Iranian Journal of Microbiology
Volume 14 Number 3 (June 2022) 395-401

ORIGINAL ARTICLE

Application of bacteriophage cocktails for reducing the bacterial load of nosocomial pathogens in hospital wastewater

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Received: July 2021**, Accepted:** April 2022

ABSTRACT

Background and Objectives: In the third world and developing countries, hospital sewage is mixed with municipal wastewater. The treated effluent contains dangerous bacteria released into the environment and used in the irrigation of agricultural products, and eventually these bacteria may endanger the human health through foods. Antibiotic-resistant bacteria are mostly found in hospital wastewater. In water and wastewater treatment plants, large amounts of toxic and polluting substances are removed and destroyed, but this process does not eliminate bacteria.
Materials and Methods: Wastewater samples from 22 hospitals in Iran were collected and in the meantime specific phages (against drug-resistant pathogenic bacteria) extracted using the bilayer agar technique. Phage amplification was performed by employing a fermenter after phage identification. Amplified phages were added to the primary sedimentation pond using New-Brunwick biofermenter BioFlo/Celligen®115 and the bacterial count was evaluated for the desired bacteria.
Results: Our phage cocktail was able to reduce 99.8%, 99.4%, 99.5%, 99.8%, 99.7%, 99.8%, 99.6% and 99.9% of *E. coli, E. faecium, E. faecalis, K. pneumoniae, A. baumannii, P. aeruginosa, S. maltophilia* and *S. aureus* counts respectively.
Conclusion: The application of phage cocktails can remarkably help improve personal hygiene, the environment, and the optimization of surface water.

Keywords: Wastewater; Ponds; Bacteriophages; Environmental pollution; Drug resistance

INTRODUCTION

Industrial residual, urban, hospital, and agricultural waste are called wastewater. The composition of wastewater varies widely depending on its source, but often is formed from water, microorganisms such as bacteria, viruses, prions, parasites, organic particles such as feces, hair, and food as well as mineral particles, large solids, pesticide, chemicals, etc (1). About 90% of the world's wastewater remains untreated, causing widespread surface and groundwater pollution, especially in low-income countries. The use of untreated wastewater, often contaminated with hospital wastewater, to irrigate agricultural lands is increasing while no alternative exists for farmers. Usually, hospital sewage contains pharmaceuticals, metabolites, biomolecules, anions, cations, radioactive isotopes, heavy metals, antibiotics, bac-

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terial pathogens, antiseptics and disinfectants (2).

Among various sources of wastewater, the hospital wastewater can be very dangerous due to the presence of a wide range of pathogenic microorganisms, endangering the health of environment, health care workers, and the society as a whole (3, 4). "Antibiotic resistance" is a well-known term in medicine and microbiology, which is one of the most serious concerns of the World Health Organization in the 21st century (5, 6).

Studies have shown that almost 70% of bacteria are resistant to antibiotics, which are now entering the agricultural water sources and municipal and hospital wastewater (5). Thus, we are encountering the evolution of more and more bacteria in the field of antibiotic resistance.

Hospitals, as a place for treatment of patients, are the main ecological sites for colonization of bacteria resistant to a wide range of antibiotics and disinfectants. Bacteria such as methicilin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli* and Extended Spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae*, which are known to cause nosocomial bacterial infections (7-12). These bacteria leave the hospital through the sewage system, but the situation even worsens when the hospital sewage enters directly into the municipal and agricultural sewage network (4).

Bacteriophages are actually viruses that infect or kill bacteria. Unlike antibiotics, phages are completely specific for the target bacteria and do not have any side effect on human cells and the human microbiome population (13). Phages are the most abundant submicroscopic infectious particle on the planet (around10^{31}) (14). Phages are necessary for controlling the population of bacteria in the environment and inside the human body (15). They are widely distributed in crowded places such as hospitals, livestock, slaughterhouses, sewage, soil, and aquatic environments (15).

