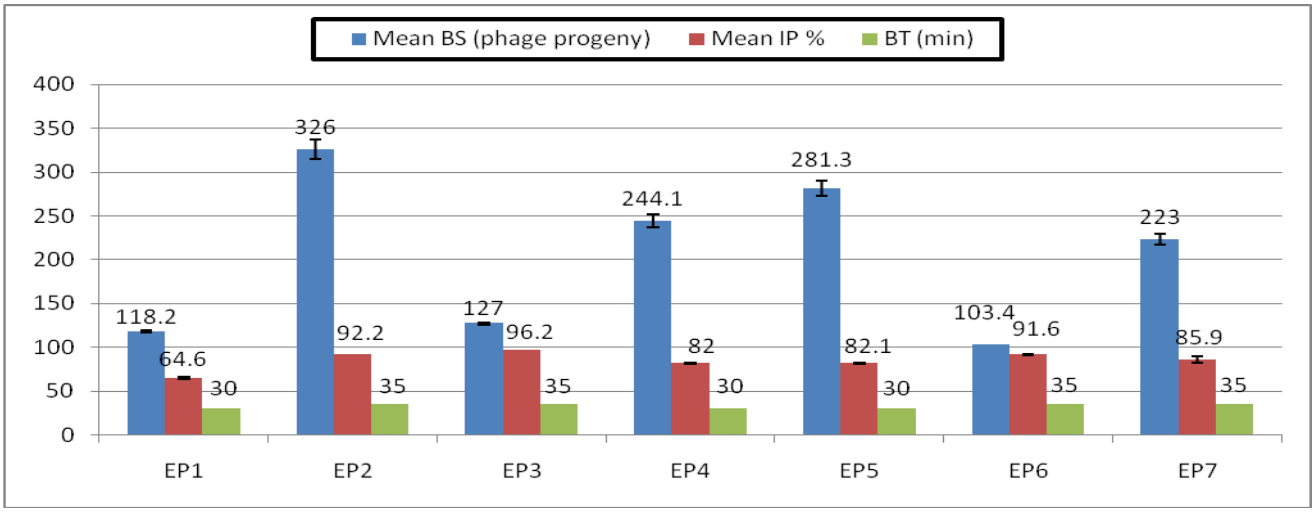

Fig. 2. Biokinetics, BS, BT, and IP%, of isolated phages to *Escherichia coli.*

Phage cocktails and challenge of developing bacterial resistance to phages:

The phage cocktails specific to each bacterial isolate were shown to remarkably minimize the bacterial resistance to individual phages. This finding solidifies our primary objective for using phage cocktails in phage therapy (Table 5).

Table 5. Resistance rate of *Escherichia coli* to phage cocktails versus individual phages.

	EP1	EP2	EP3	EP4	EP5	EP6	EP7	Cocktail
E1	$1:7.5\times10^5$	-	$1:1.5\times10^5$	-	$1:8.5\times10^5$	-	$1:1\times10^6$	$0:6\times10^8$
E2	-	$0:5\times10^7$	$1:5\times10^7$	-	-	$1:1.6\times10^7$	-	$0:1.3\times10^9$
E3	$1:2\times10^9$	-	$0:8\times10^9$	-	-	$1:8\times10^9$	-	$0:6\times10^{10}$
E4	-	$1:4\times10^5$	-	$1:2\times10^5$	-	-	$1:1.5\times10^5$	$0:7\times10^5$
E5	$1:6.6\times10^5$	-	$1:2\times10^6$	$1:7.5\times10^5$	$1:5.7\times10^6$	-	-	$0:4\times10^8$
E6	-	-	-	-	$1:8\times10^7$	$0:8\times10^7$	$1:2.6\times10^7$	$0:9.7\times10^8$
E7	-	-	-	-	$1:3.7\times10^6$	$1:1.8\times10^6$	$1:5\times10^6$	$0:8.6\times10^7$

Discussion

The emergence of antibiotic resistant strains constitutes a worsening global health problem; however, this problem is strikingly more obvious in Iraq. Globally, there are many reasons for this problem that can be explained by several hypotheses such as, chromosomal change or exchange of the genetic material by plasmids, antibiotic pressure- driven bacterial evolution, the dominant spread of mutant strains over sensitive strains, inappropriate use of antibiotics represented by the self-medication with wrong antibiotics, and the use of insufficient dosages, or subjected to unnecessary therapy. The current study revealed a remarkable MDR nature of *E. coli* isolated in Baghdad, Iraq (Table 1). Previous reports from Iraq and neiboring countries revealed similar MDR nature to what we have found in our study [7-10]. This necessitates serious work out of MDR problem in this region of the world as few studies focused on this problem are conducted in this region of the world. One of the best solutions can be taken is the use of bacteriophages as alternatives to progressively failing antibiotics.

