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Comparison of the in vitro efficacy of commercial bacteriophage cocktails and isolated bacteriophage vB_Pa01 against carbapenem resistant nosocomial *Pseudomonas aeruginosa*

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ABSTRACT: The result of a crucial rise in the prevalence of antibiotic resistance of bacteria, and the development of an inadequate number of new antibiotics, over the last decade a marked increase in interest in the studies of phages has been observed. It was aimed to determine the commercial preparation including Pyo-bacteriophage and Intesti-bacteriophage and compare their efficiency with isolated bacteriophage vB_Pa01 against carbapenem resistant *Pseudomonas aeruginosa*. Susceptibility to the bacteriophages present in each cocktail was tested using the spot test. A total of 126 carbapenem-resistant *P. aeruginosa* isolates were included in this test. Bacteriophage susceptibilities of each isolate were tested by spot test method. Confluent, semi confluent, opaque lysis was considered a sensitivity. The absence of any lysis is reported as resistance. The lytic activity of the bacteriophage was found 46% (58/126) Pyo-bacteriophage and 36.5% (46/126) Intesti-bacteriophage and 63.5% (80/126) vB_Pa01 bacteriophage. The most effective bacteriophage preparation was the vB_Pa01 bacteriophage (63.5%) and the least effective one was Intesti-bacteriophage (36.5%). 95 (75.4%) of 126 carbapenem resistant. This is the first study that investigated two commercial bacteriophage cocktails against carbapenem resistant *P. aeruginosa*. The higher efficacy of vB_Pa01 bacteriophage is promising for creating an alternative preparation especially in the treatment of infections caused by MDR pathogens that are difficult to treat.

KEYWORDS: Bacteriophage; carbapenem resistant *Pseudomonas aeruginosa*; commercial bacteriophage cocktail; lytic activity.

1. INTRODUCTION

Carbapenems are effective against most bacterial species resistant to beta lactams such as penicillin and cephalosporin. Due to resistance to many antibiotics, especially cephalosporins, carbapenems are widely used in the treatment and consequently, an increasing problem of resistance to carbapenems has been observed, among non-fermentative gram-negative bacteria. Infections caused by resistant microorganisms increase mortality, prolong hospital stay and cause problems such as increases in treatment costs. In some cases, only colistin remains an effective antibiotic. However, both nephrotoxicity and neurotoxicity have limited the clinical use of colistin (e.g, hospitalized persons) even colistin resistant strains have been reported. Multiple antibiotic resistance developing in *P. aeruginosa* strains has become a crucial problem worldwide [1-3].

With treatment failures emerging in parallel with the increasing antibiotic resistance all over the world, interest in new treatment options has increased. Bacteriophage therapy is among the prominent alternative treatment methods in studies [4, 5]. Bacterial viruses that target and kill specific bacteria defined as bacteriophages [6]. Bacteriophage described in 1915 by Frederick Twort as a factor that kills bacteria with infecting and named by Felix d'Herelle as a bacteriophage in 1917 [7, 8]. Phages, which are obligate intracellular parasites of the bacterial cell, show different life cycles such as lytic, lysogenic, pseudolysogenic and chronic infection in the bacterial host. Virulent or lytic phages infect and rapidly kill their bacterial host cell, while temperate or lysogenic phages can stably integrate into their host's genome or enter into the lytic life cycle [9]. Phage therapy is defined as the application of lytic phages directly to a patient with the aim of

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lysing the bacterial pathogen causing a clinically relevant infection [10]. Lytic bacteriophages used in the treatment of infections including upper respiratory tract, abscess, burns, and wounds infections in some countries but with the discovery of antibiotics, remained in the background. However, thereafter, with the same rapidly developing resistance, interest in bacteriophages increased again. Bacteriophages have some advantages such as protecting the natural microbiota, non-toxicity, cheap, and easily obtainable, acting independently of antibiotic resistance, and being effective on biofilms. However, having a narrow spectrum of action and the fact that the agent/bacteriophage relationship has not been defined before the bacteriophage treatment is the factor that limited clinical use [8]. Nowadays that especially personalized treatment is gaining more importance, bacteriophages are thought to be one of the promising alternatives in the treatment of infections caused by resistant bacteria.