To optimize hospital wastewater, there are currently several mechanisms such as ozonation, filtration technology, solar disinfection, UV irradiation, and heat pasteurization to reduce the bacterial load but these techniques are not enough to completely overcome the population of pathogens (3). Therefore, we aimed to use bacteriophages for reducing the microbial load of pathogenic bacteria in hospital wastewater and improving the public health. We hypothesized that cocktail of different lytic phages can reduce the pathogenic bacteria released to the hospital wastewater. The use of phage cocktails to improve hospital wastewater has not been done so far.

MATERIALS AND METHODS

Bacterial isolation and identification. Nosocomial bacteria pathogens including *E. coli*, *E. faecium*, *E. faecalis*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *S. maltophilia*, and *S. aureus* were isolated and identified using conventional protocols from the samples collected from 22 hospitals in eight cities throughout Iran (Tehran, Qazvin, Ahvaz, Tabriz, Shiraz, Isfahan, Rasht and Hamadan). These pathogens were isolated from different clinical samples including sputum, urine, blood, BAL (Bronchial Alveolar Lavage), wounds and secretions. After species identification, antibiogram test was performed by standard Kirby-Bauer (disk-diffusion method) for all identified bacteria to identify drug resistant bacteria (16). The clinical strains of different species of pathogenic bacteria including *E. coli*, *E. faecium*, *E. faecalis*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *S. maltophilia*, and *S. aureus* were used as host for isolation of lytic bacteriophages (Table 1).

Bacteriophage isolation and propagation. To find host specific lytic phages for the isolated pathogenic bacteria (strain and species), hospital wastewater samples were collected from 22 major hospitals. To increase the chance of phage isolation, all hospital wastewater specimens were mixed and then divided into eight equal parts (Fig. 1). To isolates all lytic phages even those that might have narrow host range (strain specific lytic phages) we used all recovered strains of different bacteria for phage isolation. For example, as we isolated 30 different strains of *E. coli* from the patient's samples, we added 30 different strains to 30 separate sewage samples (pooled sewage samples from all 22 hospitals) to serve as host for infection, amplification, and isolation of strain and species lytic phages. The isolated bacterial strains of each species were separately added to each 100 milliliters of Luria Bertani medium container to obtain high concentration (OD_{600}=0.5 of logarithmic phase). Then the amplified strains of each species were added separately to 500 mL of sewage sample. The mix-

Table 1. All isolated pathogenic bacteria strains from different clinical samples in this study

Samples diversity from hospitals	*E. coli*	*E. faecium*	*E. faecalis*	*K. pneumoniae*	*A. baumannii*	*P. aeruginosa*	*S. maltophilia*	*S. aureus*	Total
Sputum	1	0	0	15	2	12	0	3	33
Urine	17	4	3	2	0	0	0	5	31
Blood	8	1	0	5	2	3	1	7	27
BAL	1	0	0	3	1	3	1	2	11
Wounds	1	0	1	0	0	2	0	3	7
Etc.	2	1	1	4	2	4	3	9	26
Total	30	6	5	29	7	24	5	29	135

