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Research Article

Isolation of Bacteriophage from Guheswori Sewage Treatment Plant Capable of Infecting Pathogens

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ABSTRACT

Background: Waste water contains microorganisms which are continuously shed in the feces. These microorganisms especially bacteria might acquire antibiotic resistance and pose a significant threat to human health. Therefore, this work aims at isolating bacteriophage capable of infecting the isolated bacteria. **Methodology:** For this purpose, the grab sampling was performed at the Guheswori sewage treatment plant from the inlet in primary treatment plant and from the outlet of the secondary treatment plant. For the isolation of bacteriophage, bacteriophage in the sewage was first enriched in an isolated pathogen, then filtered and then subjected to the isolates in the nutrient agar.

Results: Pathogens like *Escherichia coli*, *Salmonella* Typhi, *Enterococcus faecalis*, *Staphylococcus aureus*, Coagulase negative *Staphylococcus* (CONS), *Citrobacter fruendii*, *Enterobacter aerogenes*, *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa* were screened. Bacteriophage was able to infect *E. coli* ($p < 0.001$), *S.* Typhi ($p < 0.001$), *E. faecalis* ($p = 0.182$); and unable to infect *S. aureus*, CONS, *C. fruendii*, *E. aerogenes*, *P. mirabilis*, *P. vulgaris*, *P. aeruginosa*. **Conclusion:** Bacteriophage are able to infect and kill pathogens like *E. coli*, *S.* Typhi, *E. faecalis* and unable to infect *S. aureus*, CONS, *C. fruendii*, *E. aerogenes*, *P. mirabilis*, *P. vulgaris*, *P. aeruginosa*. Among all other reasons of lowering bacterial load, bacteriophages could also be one of the confounding factor. Such bacteriophage able to infect and undergo lytic cycle could be used in phage typing.

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INTRODUCTION

Guheswori sewage treatment plant is located in the north-eastern part of Kathmandu valley in the bank of Bagmati river. Guheswori sewage treatment plant is a municipal wastewater treatment plant which utilizes extended aeration, activated sludge, deep oxidation ditch of Carrousal type for treatment (C1, E, C2; Figure 1). The drainage system of Gokarna, Boudha, Jorpati, Chabahil, Gaurighat and Pasuphati leads to the inlet of this plant (A1, A2; Figure 1) [1]. The main aim of this plant is remove grits and plastics, to reduce microbial load, turbidity, chemical oxygen demand, biological oxygen demand, available nutrients, direct the effluent to a safe location (B, C, D; Figure 1); as river pollution is one of the oldest existing problem in Nepal.

