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Formulation of Therapeutic Phage Cocktails to Human Isolates of *Salmonella enterica* Serovar *enteritidis*

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Abstract

Background: Antibiotics have revolutionized medicine in many respects. Regrettably, the use of these wonder drugs has been accompanied by the rapid appearance of resistant strains. *Salmonella* serotype *enteritidis* is one of the important causes of human illness; resistance of these bacteria to antibiotics prevents the success of proper therapy. Bacteriophage could be used as an alternative to conventional antibiotic therapy.

Objectives: To formulate Iraqi therapeutic phage cocktails composed of, in-lab optimized, highly specific phages against *Salmonellaenteritidis* , to evaluate and measure the rate of bacterial resistance to the formulated phage cocktails.

Materials and Methods: *Salmonellaenteritidis* bacteria were isolated from human samples and tested for antibiotics susceptibility, then specific lytic phages for these bacteria were isolated by spot lysis assay. Afterwards, phage titration was conducted and examination of phage characteristics was done by top layer plaque assay. Testing of bacterial resistance to the formulated phages cocktails was determined. **Results**: Seven phages were isolated for *Salmonella*, each phage was unique in its characteristics including clarity, shape, size, and edge of plaques formed and their titer.

Conclusion: Phage cocktails are more effective than individual phages as they show clearer plaques and less resistance rate. This study concludes that phage therapy is a very promising alternative to traditional antibiotic therapy.

Key words: *Salmonella*, bacteriophage, phage cocktails, phage therapy, alternative medicine.

INTRODUCTION

Over the last two decades, the widespread emergence and spread of antibiotic-resistant bacteria around the world has become a major therapeutic challenge [1, 2]. Antibiotic resistance is reaching a crisis situation in some bacterial pathogens where few therapeutic alternatives remain and pan-resistant strains are becoming more prevalent. Non-antibiotic therapies to treat bacterial infections are now under serious consideration and one possible option is the therapeutic use of specific phage particles that target bacterial pathogens [3].

Phage therapy is the application of bacteria-specific viruses (phages) to combat uncontrolled and undesired bacteria such as those associated with infectious disease [4]. Phage therapy has been explored extensively, with successes being reported for a variety of diseases,including dysentery, typhoid and paratyphoid fevers, pyogenic and urinary tract infections, and cholera [5].

One of the disadvantages of using phages is the emergence of phage-resistant variants which occurs rapidly if only one phage strain was used against a particular bacterium [6]. Fortunately, there is an abundance of other phage species which possess lytic ability against resist variants [7].

Accordingly, the therapeutic use of phage cocktail, which is the combining of two or more phage types to produce more pharmacologically diverse formulations, has been recently considered. The primary motivation for the use of cocktails is their broader spectra of activity in comparison to individual phage isolates: they can impact either more bacterial types or achieve effectiveness under a greater diversity of conditions. The combining of phages can also facilitate better targeting of multiple strains of a bacterial species as well as cover multiple species that might be responsible for similar diseases. In general, phage cocktails have greater potential for presumptive or empirical treatment [8].

Salmonellosis is a significant and persistent cause of diarrheal diseases among humans in developing countries [9]. Indiscriminate use of antibiotics has resulted in increasing resistance to commonly used antibiotics [10].

The emergence of plasmid- encoded multidrug resistant *Salmonella* has been a significant evolution in antimicrobial resistance [9]. In recent study, conducted in 2014, 27.5% of *Salmonella* isolates were multi-drug resistant (MDR) bacteria [11]. Hence, an escalating need has become evident for finding alternative approaches for combating serious cases of Salmonellosis.

Phage cocktails were used for reduction of *Salmonellaenteritidis in vivo* and *in vitro* by Andreatti Filho and colleagues [12]. Moreover, the efficiency of phages to treat *Salmonella enterica* serovar *enteritidis* infections in poultry was applied by aerosol spray on fertile eggs [13].

In Iraq there was no studies deals with *Salmonella* bacteriophage therapy; only two studies were deals with bacteriophage therapy with different bacterial types for example study of Specific Bacteriophage To Treated Contaminated Burns in *Pseudomonasaeruginosa* [14] and In vitro study on using bacteriophages in the treatment of pathogenic *Escherichiacoli* [15].