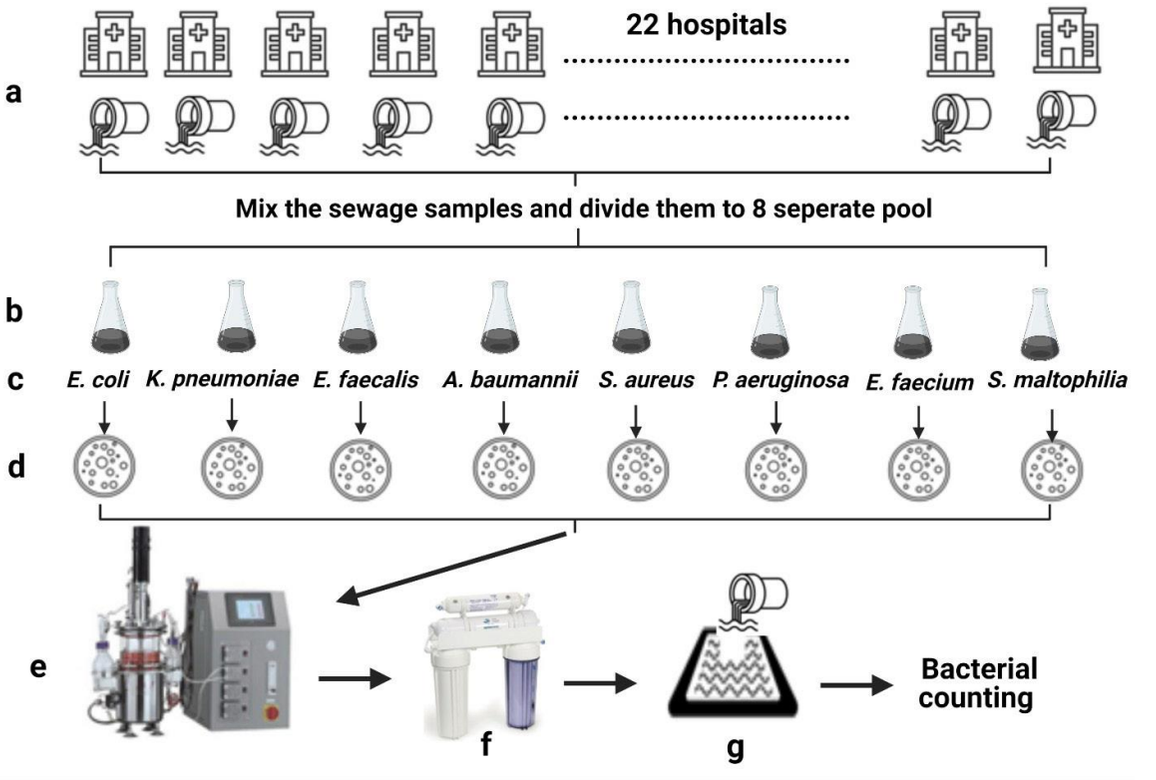

Fig. 1. Summary of steps taken in this project. a) Collection of raw sewage samples for 22 hospitals. The collected swage samples were collected and mixed together to make sewage pool. b) The sewage pool was divided into 8 same sewage samples. c) Eight different bacterial species were added to each sewage sample to amplify their own specific lytic phages. In this step different strains of each species used separately as host. d) Host strain and species-specific lytic phages for each bacterium were isolated. e) Phage cocktails proliferation was done in a fermenter. f) Phages were purified using reverse osmosis technique. g) All lytic phages were mixed to make a cocktail and then applied to the primary sedimentation pond. Then the bacterial count monitored for 6 months.

ture of sewage sample and each individual bacterium (strain) was mixed and was incubated at 35°C while shaking at 180 rpm/min for 48 hours to increase the possibility of phage infection and replication in the added bacteria as host. Following the incubation time, 50 milliliters of each wastewater samples was passed through a 0.22 µm filter. Specific phage plaques against each strain of *E. coli, E. faecium, E. faecalis, K. pneumoniae, A. baumannii, P. aeruginosa, S. maltophilia,* and *S. aureus* were screened, isolated and purified separately using the double layer plaque assay technique using each bacterial strain as host

(17). Each isolated plaque was incubated in 50 milliliters of Luria Bertani medium with specific bacteria at 35°C and while shaking at 180 rpm/min for 16 hours. Later, the electron microscopy scans of each phage sample were prepared.

Bacteriophage proliferation in large scale. After purification of the isolate lytic phages (single plaque), each of the pure phages were amplified separately on a large scale (Fig. 1) using a fermenter (New-Brunwick biofermenter BioFlo/Celligen®115, Eppendorf, Germany). We added 50 milliliters of the phage-containing liquid to 5 liters of culture medium containing the host bacteria in the logarithmic growth phase to the fermenter (pH= 7-7.4, O_2= 100 ppm, and $CaCl_2$= 15 mMol at 35°C). To separate the phage from the culture medium containing metabolites and destroyed bacteria, a Waterdrop G3 (Wayfair, USA) instrument that works based on reverse osmosis was used. Then, the phages trapped on the nano-filter, were washed with 1 liter phage buffer (18).