In this study, the commercial preparation Pyo-bacteriophage (Eliava Institute, Tbilisi, Georgia) and Intesti-bacteriophage purchased from the George Eliava Institute Pharmacy, Georgia, tested against the carbapenem-resistant *P. aeruginosa* strain isolated from intensive care patients. Also, the efficiency of a newly isolated lytic bacteriophage vB_Pa01 was also compared with these commercial preparations.

2. RESULTS

In this study, it was aimed to compare the in vitro activities of two different commercial bacteriophage preparations and a newly isolated vB_Pa01 bacteriophage against carbapenem-resistant *P. aeruginosa* strains isolated in hospital intensive care patients. Initially, 7 seemingly morphologically different phages were isolated from environmental water by enrichment with 4 strains of *P. aeruginosa*. Characteristics of these phages were determined by the diameter and turbidity of phage plaques and the presence of a halo. After characterizing the morphology of phage plaques, one newly isolated phage was selected for bacteriophage susceptibility test and named as vB_Pa01. The vB_Pa01 bacteriophage plaque morphology image is given in Figure 1.

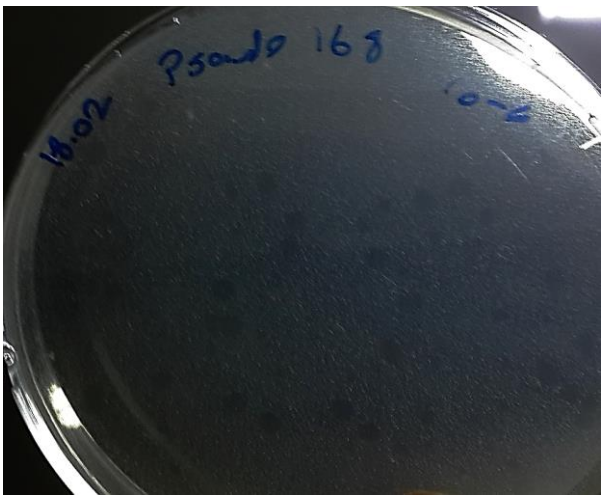

Figure 1. A newly isolated vB_Pa01 bacteriophage.

A total of 126 carbapenem-resistant *P. aeruginosa* isolates were included in this test. Bacteriophage susceptibilities of each isolate were tested by spot test method. Confluent, semi confluent, opaque lysis was considered a sensitivity. The absence of any lysis is reported as resistance. The bacteriophage susceptibility results of the pathogens are shown in Table 1. The lytic activity of the bacteriophage on the 126 carbapenem-resistant *P. aeruginosa* strains varied between 46% (58/126) Pyo-bacteriophage and 36.5% (46/126) Intesti-bacteriophage and 63.5% (80/126) the vB_Pa01 bacteriophage. The most effective bacteriophage preparation was vB_Pa01 bacteriophage (63.5%) and the least effective one was Intesti-bacteriophage (36.5%). 95 (75.4%) of 126 carbapenem resistant *P. aeruginosa* strains included in the study were found to be susceptible to bacteriophage isolated or bacteriophage cocktails. According to this; While 95 strains were found to be susceptible to at least one phage tested, 31 *P. aeruginosa* strains were found resistant to all bacteriophage tested. The Intesti-bacteriophage preparation was less effective on all *P. aeruginosa* isolates except for one strain. The Pyo-bacteriophage preparation was less effective on all *P. aeruginosa* isolates except for six strains. The vB_Pa01 bacteriophage was found to be effective in 30 of the bacterial strains.

Table 1. Bacteriophage susceptibilities of 126 carbapenem resistant *P. aeruginosa* isolates.

