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Application of bacteriophage cocktail to control multi-drug resistant *Pseudomonas aeruginosa*

Abstract

Multi–drug resistant *Pseudomonas aeruginosa*, a significant pathogen threats the public health with high mortality. The potential of phage cocktail was designed to lysis various bacterial sources. The candidate phage was isolated from soil, river water, tap water, food and human stool which belongs to Siphoviridae and Podoviridae family. The results identified that phage cocktails inhibited, lysed multi–drug resistant *Pseudomonas aeruginosa* in 30 minutes with 3 to 4 log CFU reduction. In addition, these cocktails showed effectiveness to bacterial strains isolated from wide sources including environment, food, and human. This renewed approach is contributed to overcome the dramatical increase of antibiotic resistance.

Keywords: multi–drug resistant bacteria, *Pseudomonas aeruginosa*, antimicrobial resistance, phage cocktails, phage isolations

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Introduction

Pseudomonas aeruginosa is frequently a crucial pathogen in hospital acquired and nosocomial infections. It is estimated that mortality rates fluctuated from 18–61% of nosocomial infections[1] and 21% in hospital, increasingly to 54.5% infected with multi-drug resistant strains.[2] The increasing multi-resistant strains to many classes of antibiotic such as beta-lactams, aminoglycosides and fluoroquinolones, imipenem, quinolones and third generation cephalosporins resisted against all relevant treatment and threated to patient's life.[2,3] Bacteriophages (phage) are often known as predators of their bacterial hosts that complete their evolution by lysis bacterial cell even bacteria can resist against to phage infection.[4,5] Nowadays, with globally increasing emergence of multi-drug resistance, phage therapy has renewed many optimisms for alternative methods or combined both antibiotic and phage as a strategy to combat antibiotic resistant strains (AMR).[6,7] This study aims to isolate phage in environment and apply this mixture of phage-phage cocktail for controlling multi–drug resistant *Pseudomonas aeruginosa* isolated from many sources.

Material and methods

Bacterial isolation and their susceptible profiles

All samples including stool from diarrheal patient, river water, tap water and ice water and food from local market were collected from provinces and city in the southern region of Vietnam. Then, strains were isolated by using specific agar and molecular test which certified with ISO 15189: 2012 approval. Disk diffusion test was applied to determine susceptibility profile of isolate. All strains resisted to at least one antibiotic such as meropenem, trimethoprim/sulfamethoxazole, Ceftriaxone, Ceftazidime, Augmentin, Gentamycin and Amikacin that was chosen for further test.

Phage isolation

Pseudomonas aeruginosa ATCC 27853 and *Pseudomonas aeruginosa* isolated from patients, soil was applied as indicator to phage isolation. Based on double layer agar methods (8), 300 ml of bacterial indicator solution (10^8CFU/ml) mixed with 100ml samples and 2.6ml of top agar (0.5% bacteriological agar), then poured onto TSA (ThermoFisher products). Following this, the plates were left to balance and dry on the bench for 25 minutes before inverted to incubate at 37°C overnight. Any determined plaques were harvested by sterile needle and resuspended into 3ml LB media contained indicator, then shaked in 3 hours. Next steps, all mixture filtered through membrane 0.2µm to collect pure lysate as phage. The whole procedure was repeated at least three times to obtain the single clone of phage. All phage was stored at 4°C for further test.

Transmission electron microscopy (TEM) of phage

After collecting the phage, each phage pellet was collected at high titre and sent to TEM service centre for processing the image capture.

Phage cocktail

High titre of each phage was prepared with some mixture (M) as Table 1. The high purify phage was prepared in Tryptic Soy Broth–TSB (ThermoFisher product) buffer and used immediately. The ratio between phage to bacteria was employed from 1.000 to 10.000 for these experiments. Three clones of phage with different diameter (D) were selected to make a matrix to check the effectiveness of lytic phage (Table 1).