In the current study, the phages to *Escherichia coli* were isolated from sewage, as like as other previously published studies [11-13], chicken litter [14], and sheep stool [15]. Sewage water was observed to be the best environmental source to get lytic phages with aggressive infective qualities; this might be attributed to the fact that phages from sewage tolerate hard conditions in the sewage; thus, these phages show high degree of lysis with high tolerance to harsh physical environment. Therefore, lytic phages from sewage showed larger plaques, higher titers, and clearer plaques than others.

Other sources that provided good lytic phages were chicken litterand sheep stool. The different sources of phages' isolation and the finding that each phage showed unique profile of size, shape, clarity, and margin cut of plaques provided preliminary evidence that isolated phages are unique and no phages are identical to each other.

The seven *E. coli* isolates, recognized by the primary phages at the beginning, were later recognized by more than one secondary phage. Some phages at the beginning showed very small and hazy plaques, but after serial top-layer plaque assay- based optimization, these phages showed enhanced infective characteristics to its primary host and moreover, the optimized phages showed positive infective capabilities towards bacterial hosts other than the primary host, called, secondary hosts and these phages are named secondary phages. When the bacterial isolate was recognized by more than one phage, it is a good indication to decrease or eliminate the bacterial resistance to phages; this

help confer greater chance to destroy the bacterial host.

The rate of 103-326 phage particles amplification per a cycle during 30-35min is much higher than the duplication time of bacterial hosts which is just 2 new daughter cells in approximately 25-30 min. Therefore, lytic phages showed dominant infective potential over bacterial cells. This gives a series of clues. First, lytic and specific phages to bacterial pathogens are able to eradicate bacteria in a short period of time. Second, lytic phages amplify specifically at the site of infections only while other parts of the body are spared. This is totally different from the kinetics of chemical antimicrobials where their concentration in the body is the same at both infected and non-infected sites. This criterion is considered as one of the main advantages of phages over using antibiotics and this makes therapeutic phages are largely safe to humans and animals. Third, the infection-oriented self-amplification of therapeutic phages allows administering only single dose rather than repeated doses of phages over a long course of time. Fourth, the self-amplification of therapeutic phages is followed by self-termination after eradicating the target bacterial pathogen.

An important notion from the results of the biokinetics is that each isolated phage showed unique set of biokinetic values. The results of the current study showed that each isolated phage has its own bacterial recognition profile which is different from other isolated phages. Besides, each bacterial isolate has its own set of attacking phages. This indicates that the bacterial isolates and attacking phages are all of different entities.

The successful phage cocktail must be composed of different lytic phages sharing no single receptor for bacterial recognition. Therefore, we hypothesized that each bacterial isolate which is recognized by more than one phage (several phages in addition to the primary phage) is attributed to that each bacterial isolate has more than one receptor and each receptor is specific for different phage to bind and infect. In addition, the resistance rate of bacterial isolates to the attacking phages was shown to be different to each other.

Accordingly, we picked up the resistant bacterial clone to each recognizing phage and tested its resistance rate to the other members of the phage cocktail which recognizes the same bacterial host. If the resistance was universal to all of the members of the phage cocktail, this means that phages, even though are different, share the same receptor of bacterial recognition which is not favored for the successful use of phage cocktails.

The current study showed remarkable findings that once bacterial isolate develops resistance to one member of phage cocktails, the isolate is still sensitive to other phages in the cocktail. One of the most haunting adverse effects of using phages in therapy is the rapid development of resistance by bacteria to infecting phages. The resistance rate to phages is much higher than that of antibiotics. Therefore, successful phage therapy needs to fix this weak point. Hence, this study succeeded in applying phage cocktails in reducing or eliminating the development of resistance to therapeutic phages. The current study compliments what our team achieved in a previous patented study outside Iraq where phage cocktails could minimize the development of bacterial resistance to phages [4,5]. In addition, many international studies have shown the same advantage of using phage cocktails [16-18].

In conclusion, phage cocktails are useful to tackle the problem of bacterial resistance to bacteriophages, and are good candidates for combating MDR bacteria whether used alone or in combination with antibiotics. More elaborated studies on phage cocktails are needed to find, formulate and design the proper phage cocktails to the endemic bacteria.

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Author Contribution

Marwa Bassim: conducted the sampling, phage isolation and in vitro and in vivo work. Ahmed Sahib designed the research, guided the protocols of bacteriophage related research, did the statistical analysis and finished writing and editing.

Conflict of Interest

We declare that there is no conflict of interest among authors of this study or with other authors or research teams.

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