Research Article

Isolation of *E. Coli* Bacteriophage from Raw Sewage and Comparing Its Antibacterial Effect with Ceftriaxone Antibiotic

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ABSTRACT

Bacteriophages are viruses that can infect pathogenic bacteria, as they are highly host specific. We aimed to isolate the bacteriophage which infect *Escherichia coli* from a sample of raw sewage then compare it's anti-bacterial effect with ceftriaxone antibiotic. *E. coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 19433), *Staphylococcus aureus* (ATCC 2392), and *Yersinia enterocolitica* (ATCC 9610) were prepared in order to determine the Bacteriophage host and whether it has any effect on other type of bacteria or not. Eight samples of raw sewage were prepared from a wastewater treatment plant. Sewage was mixed with BH Broth (brain, heart broth) in order to let the bacteria grow. Spectroscopy, Incubating, purification and injecting bacteria were followed repeatedly to isolate bacteriophage.

The *E. coli* bacteriophage was successfully isolated which was completely able to lyses bacterial cells and it was clear that it is effective only on the *E.coli*. At the end 1.3×10^5 pfu bacteriophages in 1ml in invitro situations had the same result as 32µg/ml of Ceftriaxone antibiotic. The bacteriophage isolated in this work may be a good substitution of antibiotics in treating diseases caused by bacteria. They can also be used for reducing bacterial jams in water and waste water treatment plants.

**Key words:** Bacteriophage, *E. coli*, Sewage, antibiotics, Drug resistance

1. **INTRODUCTION:**

Bacteriophages or phages are viruses that infect prokaryotes and are capable of killing them specially the Bacteria. Bacteriophage means bacteria eater and this name is because when they first discovered, they appeared to eat bacterial cells (Mattila, Ruotsalainen and Jalasvuori, 2015; Krukowska and Slopek, 1987). As all kinds of viruses, bacteriophages are nonliving agents that need to host cell to replicate themselves. They act too selective and each bacteriophage effect only on a specific kind of bacteria (Krukowska, Slopek, 1987; Peltomaa, López-Perolio, Benito-Peña, Barderas and Moreno-Bondi, 2015). Bacteriophages are distributed in nature especially at track bacterial host (Waldor, Friedman, Adhya, 2005; Sulakvelidze, Kutter, 2005).
past few decades, using huge amount of antibiotics has caused to resistance among various bacterial strains. This potentially dangerous situation threatens to manifest itself in modern times. Phages are thus being preferred because, unlike antibiotics, phages are highly specific and do not provide resistance from untargeted bacterial strains (Sulakvelidze, Kutter, 2005) and this is the reason that phage therapy has attracted more attention during recent years. Bacteriophages are different in shape, most of them are 24-200 nm in length. Every bacteriophage has a head, capsid or tail which contains the phage’s genetic materials like nucleic acid and it can be vary in size and shape too (Leiman, Kanamaru, Mesyanzhinov, Arisaka, Rossmann, 2003). To enter the host cell bacteriophage injects its nucleic acid into the bacterium, by using the host cell replication and genetic materials, the viral nucleic acid is replicated and integrated into the protein capsid (Beaudoin, DeCesaro, Durkee, Barbaro, 2007). In this study, we aim to isolate E. coli bacteriophage since E. coli is present in the normal micro flora of the human. However, some of these E. colistrains are able to cause disease under certain conditions, like abnormal predominance over the other gut flora, host depressed immune system or adverse environmental exposure (Zhao et al., 2005).

2. Objective: Antibiotic resistance causes many anxieties for human health in the future. In this study the phages that infect and lyses E. coli cells were tried to isolate from asample of raw sewage. For achieving this purpose special protocol was followed. Considering the interesting role of bacteriophages in lysing and destroying bacterial cells, the new treating way for bacterial deceases can be developed.

3. MATERIAL AND METHODS:
3.1 Isolation of Bacteriophage: Eight sample of sewage each containing approximately 50 ml of raw sewage was collected from a waste water treatment plant in Tehran, Iran, with a 10 minute gap between each sample collecting, 10 ml of each sample was poured in tubes. The tubes numbered as 1 to 8 and were stored in the refrigerator over a night in order to settle the main pollutions. The samples then centrifuged in 4000 RPM and the supernatants were collected in separate tubes. 10 ml of each sewage samples were filtered by a 0.22 micron filter and then Pipetted into 10 ml of BH Broth (brain, heart broth). 400 µl of E. coli (ATCC 25922) with 1.5 × 10⁵ cfu (Colony Forming unit) was inoculated in each tube and were vortexed in order to mix better. At the end, all tubes were incubated at 37°C, shaking for 24 hours.

