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Isolation of *Yersinia enterocolitica* Bacteriophage From Hospital Wastewater

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Abstract

Background: Yersiniosis is a common foodborne infection caused by *Yersinia enterocolitica*. This bacterium is frequently isolated from animals. Generally, bacteriophages (phages) are viruses that only infect bacteria, and are ubiquitous in the world including the intestinal tracts of animals and sewage. Today, due to the development of antibiotic resistance among bacteria, studies on phages have been considered as a controlling factor for bacterial infections.

Objective: The aim of this study was to identify and isolate *Y. enterocolitica,* the bacterium-specific phage, from raw sewage.

Materials and Methods: Every 10 minutes, 6 samples each containing 30 mL raw wastewater were collected from the sewage treatment center of Vali-asr hospital, Tehran-Iran. Bacteriophage was isolated from sewage samples using Double-Layer Agar method. In addition, the samples were purified and the volume required for the isolation of bacteriophage was determined. Then, we investigated the sensitivity of the isolated bacteriophage to the temperature.

Results: Lytic bacteriophages were isolated from the samples obtained from hospital sewage. This bacteriophage was largely active on *Y. enterocolitica*. It was active at 4, 22, 37, 40, 50, 60, and 70°C, however it became inactive at 80°C.

Conclusion: The results showed that *Y. enterocolitica* specific bacteriophage could be isolated from hospital sewage samples.

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Background

Yersinia is a genus of bacteria in the family of Yersiniaceae. It is a heat-sensitive bacterium which can easily be destroyed at temperatures of 60°C and higher. *Yersinia* includes 11 species out of which only *Y. enterocolitica*, *Y. pestis*, and *Y. pseudotuberculosis* have been involved in human disease.[1] *Y. enterocolitica* is a gram-negative bacillus that can be isolated from a variety of sources such as water, food, soil, and animals; it is an important cause of intestinal infections in human and animals.[2] Human and animals infected with this bacterium exhibit symptoms such as septicemia, diarrhea, and mesenteric lymphadenitis.[3] Recent studies have shown that improper and irrational use of antibiotics leads to the emergence of antibiotic-resistant strains of this bacterium. Currently, in many countries, studies have focused on an alternate method for the treatment and control of antibiotic resistance.[4,5] Bacteriophages (phages) are viruses that only infect bacteria. In recent years, with an increase in the emergence of bacterial strains resistant to several antibiotics (multidrug resistant strains), there has been an emphasis on studies conducted to utilize phages. In addition, an increasing number of studies have replaced antibiotics with phages.[6] Phages have attracted great attention of researchers because they are easily available and do not affect the normal flora in humans and animals.[7] Although several effective bacteriophages with an emphasis on *Y. enterocolitica* have been introduced, few studies have investigated the details of the morphology, host spectrum, and specific receptor. To date, bacteriophages φYeO3-12[8-10] and BYenP-AP5[11] specific to *Y. enterocolitica*, O:3, phage PY54 specific to *Y. enterocolitica* O:5,[12] and phage φR1-37 specific to *Y. enterocolitica* have been detected.[13,14] Studies have shown that the receptor for _R1-37 is *Y. enterocolitic* O:3 LPS outer core (OC) hexasaccharide.[15-19] The host receptor for phages _YeO3-12 and vB_YenP_AP5 has been defined to be the LPS O antigen of serotype O:3, comprising of the sugar 6-deoxy-L-altropyranose.[10,11,20] Virulent phages are generally preferred as biocontrol agents as they cannot integrate their genome into the bacterial chromosome to form lysogens and lyse and kill infected target bacterial cells.[15] In 2015, *Yersinia* phages were isolated from

farm samples. *Y. enterocolitica* O:3 was killed by several phages. Host ranges of these phages were evaluated with 94 *Yersinia* strains, phages-infected strains.[19] The function of *Yersinia* phage PY100 to decrease the number of Campylobacter and *Y. enterocolitica* in meat at 4°C applying different multiplicities of infections (MOIs) was studied.[21] In this study, we reported the isolation of specific phage, *Y. enterocolitica.*

Objectives

The aim of this study was to identify and isolate *Y. enterocolitica,* bacteria specific phage, from raw wastewater.

Materials and Methods

Preparation of Bacteria

Yersinia enterocolitica bacteria were obtained from Food Microbiology Department of Health Faculty, Tehran University of Medical Sciences, Tehran, Iran. The bacteria were prepared using polymerase chain reaction (PCR) method. In addition, biochemical tests were performed to further confirm the type of bacteria.