Bacteria ID	Intesti-bacteriophage	Pyo-bacteriophage	vB_Pa01 Bacteriophage
1	-	+	+++
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	+
6	+	+	-
7	-	-	-
8	-	-	+
9	-	+	++
10	+++	+++	+++
11	+++	+++	+++
12	+++	+++	+++
13	-	+++	+++
14	-	-	-
15	-	+	-
16	+	+	+++
17	+++	+++	+++
18	++	++	+++
20	+++	+++	+++
21	+	+	++
21	-	+	-
22	-	-	+++
23	+	+	-
24	-	-	-
24	-	+	-
25	-	-	+++
26	-	-	-
27	-	-	-
28	-	-	-
29	-	-	-
30	-	-	-
31	-	-	-
32	-	+++	+++
33	+	++	+++
34	+	+	-
35	+	-	-
36	+	+	+++
37	+	+	++
38	-	-	+
39	-	+++	+++
40	-	+	-
41	-	-	+++
42	-	-	+++
43	+	+	+++
44	-	-	+++
45	++	++	++
46	+++	+++	+++
47	-	+++	+++

Table 1. (Continued) Bacteriophage susceptibilities of 126 carbapenem resistant *P. aeruginosa* isolates.

Bacteria ID	Intesti-bacteriophage	Pyo-bacteriophage	vB_Pa01 Bacteriophage
48	-	-	+++
49	-	+++	+++
50	-	-	-
51	-	+	+
52	+	-	+
53	-	-	-
54	-	-	+
55	-	-	-
56	-	-	+++
57	+++	+++	+++
58	-	-	-
59	-	-	+
60	-	-	-
61	-	-	+++
62	-	-	+++
63	-	-	+++
64	-	-	+++
65	-	-	+++
67	-	+	+++
68	-	-	+++
69	++	++	+++
70	-	-	+++
71	+++	+++	+++
72	+	-	+
73	-	-	-
74	-	-	+++
75	-	-	-
76	+++	+++	+++
77	+++	+++	+++
78	-	-	++
79	-	-	+++
80	+++	+++	+++
81	-	-	-
82	-	+	+
84	-	+	-
85	-	-	-
86	+++	+++	+++
87	+++	+++	+++
88	-	-	-
89	-	-	-
90	+	++	+++
91	+++	+++	+++
92	-	-	-
93	+++	+++	+++
94	++	++	++
95	-	-	+++
96	-	-	-

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Table 1. (Continued) Bacteriophage susceptibilities of 126 carbapenem resistant *P. aeruginosa* isolates.

Bacteria ID	Intesti-bacteriophage	Pyo-bacteriophage	vB_Pa01 Bacteriophage
97	-	-	-
98	-	-	-
99	-	-	-
100	-	-	+++
101	++	++	+++
102	+++	+++	+++
104	-	-	-
106	++	++	+++
107	+	+	+++
115	++	++	+++
117	+	-	-
118	-	-	-
119	+	+	-
128	-	+	+
129	-	-	-
130	-	-	+++
132	-	-	-
133	-	+	-
134	+	+	+
135	+++	+++	+++
136	-	-	+
137	-	+	+
138	+	+	-
140	++	+	+++
141	-	-	+++
142	-	-	+++
143	++	++	+++
144	++	-	+++
145	-	-	+++
146	++	-	+++
148	-	-	+++

+++: CL (Clear Lysis), ++: SCL (Semi-Clear Lysis), +: OL (Opaque Lysis), -: No lysis.

3. DISCUSSION

There is a significant increase in the number of multi-drug resistant microorganisms worldwide due to the misuse and unconscious use of antibiotics and the ability of bacteria to transmit resistance genes, and antibiotic resistance is considered one of the most important public health threats. Difficulty/failure of treatment in infections associated with multiple antibiotic resistance causes both prolonged hospital stays and increased morbidity and mortality rates. The World Health Organization listed global priority pathogens for research and development of new antimicrobials and carbapenem resistant *P. aeruginosa* is listed in the priority 1-critical group [11]. This situation of antibiotic resistance has accelerated the research on the discovery of new antibiotics, as well as, studies related to the use of phage and aimed at reducing the virulence of bacteria.

