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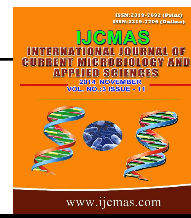

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Original Research Article

Assessment of bacteriophage cocktails used in treating multiple-drug resistant *Pseudomonas aeruginosa*

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A B S T R A C T

Extensive research is currently conducted on the use of alternative approaches for treating MDR bacteria including bacteriophages for applications in human medicine. The objective of the study is to measure the therapeutic efficacy of anti-*pseudomonas* bacteriophage (phage) cocktails to treat its multiple drug resistance. Clinical isolates of *Pseudomonas aeruginosa* were subjected to antibiotic and phages susceptibility. Characteristics of the isolated phages were determined via plaques' morphology. Phage-resistance rate was measured via phage lysis spot assay. The formulated lytic and specific phages were mixed together in what is called a phage cocktail. Five *P. aeruginosa* phages were identified; the phages were optimized and found highly lytic to MDR *P. aeruginosa* bacteria. The therapeutic phage cocktail showed higher efficacy, or faced lower bacterial resistance rate, than therapeutic separate phages. Using phages in treating MDR *P. aeruginosa* bacteria is feasible and safe and can compensate the progressively failing antibiotics.

Introduction

The occurrence of multiple drug resistant (MDR) *Pseudomonas aeruginosa* strains are increasing worldwide and limiting our therapeutic options. It is a major health concern due to increased antibiotic resistance (Strateva and Yordanov, 2009). More to the problem, MDR *Pseudomonas aeruginosa* often establishes itself in already compromised patients, such as those with cystic fibrosis or in hospitalized patients at intensive care units, resulting in life-threatening infections (Folkesson *et al.*, 2012).

In the Middle East, war-associated injuries commonly become infected and antimicrobial drug–resistant bacteria are well-described in these injuries including MDR *Pseudomonas aeruginosa* (Franka *et al.,* 2012). Accordingly, there is now a growing interest in the use of bacteriophage (phage) therapy for the control and treatment of multidrug-resistant bacterial infections (Knezevic and Petrovic, 2008). Bacteriophages are viruses that infect and rapidly destroy bacteria. The name was formed from "bacteria" and "phagein" (to

eat in Greek), almost a century ago Frederick Twort and Felix d'Herelle, independently, described entities that could destroy cultures of bacteria (Abedon *et al.,* 2011). In fact, bacteriophages were used to treat bacterial infections prior to the discovery and medical use of antibiotics in 1940s (Lu and Koeris, 2011). Phages are ubiquitous in the biosphere (estimated 10^{31} particles), which places them as the most abundant biological entity on Earth (O' Flaherty *et al.,* 2009). Phage therapy is an important alternative to antibiotics in the current era to combat of multidrug resistant pathogens, and has many advantages compared to antibiotics: they are very specific and efficient to their target bacteria, which reduces destruction of the host's natural flora; they are not pathogenic for man; and they persist only as long as the targeted bacteria are present (Azeredo and Sutherland, 2008). Recent study taking on efficacy of phage therapy in treating burn-wound associated MDR *Pseudomonas aeruginosa* sepsis (Abdul-Hassan *et al.,* 1990; Summers, 1999). The U.S. Food and Drug Administration (US-FDA) decides the fate of phage therapy in the United States and influences its adoption around the world. There are some recent exceptions, such as the (FDA) approval in 2006 of a cocktail of six phages for the control of *Listeria monocytogenes* this was the first approval granted by the FDA (FDA, 2006). There have been no reports of significant adverse reactions despite their long history of administration to humans. Even found side effects were shown to be subtle or at least relatively minor (Miedzybrodzki *et al.,* 2012). Moreover, phage dosing is simple as, unlike antibiotics, single dose of phage therapy is usually enough. The application of a phage cocktail, through phage therapy, involves the simultaneous use of more than one phage type (Chan and Abedon, 2012). Phage cocktails not only potentially provide means to circumvent resistance to a single phage (Cairns and Payne, 2008), they also allow the treatment of multiple pathogens simultaneously (Merabishvili *et al.,* 2009). Hence, the development of new therapeutic and prophylactic strategies for the control of MDR *Pseudomonas aeruginosa* infection in the region and in Iraq is highly needed. Untreatable infections have become a tragically frequent occurrence in patients infected with MDR *Pseudomonas aeruginosa* (Cryz, 1984). In addition, phages were found capable in disintegrating and eradicating biofilms of *Pseudomonas aeruginosa*. Samples for isolation of phages specific to *Pseudomonas aeruginosa* were mainly from fresh water lakes collected either from drainage of different localities as water of sewage or from feces of animal. Water of sewage is known to harbor many different bacteria and hence there is a likelihood of high prevalence of phages against different organisms. In vitro experiments have demonstrated the capacity of phage to reduce bacterial population size (Hall *et al.,* 2012).