Isolation of Bacteriophage

Every 10 minutes, 6 samples each containing 30 mL raw sewage were collected from the sewage treatment center of Valiasr hospital, Tehran, Iran. Then the samples were numbered from 1 to 6 and stored in a refrigerator for 24 hours to remove the primary sediments. Afterward, the samples were centrifuged at 4000 rpm and the supernatant was collected and stored in separate tubes. In the next step, 80 mL Brain Heart Broth (BH Broth) was prepared at an Erlenmeyer. After passing each sample from a 0.22 µm filter, 10 mL of each sample was combined with 10 mL of prepared Broth. The solution was passed through the filter (with the mentioned dimensions) to prevent the entrance of bacteria into the tube containing the Broth. After these steps, 10 mL of newly cultured *Y. enterocolitica* (which had been cultured for less than 24 hours) was added to each tube. In order to achieve a fully mixed solution, the sewage and bacteria were properly vortexed. At the end, all samples were incubated at 37°C for 24 hours. At the next step, the liquid culture was centrifuged again at 4500 rpm for 10 minutes. The supernatant was again passed through a membrane filter with a mesh size of 22 µm. Double layer method was used to confirm the presence of lytic phage in the filtered liquid. For this purpose, 100 mL of the filtered liquid together with 400 mL of the cultured bacteria were stored at 37°C for 30 minutes. Afterward, 4.5 mL of melted Brain Heart Infusion (BHI) agar, which had been cooled up to 50°C, was added to the solution. Then, the plates were stored at ambient temperature for 30 minutes to harden the agar. Finally, they were incubated at 37°C for 24 hours (the formation of plaque on plates represents the presence of lytic bacteriophage).

Purification of Bacteriophage

For this purpose, a plaque was picked using a glass Pasteur pipette and was added to Tryptic Soy Broth (TSB); it was incubated at 37°C for 24 hours. Afterward, the mixture of bacteria and bacteriophage was centrifuged at 4500 rpm for 10 minutes and was passed through a membrane filter. Then, the filtered liquid was again cleared via double layer method. The mentioned procedure was repeated 3 times to achieve 3 plaques. In the next step, bacteriophage was further purified via washing the plates by adding 5 mL of SM buffer to the tip of the plate and shaking and incubating at ambient temperature for 4 hours. Afterward, the buffer containing bacteriophage was separated from the plate and centrifuged at 4500 rpm for 10 minutes. The solution then was passed through the filter. Consequently, we obtained the bacteriophage suspension within the filtered liquid.

Determining the Host and Specificity of Bacteriophage

Spot test method was used to determine the host and specificity of bacteriophage. At this point, we used some gram-positive and gram-negative bacteria to test bacteriophage specificity. The selected bacteria were: *Y. enterocolitica*, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 2392, *Enterococcus faecalis*, *Salmonella enterica* serovar Enteritidis, and *Salmonella enterica* serovar Typhimurium. Every strain of bacteria was grown in BHI medium for 24 hours so that they entered the logarithmic phase. Then, 100 mL of culture was properly mixed with 4 mL of BHI 0.7%; the prepared mixture was used to cover the BHI agar 1.5%. After hardening, 10 µL of bacteriophage suspension was added to the top of the agar so that the tip of the sampler did not touch the surface of the agar. It was incubated at 37°C for 24 hours. The emergence of a transparent halo was the sign of sensitivity to bacteriophage.

Results

Primary Isolation of Bacteriophage

After mixing the filtered wastewater with the liquid culture containing *Y. enterocolitica*, it was stored at 37°C. Because of bacterial growth, there was much turbidity on tubes. The tube was centrifuged again and the solution was passed through the filter. Then, the bacteria were again added to the solution and it was vortexed at 37°C. Compared with the first day, there was less turbidity in the second day (Figure 1). This observation suggested the presence and the increasing amount of Yersinia phage in the tube. This procedure was repeated until there was no turbidity anymore.

Isolation of Bacteriophage

After the initial evaluation of wastewater and preparation of a solution containing phage, double layer agar method was used to confirm the presence of the phage and observe the viral plaque. One day after the study, the

phage plaques were formed on the medium (Figure 2).

Purification of Bacteriophage

After initial isolation of the phage and picking up the phage plaque, we used the method mentioned above and utilized the double layer agar method and SM and prepared the buffer of pure plaques which is shown in Figure 3. The size of plaques was about 2 mm.