MATERIALS AND METHODS

Bacterial sampling

Five strains of *Salmonellaenterica* serovar *enteritidis* were isolated (3 from stool, one from urine and one from blood). Samples were taken from patients attending Imamein Kadhimein medical city hospital, child protection teaching hospital, and Teaching laboratory of medical city in Baghdad during the period from September 2013 to May 2014. The current research was carried out according to the Helsinki agreement of ethical guidelines for biomedical research and this study has been approved by the ethical committee of College of Medicine, Alnahrain University.

Isolation, preparation and propagation of bacterial isolates

Tetrathionate broth base was used as enrichment medium for *Salmonella* present in patient samples, then these samples, after overnight incubation, were cultured on MacConkey, XLD and SS agars. The suspected single colonies from ABC streaking of each isolate were then subcultured to get pure isolates suitable for biochemical confirmatory tests. In addition, the isolates were sent to the Central Health Laboratory to attain definitive diagnosis and to exactly determine the species of isolated bacteria by using API 20E test and serotyping. After the definitive diagnosis was achieved, a single colony from each bacterial isolate was incubated in nutrient broth over night then stored in deep freeze after adding 30% glycerol as a backup sample.

Antibiotic susceptibility test

All bacterial isolates were tested for antibiotic susceptibility using commercially prepared antibiotic discs.The antibioticswere selected depending on antibiotic used routinely for *Salmonella* on clinical and laboratory standards institute and on protocols used for enterobacteriaceae in Iraq by CentralHealthLaboratory. In this test, first, a bacterial lawn was made, then 11 types of antibiotic discs were used, namely:ampicillin, Amoxicillin-clavulanic acid, ciprocifloxan, Nalidixic acid, trimethoprim, Azithromycin, Tetracycline, ceftriaxone, gentamicin, Ceftazidime , and Chloramphenicol.

Bacteriophage sampling

Bacteriophages were sought from a mixture of crude samples of chicken litters, pigeon litters, love bird litters (of genus Agapornis), cat stool, sludge pit and sewage from different places in Baghdad, particularly from AL Gazallia district, during the period from January 2014 to June 2014 . The crude samples were stored in 15- 50 ml containers, one to two days at room temperature till used.

Isolation and propagation of primary bacteriophages

Bacterial isolates were refreshed overnight in nutrient broth then mixed with crude sample, the mixture was incubated overnight at 37 C°. On the second day, the mixture was centrifuged; then the remaining bacterial cells were removed by chloroform method the resulted fluid became the supposed-to-be suspension of isolated phage(s). Bacteriophage spot lysis assay was used for the identification of isolated phage(s), if any, each bacterial isolate was refreshed in nutrient broth overnight then the lawn was made on a surface on nutrient agar plate using a sterile cotton swap, left to dry, then Ten µl of phages mixture were spotted onto the surface of each bacterial lawn. Second day, the plates were examined for the presence of lytic phage spot; if any clear plagues or clear zones were present, this means that we have isolated specific bacteriophage against target bacteria [16,17]. After that, the titer of each phage was determined.

Preparation of secondary bacteriophages

The secondary phages are those already isolated phages but later where found to be lytic to bacterial isolates other than the isolate by which the phages were isolated. Therefore, secondary phages were isolated based on certain target bacteria from the same genus, rather than the bacteria used for phage isolation, (overlapped phages specificities). This was done by testing the lytic activity of each primary phage, via using phage spot lysis assay, on all bacterial isolates involved in the current study [17].

Testing of bacterial resistance to bacteriophages

This procedure was used for determining the resistance rate of the used bacteria to each primary and secondary phages and to phage cocktail as well, that to say all specific phages for certain bacterial species. The resistant bacterial colonies in each phage lysis spot were identified as being colonies of bacteria that resist phage lysis and appear as satellite isles in the clear spot of lysis; these colonies of resistance were visually counted. In addition, the diameter of phage lysis spot was measured. A piece of bacterial lawn with a diameter identical to that of the phage spot was cut and the number of bacteria present in this piece of lawn was measured. The rate of bacterial resistance is calculated as follows:

[Number of resistant bacterial colonies appeared on the phage lysis spot: Number of bacterial colonies formed from the same size cut of bacterial lawn].