Table 1 A matrix used to test the effective of phage cocktail

	Strains (CFU/ml)	Phage inoculum (PFU)		
		D≤1mm	D=1–2mm	D≥2mm
M1	10^7	10^2	10^2	10^2
M2		10^4	10^2	10^2
M3		10^2	10^4	10^2
M4		10^2	10^2	10^4

Survival of bacteria

Double layer agar methods[8] was still applied to check the bacterial survive (CFU) at 30 mins, 2 hours, 4 hours, and overnight. The lytic phenomenon and PFU/ml were observed and counted as the effectiveness of the results.

Results

Selection and image of phage

Phage accounted for 25% (47/191) in total collective samples in which 100% of soil and tap water samples, the phage rates fluctuated from 11.11% to 33.33% in river water and food respectively, the ratio of phage isolation was smallest in stool of human (5.26%). The distribution of phage differed from provinces, but they existed in wide range of samples (Table 2). Phage isolated from samples had various size of lysis bacterial cell. Diameter of them was less than 1mm, 1–2mm and over 2mm. The number of plaques forming unit (PFU) was also different from using samples sources (Figure 1). Based on the morphologies of phage particles examined with TEM, our phage was classified to Siphoviridae family (Figure 2A), showed a head size of 56.2nm and 53.1nm of Podoviridae family (Figure 2B).[9]

Phage cocktail lysis bacteria

Three of *Pseudomonas aeruginosa* from our collection was chosen as indicator for phage cocktails. The results showed that all the mixture worked well (Table 3). Most of lytic phase damaged bacterial cell after 30 minutes and nearly whole bacterial cell was removed after overnight, from 3 to 4 log CFU reduction. Among these cocktails, mixture M2 (2:1:1) and M4 (1:1:2) were the best candidate for application on the isolates. All cocktails were prepared from high phage titre collected from soil and food and river water instead of from human, tap water due to low titre.

Table 2 Distribution of phage in different provinces

Sample	Provinces					
	LĐ	BD	TPHCM	ST	BT	AG
Soil	100	100				
Tap water		100				
River water			25	11.11	25	
Food			25			33.33
Stool - Human						5.26

Notes: LD, Lam Dong; BD, Binh Duong; TPHCM, Ho Chi Minh city; ST, Soc Trang; AG, An Giang

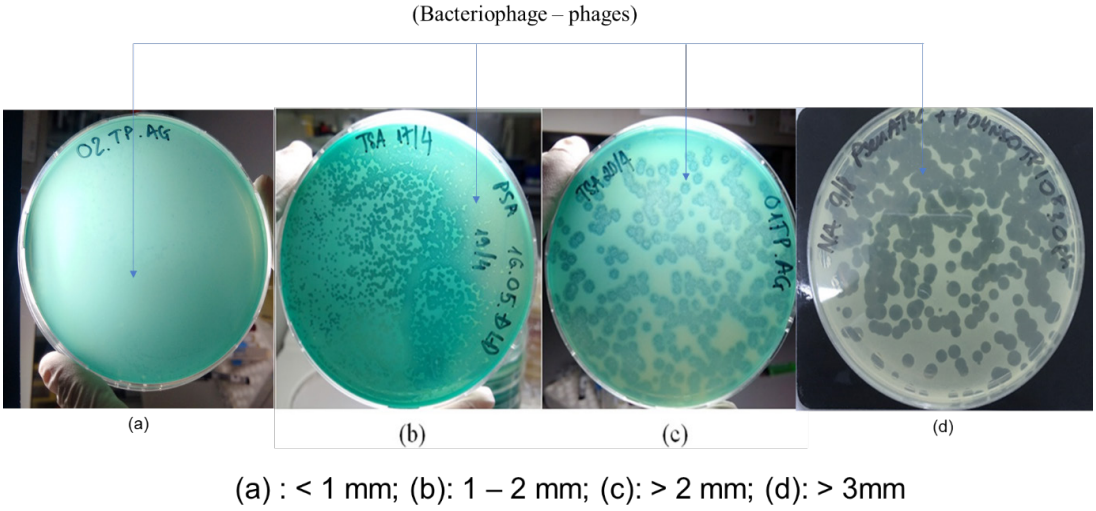

Figure 1 Size and number of PFU (plaque forming unit) on agar.