Determining the Host and Specificity of Bacteriophage

To determine the host and specificity of bacteriophage we used spot test method. According to the results, the isolated phage was active only against *Y. enterocolitica* and the other bacteria, except for *Yersinia aldovae*, were resistant to this phage. As enterocolitica and aldovae are from the same strain, they are likely to have a similar receptor. However, the growth halo of aldovae was weaker than that of enterocolitica. Overall, as the results showed, it was a phage specific to *Y. enterocolitica*. The results are presented in Table 1 and Figure 4.

Discussion

Bacteriophages present immense potential both as a source for extending new tools for bacterial diagnostics and for usage in phage therapy. This factor is also valid for bacteriophages specific for *Y. enterocolitica***.** In this study, we isolated the bacteriophage against *Y. enterocolitica* from the sewage sample and observed its effects in vitro. After several cycles of proliferation and passages, the turbidity caused by bacteria was observed in the Brain Heart Broth medium, indicating that the virus appropriately controlled the growth of bacteria. In order to evaluate the effects of this phage on the infection associated with this bacterium, a study on the animal model has been performed, whose results will be presented in a future documentation. *Y. enterocolitica* can cause acute bacterial intestinal infections and clinical symptoms such as enterocolitis, acute mesenteric adenitis lymphoma, appendicitis, and septicemia.[15] To the best of our knowledge, this is a rare study reporting isolation of the *Yersinia* bacteriophage from sewage in Iran. There are many similar studies in other countries, the results of them have shown that the specific phage of *Y. enterocolitica* has been isolated from various sources,[18] including hospital waste and animal stool. And the result of our study also showed that this phage could be separated from hospital sewage in Iran. Woolston et al[17] investigated the effects of a cocktail containing 6 lytic bacteriophages on the bacterial count of *Salmonella* on the surfaces of glass and steel. The results of the study showed that this phage product reduced the bacterial count on the studied surfaces by 99%. This indicated that salmonella phage properly controlled the

Figure 1. Results of Injecting Phage Into the tube Containing the Bacteria. Right tube: Turbidity caused by bacterial growth without being exposed to wastewater; Left tube: No turbidity after several times of exposure to wastewater (indicating the presence of phage).

Figure 2. Primary Plaques Obtained From the Isolated Phages.

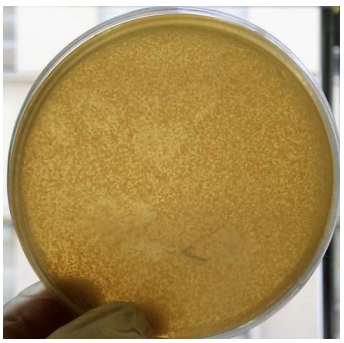

Figure 3. Purified Plaques of Phage.

Figure 4. Results of Phage Specificity Test; (A) *Yersinia enterocolitica*; (B) *Salmonella enteritidis*; (C) *E. coli;* (D) *Enterococcus faecalis*; (E) *Pseudomonas aeruginosa*; (F) *Yersinia aldovae*.

Table 1. Results of Specificity Test of Isolated Bacteriophages

Bacteria	Lysis
Yersinia enterocolitica	+
E. coli ATCC 25922	-
Pseudomonas aeruginosa ATCC 27853	-
Staphylococcus aureus ATCC 2392	-
Enterococcus faecalis	-
Yersinia aldovea	+
Salmonella enterica serovar Enteritidis	-

bacterial growth. In this study, the effect of the isolated phage on *Y. enterocolitica* was clearly observed. Jun et al extracted a specific phage which acted against *Shigella.* The phage was extracted from the water and its specificity was tested. According to the results, *Shigella sonnei* and *Shigella flexneri* were sensitive to the phage.[18] In this study, the specific phage acting against *Y. enterocolitica* was isolated from sewage. In 2015, Salem and colleagues showed that *Y. enterocolitica* specific bacteriophage was isolated from the pig stool. In this study, evaluation of the isolated phage showed that this phage was specific to different enterocolitica serotypes and other bacteria from the family and other genera were resistant to this phage.[19] The results of our study showed that the other bacteria were resistant to isolated phage. Moreover, in our study, the isolated bacteriophage was evaluated to determine the host and the sensitivity of different bacteria including *Y. enterocolitica*, *Salmonella enteritidis*, *E. coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Y. aldovae*to it. *Y. enterocolitica* and *Y. aldovae* were sensitive to this bacteriophage. Furthermore, inhibition growth zone in the spot test about*enterocolitica* was very large, but in the case of *aldovae*, this zone was slightly smaller.

Authors' Contributions

FD: isolation and identification of Yersinia bacteriophage. MMSD: Supervisor and help in the writing of the manuscript.
FH: consultant on this study.

Ethical Approval

This study dose not need to have any ethical approval .

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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