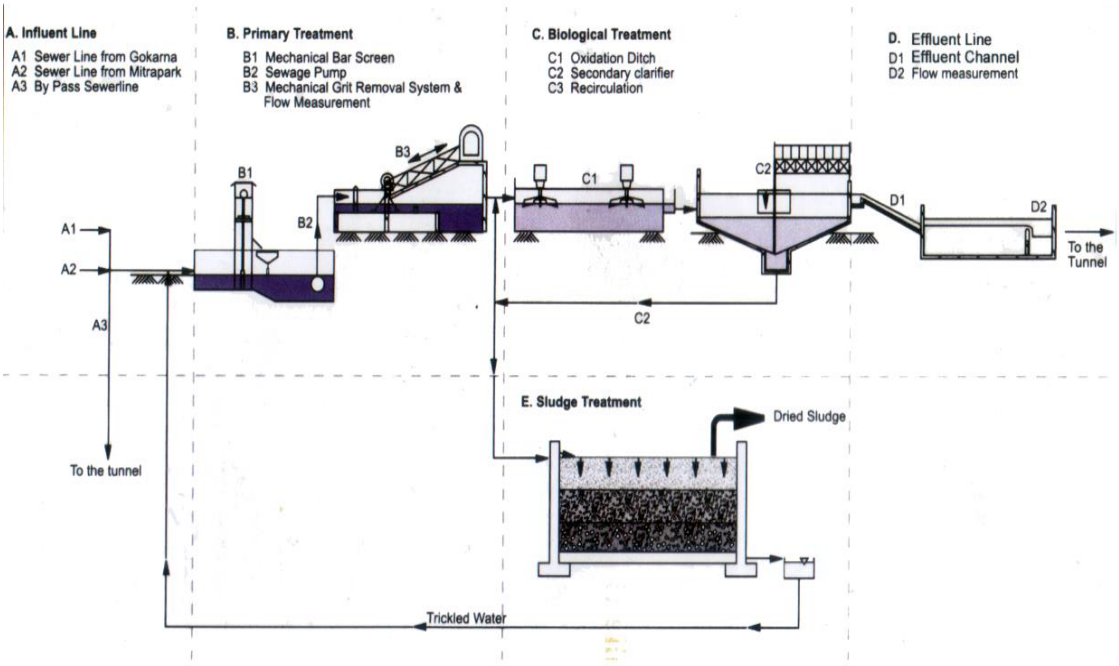

Figure 1: Flow diagram of wastewater treatment plant (**Picture courtesy: HPCIDBC, Ministry of Urban Development, Government of Nepal)**

Sewage production in developing countries is directly proportional to population growth; as there is an increment in demand of freshwater in domestic, commercial, and industrial sectors [2, 3]. Lack of education, financial and technical resources in many developing countries have led to irrigation [4]. Human health risks from wastewater irrigation include firstly people (majority female) involved in agriculture and farming followed by consumers' exposure to pathogens with the inclusion of helminthic infections [3, 5], and chemicals [6]. Microorganisms and chemicals are generally introduced into water bodies through various routes (such as industrial effluents, raw as well as treated sewage, storm-water, and animal manure runoff) [7].

The release of wastewater,(both black and gray, has increased with the increase in population and urbanization [8, 9]. It has been reported that blackwater consists of the discharges from toilets which contains intestinal flora, hormones, traces of pharmaceutical compounds, nitrogen and phosphorous in high concentrations [10, 11].

Bacteriophages are viruses that are capable of infecting or killing pathogens [12-14] when they are able to bind to a highly specific receptor of bacteria (limiting the host range to a bacteriophage) [15, 16]. Wastewater treatment processes do not remove or inactivate all pathogenic microorganisms [17, 18].
Depending on the environmental factors (such as temperature, moisture content, sunlight etc.) pathogens most commonly present in sewage sludge are bacteria (such as *Salmonella*, coliform(s), *Staphylococcus spp*, *Vibrio* etc.), viruses (such as adenoviruses, hepatitis, enteroviruses), protozoa (*Cryptosporidium*, *Giardia* etc.) and helminths (such as *Ascaris*, *Taenia* etc.) [19-21].

Government all around the globe have been struggling to remove the pathogens, chemicals etc from wastewater. The major sources of these pollutants are unplanned urbanization, lack of education, increase in industries, introducing the sewage directly into the river without any prior treatment etc. Several studies relating to microbiological load have conducted across the globe but optimization of the sewage treatment plant has rarely been done. This study focuses on bacterial load reduction, isolation of pathogens and their susceptibility to bacteriophage. This data will provide knowledge about treatment plant's role in reducing bacterial load. This research will also shed light on the importance of partial treatment of wastewater in industries of Nepal and across the globe.

In the present study, we isolated bacteriophage capable of infecting and undergoing lytic cycle in the pathogens isolated from the treated effluent obtained from the outlet of secondary treatment plant.

Methods

Sample collection and investigation

The study was conducted at Microbiology laboratory, Department of Microbiology, St. Xavier's College, Maitighar, Kathmandu, Nepal during the period of April 16 to June 16, 2017.

For the study, grab sampling was performed (50 mL of secondary treated effluent) in sterile plastic containers at 9:30 AM. The process was repeated for 20 days. The sample was kept in a mini cooler full with ice-pack and was transported to the laboratory.