Materials and Methods

Bacterial sampling, processing, and antibiotics testing

Bacterial sampling was conducted during the period from November 2013 to January 2014. Samples of bacteria were collected in Al-Imamein Al-kadhimein medical city hospital and Central teaching hospital for children. A total of 35 different *Pseudomonas aeruginosa* isolates were collected (17 ear swabs, 9 urine, 3 wound swabs and 6 blood specimens). The identification of *Pseudomonas aeruginosa* was later confirmed by routine bacteriological and biochemical assays. The bacteria were stocked in Luria–Bertani broth (LB) containing glycerol (30% v⁄v),

preserved at -20°C. Antibiotic sensitivity testing was carried out on 5 isolates of *Pseudomonas aeruginosa* to which specific phages were isolated. Antibiotic susceptibility test was carried on the bacterial isolates to determine MDR bacteria using method of disc diffusion assay (Bauer *et al.,* 1966). Accordingly, the size of inhibition zone determines whether isolated bacteria were resistant, intermediate, or sensitive. The antibiotic disks used were Imipenem (10μg), Ciprofloxacin (5μg), Gentamicin (10μg), Ceftazidime (30 μg), Cefotaxime (30μg), Ceftrixone (30μg), Trimethprime (10μg), Ticarcylin (75μg), Nalidixic acid (30μg), Nitrofurantin (100μg) and Chloromphincol (10μg).

Bacteriophages sampling, isolation, and propagation

Different crude samples for phage isolation were obtained from different regions in Baghdad including sewage (30–40ml), waste water (30–40ml), feces of sheep (20gm), chicken litter (15–20gm), swab from surgical lounge in Al-Imamein Al-kadhimein medical city hospital during the period from January 2014 to April 2014.

The procedure of isolating and propagating primary phages was done according to the methodology conducted in a recent patent of a member of our research team (Jassim *et al.,* 2010). Bacterial stocks were prepared by growing bacteria overnight on Luria broth. One hundred 100μl of 20 bacterial isolates were mixed together in a sterile 50ml test tube. Then, 2–3ml of crude samples, which were derived from sewage, cattle feces, chicken litter, mastitis discharge swabs that might contain *Pseudomonas*-specific phages were added to the mixture. Then, 2–3ml, equal volume, of nutrient broth and 2ml of Lambda buffer were added to the mixture as well. Then, the mixture was incubated overnight at 37°C with continuous shaking. Next day, 5ml of the crude mixture was dispensed into a sterile 15ml test tube, centrifuged at 1000 xg for 3 min at room temperature. One ml of supernatant was transferred to 1.5ml Eppendorf tube. Then, 1:10 v/v chloroform was added to the supernatant with gentle shaking for 7–10 min at room temperature to lyse the remaining bacteria. Centrifugation of the Eppendorf tubes at 1000 xg for 3min; the supernatant was transferred into new Eppendorf tube and equal volume of lambda buffer was added. Thus, the primary phage suspension, if any, was produced.