The studies in the literature have indicated that bacteriophages have the potential to alleviate the problem of infectious diseases, either as an alternative to antibiotics or in combination with antibiotic therapies. Although there are studies showing the clinical efficacy of phages, there is still an information gap in this area that requires additional studies. However, studies show that the use of bacteriophage especially with antibiotics is promising [12]. No study was found that performed the lytic effects of commercial bacteriophage on carbapenem resistant *P. aeruginosa* clinical isolates in our literature search but found some

studies that determined the lytic activity of these commercial phage cocktails against MRSA and ESBL positive *E. coli*. In a study conducted on 144 ESBL positive *E. coli* isolates, Gundogdu et al. performed spot test and indicated that Enko-phage was active against 87.3% of the tested strains while that ratio was 81.7% for Intesti-bacteriophage, 81.7% for Pyo-bacteriophage, and 59.2% for SES-bacteriophage cocktails [13]. Sybesma et al. found that the lytic activity of commercial bacteriophage cocktails varied between 66% (Pyo-bacteriophage) and 93% (Enko-bacteriophage) on 41 *E. coli* isolates of urinary tract infection [14]. However, in these studies in which the effects of commercial phage cocktails are investigated, researchers did not isolate the phage and therefore did not make a phage/phage cocktail comparison. The lytic activities of commercial preparations on *E. coli*, *S. aureus* are found to be higher in some studies, but similar to our study in the literature, there are also results with lower efficacy [15, 16]. Hughes et al. (2014) in their study conducted with ESBL positive *E. coli* isolates, reported the lytic activity of commercial phage cocktails as 36% and 54% for Pyo and Intesti, respectively. Also, they isolated bacteriophage from sewage water and 11 *E. coli* isolates were determined to be sensitive (82%) to this phage [14]. In our study, the efficiency of commercial preparations was found as 46% (58/126) for Pyo-bacteriophage and 36.5% (46/126) for Intesti-bacteriophage, and the efficacy of the vB_Pa01 bacteriophage (63.5%) was higher than the commercial preparations.

These results show that the efficacy of commercial preparations due to their ingredients may vary according to bacteria. Also, considering that the phage we isolated is more effective, it is thought that the regional differences between the phage in the cocktail and the bacteria tested may be reflected in the result. This situation also contributes to the importance of local phages. Also, considering that these phages have high lytic activity on some bacteria, their use potential is high when the agent can be isolated and phages can be tested before.

4. CONCLUSION

Alternative treatment methods are necessary to treat Acinetobacter, Enterobacteriaceae, Pseudomonas, Staphylococcus infections because resistance increases. Bacteriophages are being identified, purified, and developed as pharmaceutically acceptable macromolecular compounds. Thus, it appears that phage therapy will be widely available to fight multidrug-resistant infections. Although small-scale studies have demonstrated the potential of phage therapy for bacterial infections, the widespread applicability of this therapy has not been shown in clinical trials. In conclusion, the result of our study shows that the ready-to-use phage preparations have the potential to treat resistant infections and that local new phage isolates may be more effective in preventing infections. Detailed tests and in vivo experiments of the phage isolated here are required, and our study gains importance as a preliminary preparation for the studies to be carried out.

5. MATERIALS AND METHODS

5.1. Bacterial collection

To test the efficacy of commercial phage cocktails and newly isolated lytic phage vB_Pa01 were evaluated to 126 *P. aeruginosa* isolates identified as causative agents of nosocomial infections in various hospitals in Turkey and reported to be carbapenem resistant were used. Meropenem and imipenem susceptibilities of these strains were performed using the E-test (AB Biodisk, Solna, Sweden).

5.2. Bacteriophage cocktails

Two commercially available bacteriophage cocktails produced by George Eliava institutions located in Tbilisi were tested. According to the manufacturers, they are all sterile-filtrate phage lysates of different bacterial species as listed below. These cocktails were purchased from the George Eliava Institute Pharmacy, Georgia. Two liquid form phage preparations (Pyo-bacteriophage, Intesti-bacteriophage) were used for carbapenem resistant *P. aeruginosa.* The preparation lot numbers applied during the current study are indicated.