Isolation and characterization of the pathogens

With the help of sterile micropipette and sterile glass spreader, 100 µl sample (after treatment) was spread on cetrimide agar, mannitol salt agar and mac conkey agar and were incubated at 42℃, 37℃ and 44.5℃ for 24 hours respectively [22]. 10ml sample was first enriched in Selenite F broth for 5 hours at 42℃; and then was spread on salmonella-shigella agar were incubated at 42℃ for 24 hours respectively. 5 mL sample was added in 10ml azide dextrose broth containing tube and were incubated at 37℃ for 24 hours.

On the following day, colonial morphology was noted for the isolated colonies and then was subjected to biochemical tests [23].

Isolation of bacteriophage

Isolated bacteria were inoculated in 5 mL sterile nutrient broth were inoculated for 24 hours at 37℃. Following day, 5 mL of the pre-incubated bacterial inoculum, 5 mL deca-strength (10X) nutrient broth, 40 mL sewage was mixed and was incubated for 24 hours at 37℃ in a rotary shaker incubator. Among the contents, 10 mL was filtered by membrane filtration technique (filtrate was taken as enriched phage preparation). A cotton swab dipped in the bacterial inoculum (tallied with McFarland standard 0.5) and a uniform carpet culture was made on the nutrient agar [23]. Then, 100 µl of enriched phage preparation was added to the plate and incubated at 37℃ for 24 hours. Following day, plaques were observed and no of plaques were counted [24, 25]. As a control, uniform carpet culture of an organism (without introducing enriched bacteriophage) was swabbed on a nutrient agar and was incubated at 37℃ for 24 hours.

Quality control and statistical analysis

A sample was triplicated and was repeated 2 times in an interval of a week. Purity plating was performed for the media plates and equipment were calibrated. Statistical analysis was done on SPSS version 19.

Results

With the help of biochemical tests of the sewage samples obtained after treatment; *S. aureus*, CONS, *E. faecalis*, *C. freundii*, *E. coli*, *E. aerogenes*, *P. mirabilis*, *P. vulgaris*, *Salmonella* Typhi, *P. aeruginosa* were isolated. The highest frequency of bacteria being isolated were *Staphylococcus* spp (100%), *E. fecalis* (70%), *C. freundii* (100%), *E. aerogenes* (100%), *Proteus* spp (100%), *Salmonella* Typhi (85%), *P. aeruginosa* (100%). The data are presented in Table 1.

Table 1: The list of bacteria isolated from the treated sewage with their frequency of isolates

S. No.	Isolates from sewage (after treatment)		Frequency of isolates (n=20)
1	*Staphylococcus*	*S. aureus*	20
		CONS	11
2	*E. faecalis*		14
3	*C. freundii*		20
4	*E. coli*		20
5	*E. aerogenes*		20
6	*Proteus*	*P. mirabilis*	12
		P. vulgaris	8
7	*Salmonella* Typhi		17
8	*P. aeruginosa*		20

n= total sample collected days

Enriched phage solution when produced plaques after infecting isolated *E. coli* (from wastewater after treatment) ranging from 0 plaques – 17 plaques (M= 8.2, SD= 4.76). Enriched phage solution when produced plaques after infecting isolated *Salmonella* Typhi (from wastewater after treatment) ranging from 0 plaques – 19 plaques (M= 8.65, SD= 6.30). Enriched phage solution when produced plaques after infecting isolated *Enterococcus faecalis* (from wastewater after treatment) ranging from 0 plaques – 16 plaques (M= 1.64, SD= 4.36). These results are presented in table 2.

Table 2: **No. of plaque(s) counted on *E. coli, Salmonella* Typhi, *E. faecalis***

S. No.	Volume of enriched phage solution taken (µL)	No. of plaque(s) observed on *E. coli*	No. of plaque(s) observed on *Salmonella* Typhi	No. of plaque(s) observed on *E. faecalis*
1	100	9	19	2
2		8	2	0
3		2	3	0
4		9	1	0
5		7	0	0
6		5	3	5
7		10	5	0
8		15	16	0
9		0	11	0
10		6	5	0
11		15	15	0
12		8	8	16
13		17	5	0
14		13	9	0
15		8	11	
16		3	16	
17		12	18	
18		5		
19		11		
20		1		