3.2 Purification of bacteriophage: After 24 hours the turbidity of all 8 tubes was measured by spectroscopy. The least turbid tube was selected for purification. The tube was centrifuged again and the supernatant which contains bacteriophage filtered and pipetted into another sterile tube. These steps, remove any remaining bacteria. The purification of phage, expressed as plaque forming units (pfu), was determined using the DLA technique as described by Sambrook and Russel (Sambrook, Russell, 2001; Silvio B Santos et al., 2009) Briefly, 100 µl of a dilution of the phage sample was added to 400 µl of a bacterial suspension grown overnight at 37°C. This solution was added to 4 ml BHI agar 0.7%, gently homogenized, and poured into a 90 mm petri dish previously prepared with 10 ml BHI agar 1.5%. The plates were gently swirled and left for 10 min at room temperature in order to fix upper agar and then inverted and incubated at 37°C overnight. Then the plaque was cut by a scalpel and put in a tube containing BH broth and stored in 4°C until next steps.

3.3 Determination of Bacteriophage host
In the next step the aim was determination the E. coli bacteriophage host and also proving that it is selective and also does not have any special effect on other type of bacterial cells. In this step spot test method was followed. For achieving this purpose, several agarose plates and several
bacterial cultures consisting *E. coli* (ATCC 25922), *Yersinia enterocolitica* (ATCC 9610), *Staphylococcus aureus* (ATCC 2392) and *Enterococcus faecalis* (ATCC 19433) all cultured in BHI for 24 hours, were prepared. Each bacterial culture was spread on a specific agarose plate, then 10 µl of bacteriophage supernatant was poured in each plate and the plates were left for 1 hour at room temperature to fix supernatants and then inverted in 37 °C incubator for 24 hours.

3.4 Comparing Bacteriophage effect with Ceftriaxone antibiotic:

For comparing effect of bacteriophage on bacteria with antibiotic, MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bacterial Concentration) methods were considered and followed as described by Jennifer M Andrews (Andrews Jennifer, 2001) briefly.

For preparation of antibiotic stock solution, the below formula was used:

\[ \frac{1000}{P} \times V \times C = W \]

Where \( P \) = potency given by the manufacturer (µg/mg), \( V \) = volume required (ml), \( C \) = final concentration of solution (multiples of 1000) (mg/L), and \( W \) = weight of antibiotic in mg to be dissolved in volume \( V \) (ml).

**Preparation of antibiotic dilution range:**

Dilution ranges were prepared from: 4 - 128 mg/L.

- 4 µl into the container labelled 1
- 8 µl into the container labelled 2
- 16 µl into the container labelled 3
- 32 µl into the container labelled 4
- 64 µl into the container labelled 5
- 128 µl into the container labelled 6

The samples then stored in 37 °C for 24 hours to determine MIC result.

**MBC:**

To determine the MBC result, after obtaining MIC, a sample of tubes, which were without turbidity was cultured in, plates containing EMB culture media. The first sample, which bacteria did not grow in it, was considered as MBC result.

3.5 Preparation of Phage serial dilution:

For preparation of phage serial dilution, the method was similar to the MIC method with some differences: Eight sample and 7 different dilutions were prepared. The first tube contained original phage solution and the rest of the tubes were dilutions. In the first tube 1 ml of isolated bacteriophage was added to 9 ml of Muller Hinton Agar so the dilution factor would be \( 10^1 \) and the other samples \( 10^2, 10^3 \) and... \( 10^9 \) respectively. Then 1 ml of *E. coli* (ATCC 25922) was added into first tube and after mixing by vortex 1 ml of the solution was added to other tube respectively.

Number of Viruses in the sample can be counted by:

\[ \text{Number of Viruses (phages) in 1 ml} = \frac{\text{Number of plaques}}{\text{Volume of phage solution}} \]

4. RESULTS:

4.1 Purification of Bacteriophage:

The results of spectroscopy were 0.246, 0.354, 0.033, 0.12, 0.416, 0.512, and 0.093. The least turbid tube was considered as main tube for purification. After the phages have purified the plaques can be seen on the surface of the plate, distinct zones are the phage plaques, which have lysed the *E. coli*. Fig (1)

4.2 Bacteriophage act selective

Different plates with different type of bacteria, but consisting one kind of bacteriophage, have shown the effect of bacteriophage on its specific bacteria. *E. coli* bacteriophage inhibited the growth of *E. coli* in the plate, but almost had no special effects on other type of bacteria on the other hand *E. coli* bacteriophage had lysed *E. coli* cells. So that the *E. coli* bacteriophage was successfully isolated and have shown effective (Steele, 1971). The results can be seen in Figure 2.

4.3 Obtaining MIC and MBC test results:

After 24 hours incubation, the tube number 4 was shown less turbid so the Minimum Inhibitory Concentration (MIC) for antibiotic obtained 32 µg/ml, which means this amount of antibiotic inhibited growth of bacteria in the sample. The
result can be seen in Figure 1. For obtaining MBC result, a sample of tubes, which were without turbidity, was cultured in EMB culture media. After 24 hours, incubation in 37°C tube number 4 has shown no bacteria growth. So the MBC result was the same as MIC result.

The MIC and MC results can be seen in table (1).