Treatment in a hospital pond. A hospital was selected as a pilot location to study the effectiveness of the phage cocktail to eliminate targeted pathogenic bacteria. The selected hospital had two same primary sedimentation ponds containing a total of 80 cubic meters of raw wastewater. These two primary sedimentation ponds had the same volume input of hospital sewage waste. The bacterial count of *E. coli*, *E. faecium*, *E. faecalis*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *S. maltophilia*, and *S. aureus* were monitored for each bacterial species from the raw wastewater samples collected from both primary sedimentation ponds according to standard methods every other day for a duration of 6 months (March to August 2019) (16). On September 1, 33 liters of phage cocktails (10^8 pfu/mL of each phage in phage buffer containing 10 mMol $MgSO_4$ and 15 mMol $CaCl_2$) were added to one of the primary sedimentation pond against the eight bacterial species and then the bacterial concentration were mentioned in both ponds every other day for the next 6 months (September 2019 to February 2020) according to standard methods (16). The load of the targeted bacteria in treated and untreated sedimentary ponds were completed to measure the effectiveness of the phage cocktail to eliminate bacterial pathogens. All identified phages were evaluated for the absence of common antibiotic resistance genes and toxin-producing genes by PCR.

RESULTS

In total, 135 pathogenic bacterial strains were isolated from the samples collected from 22 hospitals. Table 1 summarizes the clinical samples and the pathogenic bacteria recovered from each sample.

Antibiogram results showed that all the bacteria identified were resistant to routine antibiotics. For example, 65% of *S. aureus* were resistant to methicillin, 77% of *K. pneumoniae* produced ESBL, 92% of *E. coli* found to be ESBL positive, and 91% of *P. aeruginosa* demonstrated resistance to many antibiotics (Multi-Drug Resistance) including colisitin. After performing the two-layer agar technique, for each strain of eight different bacterial species a total of 42 lytic phages were isolated (Fig. 2a). Following several courses of purification and amplification, each plaque was sent to an imaging center for electron microscopy study. At this stage, 42 phage were isolated against nosocomial infections, which included six phages against *S. aureus*, eight phages against *E. coli*, four phages against *P. aeruginosa*, five phages against *K. pneumoniae*, five phages against *S. maltophilia*, six phages against *E. faecium*, three phages against *A. baumannii*, and five phages against *E. faecalis*. Based on images obtained by transmission electron microscopy (TEM), all isolated phages belonged to the *Podoviridae*, *Siphoviridae* and *Myoviridae* families. Fig. 2b shows the images of some different phages. All 42 isolated phages were purified and amplified and finally were combined (10^8 pfu/mL of each phage) as phage cocktail for treatment. The phage cocktail was added to one of the primary sediment pond to evaluate the phage cocktail efficiency to remove the pathogenic

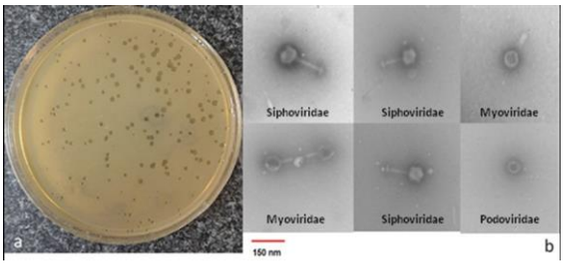

Fig. 2. a) Types of plaques obtained from hospital sewage. b) Types of phages obtained from hospital wastewater. *Siphoviridae*: an icosahedral head and a long noncontractile flexible tail, *Myoviridae*: isometric heads of 87-94 nm in diameter and conspicuous capsomers, striated 140-219 nm long tails and a double base plate and *Podoviridae* have short and noncontractile tails

bacteria from hospital sewage. The results of bacterial counts from March 2019 to the end of August 2019 and also from September 2019 to the end of February 2020 are shown in the supplementary Table 2.