Top layer plaque assay

This test is used for applying phage passage in vitro and determining the size, clarity, shape and margin of the phage's plaques. Moreover, top layer plaque assay provides significantly greater titer of phages than other methods of virus preparation and quantification.

RESULTS

Isolated bacteria

Five bacteria were isolated and diagnosed as *Salmonellaenteritidis.Salmonella* isolates along with the clinical specimens from which they were isolated are shown in correspondence with age and gender of different patients with diarrheal and urinary diseases (Table 1).

The patients' age ranged from 1 to 30 years, the children were 3/5(60%) and the adult were 2/5 (40%). Gender of patients was 4/5 (80%) male and 1/5 (20%) female. Accordingly, all age groups and both sexes were infected with Salmonella bacteria.

Table 1. Samples of *Salmonella* spp. isolates in correspondence with age and gender of patients from which they were isolated.

Genus of bacteria	Isolate number	Species	Type of specimen	Patients age (year)	Gender
Salmonella	1	*enteritidis*	Urine	23 year	Female
	2	*enteritidis*	Stool	30 year	Male
	3	*enteritidis*	Stool	1 year	Male
	4	*enteritidis*	Stool	2 year	Male
	5	*enteritidis*	Blood	3 year	Male

Antibiotic susceptibility test

The results of antibiotic susceptibility test indicated different antibiotic profiles (Table 2). The resistance rates were as follows: 2/5 (40%) ampicillin, 4/5 (80%) amoxicillin-clavulanic acid with 1/5(20%) intermediate response, 0/5 (0%) ciprocifloxan, 2/5 (40%) Nalidixic acid, 1/5(20%) trimethoprim, 0/5 (0%) Azithromycin, 1/5 (20%) Tetracycline, 1/5 (20%) ceftriaxone, 0/5 (0%) gentamicin, 1/5 (20%) Ceftazidime with 2/5 (40%) intermediate response, and 1/5 (20%) Chloramphenicol. 2/5 (40%) of bacteria was MDR.

Table 2. Antibiotic susceptibility test of *Salmonella enteritidis* .

Antibiotics	Bacterial isolate				
	1	2	3	4	5
Ampicillin (10mcg)	R	S	R	S	S
Amoxicillin-clavulanic acid (20/10mcg)	R	I	R	R	R
Ciprofloxacin (5ug)	S	S	S	S	S
Nalidixic acid(30mcg)	S	S	S	R	R
Trimethoprim (5 mcg)	S	S	R	S	S
Azithromycin (15mcg)	S	S	S	S	S
Tetracycline (10mcg)	S	S	S	R	S
ceftriaxone (30mcg)	S	S	R	S	S
Gentamicin (10 mcg)	S	S	S	S	S
Ceftazidime (10mcg)	S	I	R	S	I
Chloramphenicol (10mcg)	S	S	S	R	S

Primary phages

In this study, 4 out of 5 isolates of Salmonella enteritidis yielded seven specific primary phages. One isolate had more than one primary phage (Table 3). Plaque assay revealed plaques characteristic of primary phages in that the plaque size of phages ranged from 0.5 mm to 16 mm; the size mean was 6.14 and the median was 3. The margin edge was regular in 3/7 (42.8%) and irregular in 4/7 (57.1%). plaques were shown to be clear in 1/7 (14.2%), semi clear in 1/7 (14.2%), clear edge with turbid center in 2/7 (28.5%), semi turbid in 2/7 (28.5%) and turbid in 1/7 (14.2%). The shape of plaques was ranging from circular 5/7 (71.4%) to oval 2/7 (28.5%) (Table 3) and (figure 1).

Titer of the isolated primary phages

The titer of each specific phage isolated and optimized so far was measured by using phage serial dilution method of top layer plaque assays; the phage titers are shown in table 4.