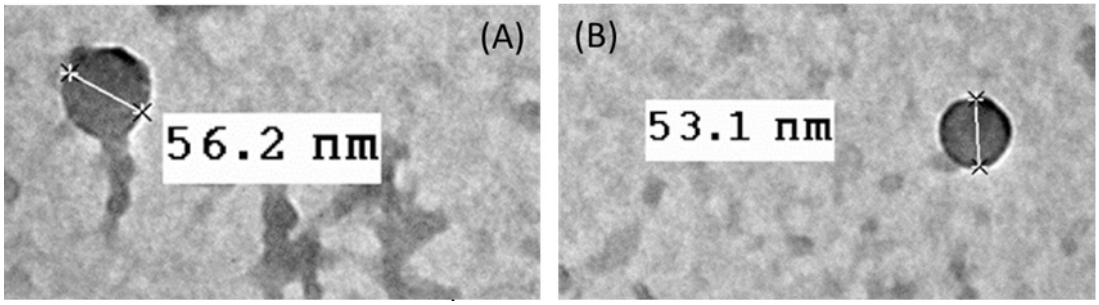

Figure 2 Phage particle was presented by TEM.

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Table 3 Results of effective lytic phage to different indicator

Strains (10^7 CFU/ml)		Phage inoculum (PFU)			Plaque forming unit (PFU/ml)
		D≤1mm	D=1–2mm	D≥2 mm	
M1	Reference strain – ATCC 27853	10^2	10^2	10^2	>1000
M2		10^4	10^2	10^2	>1000
M3		10^2	10^4	10^2	>1000
M4		10^2	10^2	10^4	>1000
M1	Strain isolated from patient	10^2	10^2	10^2	600
M2		10^4	10^2	10^2	>1000
M3		10^2	10^4	10^2	400
M4		10^2	10^2	10^4	800
M1	Strain isolated from soil	10^2	10^2	10^2	>1000
M2		10^4	10^2	10^2	>1000
M3		10^2	10^4	10^2	>1000
M4		10^2	10^2	10^4	>1000

In our collective bacterial strains, 30 of them resisted to at least one type of antibiotic or multi-drug resistant strains to meropenem, trimethoprim/sulfamethoxazole, Ceftriaxone, Ceftazidime, Augmentin, Gentamycin and Amikacin. Among that, wild type resisted from one to four antibiotic was 10%, 30%, 56.7% and 3.3% respectively. Whilst there was a little difference between lytic capacity of phage to strains isolated from tap water and iced water, most of phage killed the bacterial cell (Table 4). The number of PFU was over 1000 and nearly reduced all colony forming unit (CFU) of bacteria.

Table 4 Results of effective lytic phage to isolates

STT	Strains	Source	Susceptibility profiles							Mixtures	
			MRP	SXT	CRO	CAZ	AMC	CN	AK	M2	M4
1	16.01	River water	S	R	R	S	R	S	S	+++	+++
2	16.02	River water	S	R	I	S	R	S	S	+++	+++
3	16.03	River water	S	R	R	S	R	S	S	+++	+++
4	16.04	River water	S	R	R	I	R	R	S	+++	+++
5	16.05	River water	S	R	S	S	R	S	S	+++	+++
6	17.06	River water	S	R	R	I	R	S	S	+++	+++
7	16.03	Tap water	S	R	R	S	R	S	S	+++	+++
8	16.06	Tap water	S	S	S	S	R	S	S	+++	+++
9	16.08	Tap water	S	R	S	S	R	S	S	+++	+++
10	17.03	Tap water	S	R	R	S	R	S	S	+++	+++
11	17.05	Tap water	S	R	R	S	R	S	S	+++	+++
12	17.06	Tap water	S	R	R	S	R	S	S	++	++
13	16.01	Iced water	S	R	I	S	R	S	S	++	++
14	16.02	Iced water	S	R	I	S	R	S	S	++	++
15	16.03	Iced water	S	R	I	S	R	S	S	++	++
16	16.04	Iced water	R	S	S	S	S	S	S	++	++
17	17.01	Iced water	S	R	R	S	R	S	S	++	++
18	17.05	Iced water	S	R	R	S	R	S	S	++	++
19	16.01	Food	S	R	R	S	R	S	S	+++	+++
20	16.02	Food	I	R	I	S	R	S	S	+++	+++

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Table Continued...