Our bacterial count monitoring revealed a consistent high level of each targeted bacterial pathogens in untreated pond from March 2019 – February 2020, while adding the phage cocktail reduced the count of pathogenic bacteria noticeably (near to zero count for September, October, November and December 2019) (Table 2, Fig. 3). More interestingly, the phage cocktail was able to fully eliminate the pathogenic *E. faecium, E. faecalis, K. pneumoniae, A. baumannii* and *S. maltophilia* (zero counts for 1-4 months after treatment, Table 2). The phage cocktail also was able to decrease the count of *E. coli, S. aureus* and *P. aeruginosa* but was not able to fully elimination of them at the beginning of treatment. The count of all targeted bacterial species increased in January and February (Table 2, Fig. 3) after phage treatment. Our phage cocktail was able to reduce 99.8%, 99.4%, 99.5%, 99.8%, 99.7%, 99.8%, 99.6% and 99.9% of *E. coli, E. faecium, E. faecalis, K. pneumoniae, A. baumannii, P. aeruginosa, S. maltophilia* and *S. aureus* counts respectively (Table 2).

Table 2. The results of bacterial counts from March 2019 to the end of February 2020

		Treated pond							Untreated pond					
Bacterial species	**March**	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**	**January**	**February**
E. coli	133000	112000	120000	140000	125000	130000	126000	120000	125000	112000	115000	100000
E. faecium	8200	6000	7500	6000	7300	8000	7700	7000	7600	6200	7000	6500
E. faecalis	6700	11000	9000	9000	9500	6800	9000	8500	7000	6900	7000	6900
K. pneumoniae	72500	107000	102000	120000	120000	122000	100000	110000	110000	115000	116000	110000
S. aureus	177000	168000	158000	180000	191000	200000	198000	190000	170000	161000	155000	150000
A. baumannii	2100	2000	1700	1500	2000	1700	2000	1900	1400	1000	1000	800
S. maltophilia	550	700	900	900	1000	1500	1800	1000	1600	1100	1000	900
P. aeruginosa	420000	500000	450000	500000	490000	500000	477000	475000	400000	420000	420000	440000
E. coli	125000	132000	127000	137000	131000	129000	170	150	200	220	270	330
E. faecium	6500	7400	8800	5000	6200	7300	0	0	10	50	80	100
E. faecalis	7800	10000	8100	8400	9000	7000	0	0	30	60	80	100
K. pneumoniae	98000	110000	100000	122000	116000	125000	0	100	140	220	240	350
S. aureus	155000	170000	160000	184000	200000	220000	100	100	550	620	900	1100
A. baumannii	1200	1500	1400	1400	1700	1500	0	0	0	5	5	10
S. maltophilia	900	800	1000	1000	900	1200	0	0	0	0	10	10
P. aeruginosa	500000	550000	480000	550000	600000	580000	80	100	120	110	150	170

DISCUSSION

The use of phage helps a lot to improve the municipal sewage system for environmental health (19). Phages kill bacteria and the use of phage cocktails prevents phage resistance (19). In this project, our results showed that phages cocktail (lytic phages), noticeably reduced the pathogenic bacteria in the hospital sewage effluent.

In January to February 2020, there was a slight increase in all bacterial population in the treated pond. This may be due to the flow of the hospital wastewater or increase in phage resistant bacteria. It seems that the phage cocktail eradicated the *E. faecium, E. faecalis* and *K. pneumoniae* in the hospital wastewater on March 2019 to August 2019 (Table 2). *E. faecium, E. faecalis* and *K. pneumoniae* are considered as important sources in transmission of antibiotic resistance genes in Gram-positive and Gram-negative bacteria. Therefore, the phage cocktails have the potential to significantly improve the quality of hospital wastewater (Table 2, Fig. 3). On the other hand, *A. baumannii* and *S. maltophilia* were almost eradicated