### Table 1: MIC and MBC results

<table>
<thead>
<tr>
<th>Antibiotic Concentrations</th>
<th>MIC and MBC results</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>+</td>
</tr>
<tr>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>128</td>
<td>-</td>
</tr>
</tbody>
</table>

+: Growth of Bacteria   - : Bacteria did not grow

### Table 2: Growth of bacteria in different amount of bacteriophages

<table>
<thead>
<tr>
<th>Sample number containing bacteriophage</th>
<th>Dilution factor</th>
<th>Growth/In growth of Bacteria</th>
<th>Count of bacteriophages(pfu)</th>
<th>Count of bacteria (cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$10^3$</td>
<td>-</td>
<td>$1.5 \times 10^8$</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>$10^2$</td>
<td>-</td>
<td>$1.35 \times 10^7$</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>$10^1$</td>
<td>-</td>
<td>$1.22 \times 10^6$</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>$10^0$</td>
<td>-</td>
<td>$1.3 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>$10^{-1}$</td>
<td>+</td>
<td>$1.3 \times 10^5$</td>
<td>$1.1 \times 10^3$</td>
</tr>
<tr>
<td>6</td>
<td>$10^{-2}$</td>
<td>+</td>
<td>$1.3 \times 10^5$</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>7</td>
<td>$10^{-3}$</td>
<td>+</td>
<td>$1.3 \times 10^5$</td>
<td>$1.4 \times 10^5$</td>
</tr>
</tbody>
</table>

+: Growth of Bacteria   - : Bacteria did not grow

Figure 1: E. coli bacteriophage lysed the E. coli cells. Plaques can be seen and are the parts that bacteriophage exist
Isolation of E. Coli Bacteriophage from Raw Sewage and Comparing Its Antibacterial Effect with Ceftriaxone Antibiotic

Figure 2: E. coli bacteriophage lysed the E. coli cells. Distinct zones are the parts that bacteriophage exist. Since the bacteriophages are too selective it had no effect on other type of bacteria. E. coli (ATCC 25922) at the center, Yersinia enterocolitica (ATCC 9610) in top of the figure, Staphylococcus aureus (ATCC 2392) in the right side and Enterococcus faecalis (ATCC 19433) can be seen in the bottom of figure.

5. DISCUSSION:
In this study E.coli ATCC (25922) Bacteriophages have isolated successfully and Plaques with 1mm diameter have observed which they did not observed in other types of bacteria in the test and this might be because of lack of it’s receptors so that selectivity of bacteriophages have obtained. In the other hand 1.3×10⁵ pfu bacteriophages in 1ml in invitro situations had the same result as 32µg/ml of Ceftriaxone antibiotic. In addition, in the bacteriophage tube number 4 no bacteria have grown after 24 hr. which has shown the same result as MBC test.

Concerns over the consequences of the mechanismsof the bacteria that prevent the inhibitoryeffects of the antibiotics in the treatment of animalsis widespread (Schwarz, Chaslus-Dancla,2001;W.E Huff, G.R Huff, Rath, Balog and Donoghue,2004). The antibiotic capacity that causes the resistant of bacteria is an important problem for public health. Antibiotic residues can be found in the environment for long periods of time after treatment (Levy,2001). Raghu et al. Have discussed about several roles that bacteriophages play in the environment, biofilm control and water treatment (Raghu, Gaare, Manjunatha, Mishra and Sawale,2012). There are a few reports suggested that the presence of bacteriophages in sewage could be useful in wastewater treatment especially in procedure of activated sludge. (Hantula, Kurki,Vuoriranta,Bamford, 1991;Hertwig, Popp, Freytag, Lurz and Appel, 1999;Khan, Satoh, Mino, Katayama, Kurisu and Matsuo, 2002). In addition, it has suggested that phages can be used as biological tracers of pathogenic bacteria in water and wastewater treatment (Borrego, Morinigio, De Vicente, Cornax, and Romero, 1987. Abdulla, Khafagi, El-Kareem andDewedar, 2007). Zumstein et al. studied the interactions of bacterial populations and bacteriophages in anaerobic wastewater treatment using laboratoryanaerobic digesters. They suggested that bacteriophages could be effective on the
dominance of bacterial strains during the process (Zumstein, Moletta and Godon, 2000). Periasamy and Sundaram have reported the potential of bacteriophages for the removal of bacterial pathogens including E. coli in hospital wastewater. They showed that the specific phages of E. coli could destroy the bacterial host after 14 hours of incubation (Periasamy and Sundaram, 2013).

Meanwhile, using bacteriophages which have shown same results as antibiotics can be a substitution of themin cases that antibiotic makes resistance and can reduce the investment costs of treating bacterial diseases than producing antibiotics, which are so hard to find and need high level technologies. In addition they can be directly used in wastewater treatment plants to reduce bacteria jams so they are potentially useful for environmental sciences too.

ACKNOWLEDGMENTS:
This paper is part of a research project approved by the Food Microbiology Research Center, Tehran University of Medical Sciences and Health Services Contract No. 31123, Tehran, Iran. Many thanks to Mr. S. Khanjani for his supports and also comments during the research.

6. REFERENCES:
11. Silvio B Santos, Carla M Carvalho, Sanna Sillankorva, Ana Nicolau, Eugénio C Ferreira and Joana Azeredo. (2009). The use of antibiotics to improve phage detection and enumeration by the double layer agar technique, Journal of BMC Microbiology. 9; 148