Table 3. Plague characteristics of the isolated primary phages to Salmonella enteritidis

Bacterio phage name	Phage size (mm)	Margin edge	Plagues clarity	Plagues shape
1p	0.5	Regular	Clear	Circular
2pa	3	Irregular	Semi turbid	Oval
2pb	2.5	Irregular	Semi turbid	Circular
2pc	2	Irregular	Clear edge with turbid center	Circular
2pd	7	Regular	Turbid	Oval
3p	12	Regular	Clear edge with turbid center	Circular
4p	16	Irregular	Semi clear	Circular

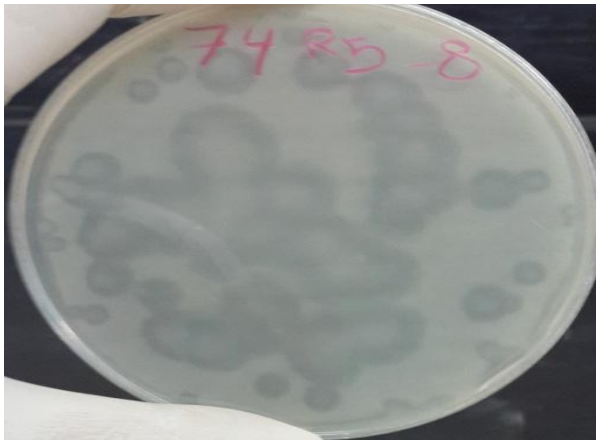

Figure 1. An example of top layer plaque assay for phage 3p showing circular, 12 mm diameter, plaques with regular and clear edges but with characteristic turbid center.

Secondary phages

It was found that one secondary phage for isolate number 1, one secondary phage for isolate number 2, two secondary phages for isolate number 3, and no secondary phage for isolate number 4 were detected (Table 5).

Assessment of the bacterial resistance to infecting phages

The resistance rates of Salmonella spp. to individual phages and to phage cocktails were measured by observing

the number of bacterial colonies resistant to phage-based lysis per each phage lysis spot on bacterial lawns as shown in Table (6). The resistance rate to the phage cocktails was lower than that to individual phages.

Table 4. Phage titers by top layer plaque assay

Bacteriophage number	Titer (PFU/ml)
1p	1.8×10^7
2pa	4.2×10^9
2pb	7×10^{10}
2pc	1×10^{11}
2pd	1.6×10^8
3p	1.1×10^9
4p	1.5×10^5

DISCUSSION

The antibiogram results of Salmonella isolates indicate that all of the isolates were resistant to one or more antibiotics except one isolate; this was in agreement with other studies [18, 19]. Susceptibility of the isolates to ciprofloxacin, gentamicin and ceftriaxone is in harmony with the findings of a recent study [20], while resistance to chloramphenicol, ampicillin, amoxicillin and nalidixic acid was found in a recent study done in 2012 [21] which showed Salmonella resistance of 37.7% to chloramphenicol, 38.4% to ampicillin, 50.7% amoxicillin and 91.1% to nalidixic acid. However, the findings of Salmonella antibiogram in the present study disagree with a study done in Greece [22] in which Salmonella bacteria showed a very low resistance rate to ampicillin, amoxicillin/clavulanic acid, chloramphenicol, tetracycline,

Table 5. Anti-*Salmonella* primary and secondary bacteriophages

Bacterial isolate	Bacteriophage number						
	1p	2pa	2pb	2pc	2pd	3p	4p
1	**PRI**	NIL	NIL	NIL	NIL	NIL	**SEC**
2	**SEC**	**PRI**	**PRI**	**PRI**	**PRI**	NIL	NIL
3	NIL	**SEC**	NIL	NIL	**SEC**	**PRI**	NIL
4	NIL	NIL	NIL	NIL	NIL	NIL	**PRI**

*: PRI= primary phage, SEC= secondary phage, NIL= no clear zone

Table 6. The resistance rate of Salmonella spp. to individual phages and to phage cocktail.