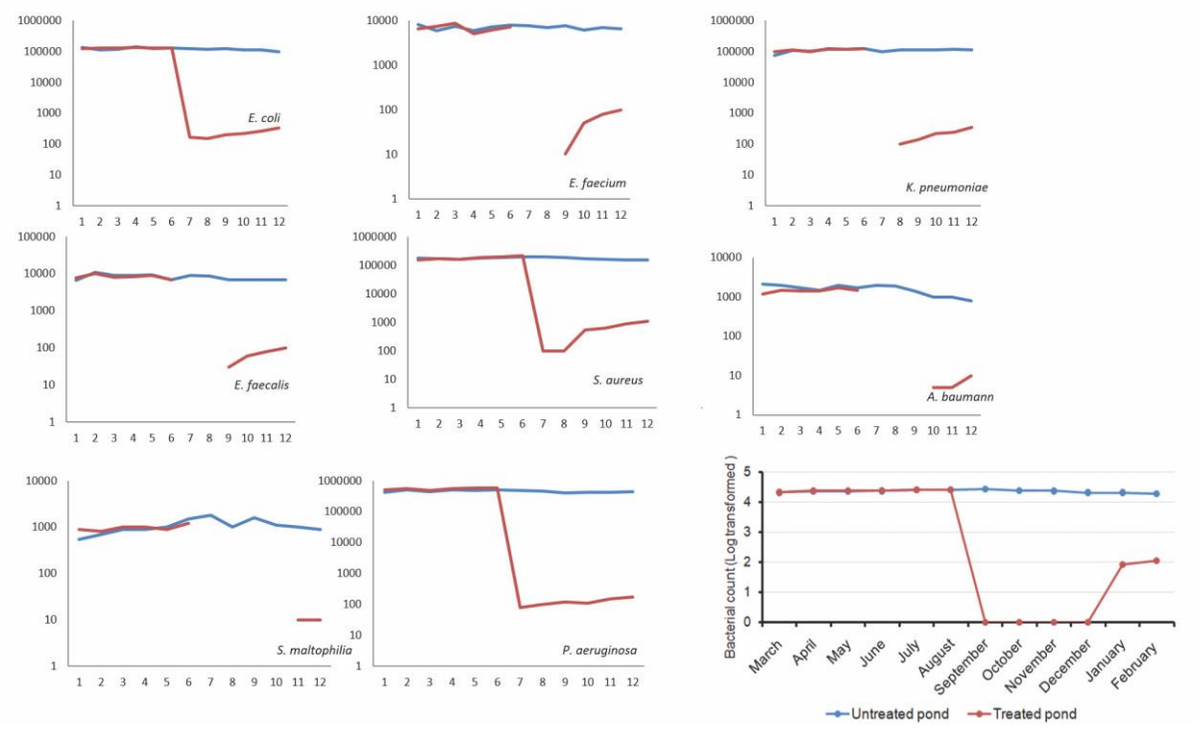

Fig. 3. a) Mean of bacterial count (log transformed) from two ponds (untreated and treated with phage cocktail). Both ponds were monitored from March to August 2019 for eight bacteria species. While untreated pond was monitored for eight bacteria species from September 2019 to February 2020, treated pond were exposed the phage cocktail and was monitored from September to February for eight bacterial species from September 2019 to February 2020 in treated pond. b) Mean of bacterial count (log transformed) from two ponds for each bacteria [untreated (blue line) and treated with phage cocktail (red line)]

using the phage cocktails. In particular, the stability of phage cocktails against these two bacteria in hospital wastewater was very stable. Phage cocktails caused a significant reduction in the population of *E. coli, P. aeruginosa* and *S. aureus.* It seems that there are two ways to further reduce the *E. coli, P. aeruginosa* and *S. aureus* populations. The first way is to create a primitive storage pool (pre-pool storage room), before the main primary sedimentation pond, for the infinite source of phage cocktails and the second way is to use higher concentrations of phage cocktails against *E. coli, P. aeruginosa* and *S. aureus.* So far, no comprehensive study has been performed to reduce all the causes of nosocomial infections with phage in hospital wastewater. Therefore, no comparison of the results can be made.

CONCLUSION

Obviously, by reducing the load of dangerous bacteria in hospital wastewater, it is possible to prevent the transmission of these bacteria to the environment. This is true when the hospital wastewater somehow finds a way to come into contact with the municipal wastewater, especially in developing countries and the third world. So, using phage cocktails can greatly reduce the transmission of antibiotic-resistant infectious agents and decrease the risk of diseases in humans, livestock, and agricultural products contaminated with hospital wastewater.

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