The results of t-tests indicated that the enriched phage solution infecting *E. coli* and *Salmonella*, demonstrated a statistically significant difference ($p < 0.001$ at 95% confidence interval) while the enriched phage solution infecting *Enterococcus* didn't demonstrate a statistically significant difference ($p = 0.182$ at 95% confidence interval). Among isolated bacteria, bacteriophage was able to infect *E. faecalis, E. coli*, and *Salmonella* Typhi and unable to infect *S. aureus*, CONS, *C. fruendii, E. aerogenes, P. mirabilis, P. vulgaris, P. aeruginosa*.

Discussion

The isolated bacteria from the effluent after treatment were *S. aureus*, CONS, *E. faecalis, C. freundii, E. coli, E. aerogenes, P. mirabilis, P. vulgaris, Salmonella* Typhi, *P. aeruginosa*. These bacteria might have been infected with bacteriophage but instead of the lytic cycle, the lysogenic cycle could have been initiated [21], antibiotic resistance could also have been gained by these bacteria [18, 26].

Among isolated bacteria, bacteriophage was able to infect *E. faecalis, E. coli* and *Salmonella* Typhi. Table 1 shows that bacterial load has significantly been reduced, which could be due to the interaction between bacteria and bacteriophage [14, 21, 27]. The lack of plaque after the introduction of bacteriophage in the test microorganism (Table 2) could be the result of a lysogenic cycle of bacteriophage [26, 28] which might prove to be harmful in the long run if they acquire resistance or mutation took place [21, 26].

Due to matched unique receptor of bacteriophage and bacteria, the findings of this study agree with the findings of Bonilla et al (2010), Mandilara et al (2006), Gillespie et al (2005), Shin et al (2012), Trampuz et al (2017), Mandilara et al (2006), Namura et al (2008), where isolated bacteriophage possessed the ability undergo lytic cycle in *E. faecalis*, *Salmonella* enterica serovar Enteritidis and *Salmonella* enterica serovar Typhimurium, methicillin resistant *S. aureus*, *E. coli*, *Enterococcus* spp.

Due to undergoing of lysogenic cycle [21, 26, 29] or unmatched unique receptor of bacteriophage [12, 21, 30], the result of this investigation disagree with the result of the investigation of Li et al (2016); Sana et al (2016); Melo et al (2016); Gutie´rrez et al (2015) as the isolated bacteriophage could infect and undergo lytic cycle in multidrug resistant *E. aerogenes, C. frundii, P. mirabilis, S. epidermidis* respectively.

A similar investigation by Huff et al (2002) revealed that mortality rate of infected chicken decreased after spraying bacteriophage aerosol on the chickens which were infected with *E. coli* infected chickens (without administering antibiotics).

In a similar study by Lavigne et al (2003), bacteriophage T7 possessed the ability to complete lytic cycle in both *E. coli* and *P. aeruginosa*. If such novel viruses are characterized in the near future then phage typing will be preferred in the multidrug resistant pathogens ridden future [9, 12, 21, 26, 29-32]. The bacteriophage can be used to

classify bacteria and can even be used as antibacterial agents to control pathogens [9, 26, 29, 30, 32].

In an investigation conducted by Tyrrel and Quinton (2003), wastewater containing pathogens of fecal origin were used in irrigation increasing the threat to the public masses consuming agricultural goods. The main reason for collecting the sample at a specific time was due to the sunlight inactivation of microorganisms present in wastewater [33].

Conclusions

The results obtained in this research can be summarized as follows:

1. Plaques observed on the nutrient agar plate means matching of unique receptor between bacteriophage and bacteria and undergoing lytic cycle.
2. The bacteriophage isolated from the sewage could undergo lytic cycle for a specific bacterium but could not infect multiple bacterial strains.
3. In some bacterial isolates, plaques were not formed this could imply that the unique receptor didn't match or the bacteriophage underwent lysogenic cycle which may end up making the bacteria mutate and even lead to more resistant strains.

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