5.2.1. Pyo-Bacteriophage

Pyo-bacteriophage (lot # M1-1001; Eliava BioPreparations, Tbilisi, Georgia) is a mix of sterile filtrates of phage lysates active to: *Staphylococcus spp., Streptococcus spp*., Different types of *E. coli*, *P. aeruginosa*, *Proteus mirabilis* and *Proteus vulgaris*.

5.2.2. Intesti-Bacteriophage

Intesti-bacteriophage (lot # M2-1001; Eliava BioPreparations, Tbilisi, Georgia) is a mix of sterile filtrates of phage lysates active to: Shigella (*Shigella flexneri* serotype 1,2, 3, 4 and *Shigella sonnei*), Salmonella (*S. paratyphi* A, *S. paratyphi* B, *S. typhimurium*, *S. enteritidis*, *S. cholera suis*, *S. oranienburg*), Different types of *E. coli*, *Proteus mirabilis, Proteus vulgaris*, Staphylococcus (*S. aureus*), *P. aeruginosa* and *Enterococcus spp*.

5.3. Bacteriophage isolation

For bacteriophage isolation, phage enrichment was performed firstly. The environmental water samples taken were centrifuged at 10.000 rpm to remove particles and passed through a 0.45 µm membrane filter to the supernatant. Overnight fresh bacterial cultures were inoculated into x2 Luria Bertani Broth (Merck) medium enriched with $CaCl_2$ and $MgSO_4$ and incubated for 1 night at 37 °C. On the second day, the suspension was centrifuged for 10 minutes at 10.000 rpm and the bacteria remaining in the supernatant were killed by adding 10% chloroform [17, 18]. The spot test method was used to determine the possible presence of bacteriophage. Strip cultivation was made from fresh bacterial culture and 10 µl of bacteriophage suspension was dropped to the cultivation areas. After overnight incubation, the lysis occurred in the cultivation areas, and was evaluated [19]. The double layer agar method was used to determine the presence of bacteriophage in samples with lysis as a result of the spot test. The supernatant was mixed with fresh bacterial culture and soft agar (0.7% agar) and spread on a pre-poured agar plate (1.5% agar) and incubated for 1 night at 37°C. The next day, petri dishes were evaluated for the presence of bacteriophage plaques [20].

5.3.1. Single plaque isolation

Single plaque isolation was made for purification in petri dishes with bacteriophage plates. The formed plates were cut from their areas with a sterile pasteur pipette and taken into a 3 mL Luria Bertani broth (Merck) medium. Fresh bacterial culture was added and left for 10 minutes of phage-bacteria adsorption and again Luria Bertani broth was added and incubated overnight at 37°C. The next day, the bacteriophage suspension was centrifuged and filtered through a 0.22 µm membrane filter. Double layer agar method was applied to each dilution. This stage was repeated at least 5 times. The samples that did not show uniform plaque on the Petri dish, the process was continued until a uniform type was seen [21, 22].

5.3.2. Bacteriophage enrichment

To obtain the concentrated bacteriophage suspension, a double layer agar was applied to the dilution where the phage and its host achieve the most appropriate growth together. The next day, the soft agar part was taken with a Dragalski spatula and the bacteria were killed by adding chloroform and vortexing it. The bacteriophage titer was determined as a 'plaque forming unit' (PFU) by making 10-fold serial dilutions.

5.4. Bacteriophage susceptibility

Spot test (instillation) were applied to determine the lytic spectra of vB_Pa01 bacteriophage and two commercial bacteriophages. The vB_Pa01 bacteriophage stock was diluted 10^8 PFU/mL with LB broth. From the fresh cultures of the hospital isolates, 10^8 CFU/mL concentration was streaked and 10 µl of diluted bacteriophages were dropped to these areas. At the end of an overnight incubation, the areas where no growth was observed in the sowing areas were evaluated as; CL (Clear Lysis), SCL (Semi-Clear Lysis), OL (Opaque Lysis), or No lysis. The results were replicated three times and were consistent for all strains tested [23].

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