Phage number		Bacterial isolate			
		1	2	3	4
1p	Clarity	Semi clear	turbid	No reaction	No reaction
	Resistance rate (CFU)	1: 5.4×10^5	None		
2pa	Clarity	No reaction	Semi clear	Semi turbid	No reaction
	Resistance rate (CFU)		1: 5×10^6	1: 5.3×10^6	
2pb	Clarity	No reaction	Semi turbid	No reaction	No reaction
	Resistance rate (CFU)		None		
2pc	Clarity	No reaction	turbid	No reaction	No reaction
	Resistance rate (CFU)		None		
2pd	Clarity	No reaction	turbid	Turbid	No reaction
	Resistance rate (CFU)		None	None	
3p	Clarity	No reaction	No reaction	Turbid	No reaction
	Resistance rate (CFU)			None	
4p	Clarity	Semi clear	No reaction	No reaction	clear
	Resistance rate (CFU)	None			None
Phage cocktail	Clarity	Semi clear	Semi clear	Semi turbid	Clear
	Resistance rate (CFU)	1: 1.6×10^7	1: 1.5×10^7	1: 2.2×10^7	None

and cotrimoxazole, 9.3, 4, 2, 15.3, and 8.7%, respectively. In the current study, different sources for phage-containing specimens were pursued; most of Salmonella lytic phages were isolated from chicken and pigeon litters. Other sources also provided good lytic phages such as love bird, genus Agapornis, litters, cat stool, and sewage. The isolation of Salmonella phages from poultry and sewage agrees with a study done in Italy in 2007 [12]. The results of the top layer plague assay showed different infective characteristics among phages studied. All of the phages were of unique virulence properties, and differ from each others in terms of plaques' size, clarity, margin, and shape. These findings are important because the pillar of a successful phage therapy is to use unique virulence characteristics of phages mixed in a single therapeutic phage cocktail. Such mixture can result in an increased potential for phage formulations to be used presumptively and an increased breadth of utility for individual formulations. The latter will probably be crucial to the commercial as well as clinical success of phage therapy [23]. Also, phage cocktail therapy was more effective in counteracting bacterial mutations than monophage therapy suggesting that phage cocktail has great therapeutic potential for multidrug-resistant bacteria infection [24]. Some of the Salmonella primary phages acted as secondary phages on other bacteria .This phenomenon is essential in formulating phage cocktails in which group of phages can lyse together a target bacterial strain. The results of the current study showed that the titer of primary phages for Salmonella were different and these phages were of different plaque characherisitics; this indicated that each primary phage, despite being a secondary phage to other bacterial isolates, is unique in its characteristics. Several previous studies formulated phage cocktails to treat certain bacterial infections [13, 25]; their results were good but not exquisite for a reason. They used mixtures of phages to certain bacterial species but they did not design a phage cocktail where different bacterial strains are covered by more than one phage. Our strategy is quite different. We were keen to formulate a phage cocktail that each of treated isolates must be covered by two or more different phages. The resistance rates of Salmonella spp. to individual phages and to phage cocktails were measured in this research. The resistance rate to phage cocktails was lower than the resistance to individual phage in all bacteria. This result renders the phage cocktail more beneficial than mono-phage therapy. Antibiotic resistance can be achieved by horizontal acquisition of resistance genes (carried by plasmids or transposons), by recombination of foreign DNA into the chromosome, or by mutations in different chromosomal loci [26]. The probability of the apparition of a plasmid and chromosomal mutation for one character is 1:106 and 1:109 CFU, respectively [27]. In this case, the performance of phage cocktail was equal or sometimes better than that of antibiotics. Phage cocktails used in the current study provided evidence that phage cocktails can broaden the host range as well as lowering the chances of bacteria to resist attacking phages. However, the narrow host range of phages is a good and bad criterion at the same time. Many phages are known to be highly specific for certain bacterial receptors and are therefore characterized by a narrow host range, limiting their infectivity to a single species or to specific bacterial strains within a species [28]; hence, phages only minimally impact health-protecting normal flora bacteria [29]. It is good if the bacterial pathogen is well known and well analyzed. On the other hand, not well characterized life-threatening bacterial infections need highly effective and broad spectrum antimicrobials; in this instance, phage cocktails are much more preferred over mono-phage therapy. Taken together, using therapeutic phages, in general, and phage cocktails, in particular, composed of specific lytic optimized phages against Salmonella enteridis bacteria seems a promising alternative antimicrobial approach. Studies with broader phage cocktails are needed to be done in order to standardize this approach for combating bacteria where traditional chemical antimicrobials are failing.

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Author Contribution

Rafal Nazar : conducted the sampling, phage isolation , optimization and in vitro work on targeted bacteria. Ahmed Sahib designed the research, guided the protocols of bacteriophage related research, 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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