Phage spot lysis assay

Virulent phages were screened by phage spotting test on a nutrient-agar. Phage spotting can be used to provide a first approximation of the ability of a phage to lyse certain bacterial isolates. The formation of clear zones suggested the presence of lytic phages (Jassim *et al.,* 2010). At the first, the target bacteria were refreshed in Luria broth at 37°C for 24h. After overnight incubation, one ml of the bacterial broth was poured on to nutrient agar plate in order to make bacterial lawn. After 20–30 min, the lawn should have been dried. Using a mechanical pipette, 10μl of primary phage suspension were dropped on to the surface of the bacterial lawn and were allowed to dry before incubating at 37°C for 24h. On the next day, a lytic and specific phage can be discovered for the target bacteria if zone of lysis was developed at the spot where the primary phage suspension was applied. Then, specific lytic phages to MDR *Pseudomonas aeruginosa* were picked up by sterile loop and put into 1ml of Lambda buffer in 1.5ml sterile Eppendorf tubes with gentle shaking for 5 min. About 1:10 v/v chloroform was added to the lysate with gentle shaking for 5-7 min at room temperature. Host cell debris were pelleted by centrifugation at 1000 xg for 3 min, and the

supernatant containing phages was transferred to 1.5 ml sterile Eppndorf tubes and stored at 4°C. The supernatant was called transient phage stock suspension (Knezevic and Petrovic, 2008).

Top layer agar plaque assay

Serial dilutions of the phage preparation were mixed with a permissive host bacterium and dispersed evenly onto solid medium. Ten folds serial dilutions (10^1 to 10^7) were made with Lambda buffer for the phage stock solutions by taking 100 µl of the phage solution into 900 µl of lambda buffer. One hundred (100) µl of each dilution were transferred for each phage stock solution into 15ml volume sterile plastic container containing 100µl of 10^9 CFU/ml culture of targeted bacteria. After 10 min incubation at 37°C, 2.5 ml of top layer agar (soft agar or semi-solid agar of concentration 0.5% cooled at 45°C) were added. Immediately, the resulting mixture was poured over nutrient agar plates. This entailed the addition of 100µl of the different phage dilutions to 100µl of an overnight culture of their host strain. The soft agar overlay was allowed to solidify; the plates were incubated overnight at 37°C for 24h in an incubator. Next day, plaques are counted relying on the best dilution that yields the best rational number of plaques with clear margins and without merging to each other. The plaques were counted to determine the phage titer. In addition, plaques morphological characteristics, size, edge, and clarity, were determined. Moreover, top layer plaque assay is the best assay used for getting highest titers of phage suspensions (Budzik *et al.,* 2004). The phage titer was calculated by using the following formula:

Phage titer = number of plaques per plate x 100 x dilution factor

Accordingly, the clearest and largest plaques were selected; moreover, small or turbid plaques were subjected to optimization by conducting serial passage in top layer plaque assays; at each run, the best of the best plaques, in terms of the above mentioned parameters, were selected in order to acquire better virulence characteristics of the isolated lytic phages.

Preparation of secondary phages

In this approach, primary phages, after optimization, were tested for their ability to infect other isolates, other than the primary isolate to which the phage was isolated. Any newly detected lytic reaction was considered to be caused by secondary phages. All of the bacterial isolates were refreshed in Luria broth, and then incubated at 37°C for 24h. After overnight incubation, one ml of the bacterial broth was poured on to nutrient agar plate in order to make bacterial lawn. After 20–30 min, the lawn should have been dried. Using a mechanical pipette, 10 µl ofeachof the Five primary phage suspensions were dropped on to the surface of five bacterial lawns and were allowed to dry before incubating at 37^0C for 24h. On the next day, lytic secondary phages can be discovered for the target bacteria if zone of lysis was discovered. Also, plaques morphological characteristics, size, shape, edge and clarity, were determined.

Formation of the optimized ultimate phage stocks

In this endeavor, certain phage dilutions were used to obtain confluent plaques which were used to prepare concentrated phage suspensions by using sterile L-shape rod to scrape the plaques-filled top soft layer agar along with pouring 5ml of lambda-buffer on the scraped plate. Then, the scraped layer in lambda buffer was poured into 15ml sterile tube. Chloroform 1:10 v/v was added to the lysate to separate phages from the bacterial

cells. Tubes were shaken for 10min at room temperature. Then, centrifugation at 1000 xg was done for 5 min. The supernatant was collected that represents purified stock of phages with higher titer. The phage stocks were maintained in lambda-buffer at 4°C (Jassim *et al.,* 2010).