STT	Strains	Source	Susceptibility profiles							Mixtures	
			MRP	SXT	CRO	CAZ	AMC	CN	AK	M2	M4
21	16.03	Food	R	R	R	I	R	S	S	+++	+++
22	16.04	Food	R	R	S	S	R	S	S	+++	+++
23	16.05	Food	S	R	S	S	S	S	S	+++	+++
24	17.02	Food	S	R	R	S	R	S	S	+++	+++
25	17.07	Food	R	R	I	S	R	S	S	+++	+++
26	17.03	Stool – human	S	R	R	S	R	S	S	+++	+++
27	17.04	Stool – human	S	R	R	I	R	S	S	+++	+++
28	17.05	Stool – human	S	R	I	S	R	S	S	+++	+++
29	17.06	Stool – human	S	R	I	S	R	S	S	+++	+++
30	17.13	Stool – human	S	R	R	S	R	S	S	+++	+++

Note: ++, PFU=>1000; +++, PFU=>2000; R, resistance; S, sensitive; I, intermediate

Discussion

Pseudomonas aeruginosa, one of three pathogens required urgent action can be found in various surfaces and aquatic habitats with high resistant profiles such as ceftazidime (63.9%), piperacillin (58.3%), cefepime (55.6%), imipenem (50%), piperacillin/tazobactam (47.2%), meropenem (41.7%).[10,11] In Vietnam, the resistant ratios of bacteria fluctuated on specific antibiotics such as ceftazidime (42.9%–45.8%), meropenem (37.1%–38.4%), gentamycin (39.7–41.8%), Amikacin (17.4 %–18.24%), ceftriaxone (4.72%), levofloxacin (41.3%–43.9%), ciprofloxacin (40.5–42.7%).[12,13] Most of strains, however, isolated on patients and it is rarely to report on food or environment. Our collective strains presented in numerous sources with high resistance profiles. This showed that bacterial antibiotic resistance was not only hospital acquired infections, but nosocomial infections was dangerous as well. This wild type strains have apparently transmitted to food manufacturing or food cycle and threated to public health that must be more attention now.

The data obtained from this study showed that various circulation of phage in environment and stool of human. Nevertheless, few numbers of phage isolated from human hypothesized that the bacteria isolation on human was more common than other sources because the inhibited–function of phage can control the bacterial growth and dissemination.[14] This observation has also pointed in some places where phage circulated, the less bacteria had been found in another our research.[8] Importantly, the different distribution of phage from provinces could be affected by environmental condition, in which temperature was a key factors.[15]

Interestingly, our lytic phage titre was high and promised to control the increase of multi- drug resistant *Pseudomonas aeruginosa.* To combine some phage species into one cocktail, this effectively enables to inhibit, kill, and reduce most of multi-drug resistant *Pseudomonas aeruginosa* which come from many numerous sources. Despite this only was in vitro test, but it's fully applicated to in vivo. Whereas, phage may produce toxin and effect to treatment therapy,[5] phage therapy, phage cocktails are going to application and many successful clinical trial has been reported.[7,16] In our experiment, with two mixtures was the same potential to apply to decontaminate *Pseudomonas aeruginosa* as promising therapeutic or spray decontamination. Notably, antibiotic needs to compulsory and failure to control multi–drug resistance bacteria, phage was easy to isolate from environment and their effectiveness has been proved.

Conclusion

In summary, the application of phage cocktails renewed approach to combat the multi-drug resistant *Pseudomonas aeruginosa*. In such data, the initial dose of phage in short time, just 30 minutes was sufficient to treatment without waiting the replication of phage. Their broad spectrum to all bacterial isolates from many sources has been promisingly applied as phage therapy or disinfectant agents to decontamination of multi-drug resistant *Pseudomonas aeruginosa.*

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Conflicts of interest

I declare no conflict of interest.

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