Resistance rate of *Pseudomonas aeruginosa* to infecting bacteriophages

In this approach, 10µl of each phage were spotted on the target bacterial lawn and phage spot lysis test was used to check the sensitivity of bacteria to the corresponding phage. Resistant bacterial colonies were identified by observing scattered bacterial colonies within the phage lysis spot. Number of phage-resistant colonies differed from spot to another depending on the bacterial isolate resistant to each phage (Jassim *et al.,* 2010).

The resistance rate of bacteria to infecting phages was measured. A piece from the same bacterial lawn of the target bacteria that is equal in diameter to phage lysis spot was cut by a sterile loop and put in 1.5 ml sterile Eppendorf tube containing one ml of normal saline. This approach is to obtain the same number of bacteria that was present in the phage spot lysis zone. Then, to dislodge bacteria from the cut piece of agar, the tube was subjected to periodic shaking for 5 min. Then, tubes were centrifuged at 1000xg for 3min at room temperature. Afterwards, the supernatant was removed and the precipitate was resuspended in one ml of normal saline. Ten fold serial dilutions of the resulting bacterial suspension (10^{-1}–10^{-5}) were made with normal saline by taking 100µl of bacterial suspension added onto 900µl of normal saline. Then, 10µl drop of the bacterial suspension was spotted on a nutrient agar plate inclined 45 degree in one direction in order to spread the drop to one direction forming lines at which counting of bacterial colonies becomes much easier. The plates were incubated at 37°C for 24h. The bacterial resistance rate was calculated as the following:

Resistance rate = Number of resistant colonies per phage lysis spot / number of bacterial colonies formed from the same size cut of bacterial lawn.

Results and Discussion

A total of 21 bacterial isolates of *Pseudomonas aeruginosa* were collected from hospitals. Among these clinical isolates, 5 isolates of bacteria were involved in this study after performing initial bacteriological and phage tests showing that these 5 bacteria did establish specific lyticphages as shown in Table 1.

Antibiotic susceptibility test

Antibiotic susceptibility testing was performed on Muller Hinton agar by Kirby-Bauer disk diffusion method against 11 different antibiotics and was interpreted according to CLSI (Clinical and Laboratory Standard Institute). It revealed that all of the tested isolates were resistant to three and more antibiotics which were designated as multidrug resistant (MDR) bacteria as shown in Table 2. Accordingly, all of the phage-susceptible isolates (5/5) were MDR bacteria.

Characteristics of the isolated and optimized primary phages

In this study, five phages active against *Pseudomonas aeruginosa* were isolated and purified. All of the isolated phages formed visible plaques in the early stage when tested on bacterial lawn of specific MDRs *Pseudomonas aeruginosa*. These phages were dubbed as primary phages. Primary

phages are those phages that were isolated directly from environment by showing lysis on bacterial lawns of MDR *Pseudomonas aeruginosa*. Primary phages were isolated from sewage 1/5 (20%), chicken litter 3/5 (60%), and sheep stool 1/5 (20%) and shown in Table 3.

Secondary phages to MDR *Pseudomonas aeruginosa*

When the primary phages, after *in vitro* optimization, show lytic specificity to the closely related strains of the same bacterial genus, they are called secondary phages. The characteristics of plaques of the secondary phages showed that these phages were generally less virulent than primary phages of the same bacterial isolate. The results are shown in Table 4.

The titer of the specific phages to MDR *Pseudomonas aeruginosa*

High titers of specific and lytic phages isolated and optimized to the studied bacterial isolates were obtained by using top layer plaque assay which was used to also further screen, amplify, and measure phages. Data were collected by visual examination of the plates and by manual plaques counting. The titers of the isolated and optimized phages are shown in Table 5.

Resistance rate of MDR *Pseudomonas aeruginosa* to phage cocktails versus individual phages

The current findings indicated that all of the primary and secondary phages were under effect of resistance development by the bacterial isolates tested. And phage cocktails showed much lower rate of bacterial resistance when compared to that of individual phages. This provided evidence on the additive effect of combinational phage therapy by using phage cocktails composed of a number of specific and lytic phages to certain bacterial isolate as shown in Table 6.

We chose *Pseudomonas aeruginosa* in this study because it is a dangerous opportunistic human pathogen that infects individuals with weakened immune systems, such as hospitalized patients and those suffering from severe burns or other traumatic skin damage (Kerr and Snelling, 2009). We focused our efforts on MDR *Pseudomonas* because we anticipated that positive results would demonstrate the potential of this form of phage therapy in situations where few alternatives are available nowadays.

The antibiotic resistance/susceptibility profile of *Pseudomonas* isolates revealed that most of the isolates were resistant to one or more tested antibiotics. This agrees with several previous studies conducted on *Pseudomonas aeruginosa* (Gales *et al.,* 2002; Gaynes and Edwards, 2005; Agarwal *et al.,* 2005). The reason that *Pseudomonas aeruginosa* is a vital target for phage therapy that it routinely exhibits efficient and multiple mechanisms of antibiotics resistance, including efflux pumps, antibiotic degrading or modifying enzymes of a variety of types, and limited membrane permeability (Wright, 2005; Nikaido, 2003; Li and Nikaido, 2009). Added to these, its growth occurs in dense biofilms, reducing the efficacy of multiple antibiotics (Davies, 1998). Phages use different mechanisms of bacterial killing than antibiotics making them potentially good candidates for alternative antibacterial therapy (Matsuzaki, 2005).

Nevertheless, phages are not excluded from bacterial defense mechanisms. Bacterial resistance to phages, that is acquired by mutations, typically involves loss or alteration of the bacteria-encoded phage

receptor molecules that are found on the surfaces of bacterial cells (Sturino and Klaenhammer, 2006). Actually bacteria develop resistance to phages more easily and quickly than do with antibiotics. But, the pipeline of discovering, optimizing and preparing new therapeutic phages is of endless feed (Devasahayam *et al.,* 2010).

The five phages obtained in this study displayed potent lytic activity and stability in laboratory; this might be explained that these phages were isolated from harsh environment, for example sewage.

The experiments in the current study presented solutions to many of the problems that hindered the prior applications of phage therapy. For example, the relatively narrow host range of most phages, which caused many of the early attempts of phage therapy to fail, can be overcome by isolating different phages and mix them together making phage cocktail with a broad host range within the species being targeted. Moreover, phage cocktails are of much lower tendency to be prone for bacterial resistance (Pirnay *et al.,* 2011).

The findings of the current study are in harmony with a previous report stating that phage cocktails could increase the effective host range and further reduce biofilm formation (Abedon, 2011; Hanlon *et al.,* 2001; Hughes *et al.,* 1998).

Moreover, our study reveals that if once bacterial isolate develops resistance to one phage in phage cocktail, the other phages in phage cocktail are still lytic for bacterial isolate; this observation is essential in establishing successful and effective phage therapy where minimal chances of host bacteria to resist phage-based killing. This notion is supported by a previous study (Skurnik and Strauch, 2006), which stated that a mixture of unique RBP-bacterial receptor phages are necessary in the success of phage therapy to any given host bacteria. Previous studies indicated that the phage cocktail can setback the appearance of phage-resistant variants and enhance treatment efficacy (Kudva *et al.,* 1999), (Tanji *et al.,* 2004).

In this concept, the phage cocktail used in the current research did not exhibit broad lytic range only, but also ensured that bacteria resistant to one phage remain susceptible to other phages and this potentially provides the main resource to evade resistance to a single phage (Cairns and Payne, 2008), (Kunisaki and Tanji, 2010). These results suggest that the use of a phage cocktail containing phages that use different receptors may have prevented the emergence of phage-resistant mutants and that the therapeutic phages had found their host and multiplied. Therefore, it seems that the potential obstacles to the use of this treatment including narrow host range of phage, resistance of host bacteria to phage, potential for inactivation by the patient's immune system, and safety of phage preparations in humans (Donlan, 2009), can be partially overcome by using unique receptor-specific phage cocktail.

Besides, the use of phage cocktail might have prevented the emergence of phage-resistant mutants which usually dominate over susceptible non-mutant bacteria causing phage therapy to fail.

Table.1 Characteristic features of *P. aeruginosa* to which lytic phages were isolated

Bacterial isolate	Site of isolation	Patient's age (year)	Patients sex	Disease
P1	Blood	16	Male	Septicemia
P2	Urine	35	Female	UTI
P3	Wound	45	Female	Wound infection
P4	Ear swab	25	Male	Otitis media
P5	Ear swab	45	Female	Otitis externa

Table.2 Antibiotic susceptibility test to phage-susceptible *P. aeruginosa*

Antibiotic	P1	P2	P3	P4	P5
Chloramphenicol(C)	R	R	S	S	S
Nitrofurantoin(NIT)	R	R	R	R	R
Cefotaxime (CEC)	R	R	S	S	R
Gentamycin(CN)	R	S	S	S	R
Ciprofloxacin(CIP)	S	S	R	S	S
Imipenem(IPM)	R	S	S	S	S
Ceftazideme(CAZ)	S	S	S	S	S
Ceftriaxone(CRO)	R	R	S	R	R
Nalidixic acid(NA)	R	R	S	R	R
Ticarcillin(TI)	R	R	S	R	S
Trimetheprime(TMP)	R	R	R	R	R

Table.3 The source of phage specimens and plaque characteristics of the isolated phages to MDR *P. aeruginosa* bacteria

Bacteriophage isolates	Source of phage specimen	Plaques Size (mm)	Margin cut	Plaques clarity	Plaques shape
PP1	Sewage	1.1	Irregular	Clear	Oval
PP2	Chicken litter	0.9	Irregular	Semi-clear	Circular
PP3	Sheep stool	0.8	Regular	Semi-clear	Circular
PP4	Chicken litter	1.3	Regular	Clear	Circular
PP5	Chicken litter	2.6	Regular	Clear	Oval

Table.4 Plaque characteristics of the secondary phages to *P. aeruginosa*

Bacterial isolate	Secondary phage	Plaques size (mm)	Margin cut	Plaques clarity	Plaques shape
P1	PP4	0.5	Regular	Semi-turbid	Oval
	PP5	0.4	Regular	Semi-turbid	Circular
P2	PP4	0.5	Regular	Semi-turbid	Oval
	PP5	0.4	Regular	Turbid	Circular
P3	PP1	0.7	Regular	Semi-turbid	Circular
	PP2	0.6	Regular	Turbid	Oval
	PP4	0.6	Irregular	Turbid	Circular
	PP5	0.5	Regular	Turbid	Oval
P4	PP3	0.3	Regular	Semi-turbid	Oval
	PP5	0.6	Regular	Clear	Oval
P5	None				

Table.5 The titer of specific and lytic phages to MDR *P. aeruginosa* using top layer plaque assay

Bacteriophage isolate	Titer (PFU/ml)
PP1	4.8×10^{10}
PP2	1.2×10^{10}
PP3	1×10^{9}
PP4	1.6×10^{10}
PP5	6.4×10^{8}

Table.6 Resistance rate of MDR *P. aeruginosa* to phage cocktails versus individual phages

	PP1	PP2	PP3	PP4	PP5	Cocktail
P1	$1:4.6 \times 10^{7}$	0	0	$1:6 \times 10^{7}$	$1:1 \times 10^{6}$	$0:4.2 \times 10^{9}$
P2	0	$1:1.9 \times 10^{6}$	0	$1:1.9 \times 10^{6}$	$1:1.6 \times 10^{7}$	$0:7.8 \times 10^{7}$
P3	$1:1 \times 10^{7}$	$1:1.4 \times 10^{7}$	$1:2.7 \times 10^{7}$	$1:1.5 \times 10^{7}$	$1:1.6 \times 10^{7}$	$0:6.2 \times 10^{7}$
P4	0	0	$1:2 \times 10^{6}$	$1:2.1 \times 10^{7}$	$1:2.4 \times 10^{7}$	$0:6.4 \times 10^{8}$
P5	0	0	0	0	$1:1.7 \times 10^{8}$	$0:2.3 \times 10^{8}$

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