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Sewage as a rich source of phage study against *Pseudomonas aeruginosa* PAO

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ABSTRACT

Pseudomonas aeruginosa is a ubiquitous organism which has emerged as a major public health threat in hospital environments. Overuse of antibiotics has significantly exacerbated the emergence of multi-drug resistant bacteria such as *P. aeruginosa*. Phages are currently being utilized successfully for aquaculture, agriculture and veterinary applications. The aim of this study was to isolate and characterize of lytic *P. aeruginosa* phage from sewage of Ilam, Iran. Phage was isolated from sewage that was added to the enrichment along with the host and subsequently filtered. Plaque assay was done by using an overlay method (also called the double agar layer method). Purified plaques were then amplified for characterization. Finally, RAPD-PCR method was conducted for genotyping and Transition electron micrograph (TEM) recruited to determine the morphology and phage family. The phage had high concentration and tremendous effects against a variety of clinical and general laboratory strains (ATCC15693) of *P. aeruginosa*. Among a set of primers in RAPD panel, only P2 and RAPD5 primers, were useful in differentiating the phages. TEM images revealed that the isolated phages were members of the *Siphoviridae* family. The phage effectiveness and specificity towards target bacteria and potential to control biofilm formations will be investigate in our further studies.

1. Introduction

Pseudomonas aeruginosa is considered as the most frequently isolated Gram-negative organism in the blood stream, wound infections, pneumonia and intra-abdominal and urogenital sepsis. It is also a serious problem, infecting immune-compromised patients with cystic fibrosis (CF), severe burns, cancer, acquired immune deficiency syndrome (AIDS), etc. [1]. One of the most worrying characteristics of this bacterium is its low antibiotic susceptibility, which can be attributed to a concerted action of multidrug efflux pumps with chromosomally-encoded antibiotic resistance genes and the low permeability of the bacterial cellular envelopes [2]. Overuse of broad-spectrum antibiotics has also significantly increased the emergence of multi-drug resistant bacteria; consequently, most chronic *P. aeruginosa* infections with antibiotics are notoriously difficult to treat [3]. Additionally, *P. aeruginosa* has an innate ability to adhere to surfaces and form virulent biofilms making them persistent and particularly difficult to eradicate [4]. Thus, new alternative strategies to antibiotic therapy are in high demand by the worldwide medical and scientific communities.

Bacteriophages (phages) are an order of viruses that are able to infect bacteria, resulting usually in propagative lysis (lytic cycle) or lysogenization (lysogenic cycle) of the infected cell [5,6]. Depending on the species of the phage and host, conditions of the infection and the composition of media, phages can produce burst sizes between 50 and 250 progeny per cell per infective life cycle. After infecting, each phage in a host will produce 40,000 particles at the end of the second cycle. This will result in 8 million progeny at the end of the third cycle and 1.6 billion at the end of the fourth cycle [7]. Phages are strongly specific to their target bacteria and if

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prepared correctly are safe for use with humans since they show no negative activity against eukaryotic cells. Recruiting phage as a therapeutic agent was initiated in 1919; only a few years after Felix D'Herelle discovered it independently from Fredrick Twort, to treat dysentery and continued until the 1940s. Over this time, phages were used to treat various infections. With the recent increase in antibiotic resistance and poor efficacy of antibiotics against bacterial biofilms, there is renewed global interest in phage applications as a potentially powerful alternative to antibiotics [8].

Phage therapy is based on the use of lytic phages to combat multi-drug resistant bacteria, such as *P. aeruginosa*, and has many advantages compared to antibiotics: phages are very specific and efficient for their target bacteria, which mitigates the destruction of the patient's natural flora; they are not pathogenic to humans; and they persist only as long as the targeted bacteria are present [2,9]. Moreover, with regard the partial development of bacterial resistance to phages, bacteriophages might be suggested as valuable and may be the only efficient antimicrobial agent against some bacteria in specific situations. It is undeniably time to re-evaluate the possibility of phage therapy as a capable agent to control multidrug-resistant bacteria [10].

Lytic bacteriophages of *P. aeruginosa* belong to main family of phages; *Myoviridae*, *Siphoviridae*, and *Podoviridae*. They are dsDNA by 20–200 nm length [5,11]. These phages are considered to be economical, safe, self-replicating and effective bactericidal agents. This study was planned to evaluate the isolation, and characterization of *P. aeruginosa* phage in Ilam, a western province of Iran.

2. Materials and methods

2.1. Phage isolation

Samples were collected from sewage of Ilam University of Medical Sciences. Isolation was done according to a modified version of Martha Clokie's protocol [12]. 2 ml Mg_2SO_4, 5 CC of overnight bacteria and 20 CC of sewage were added to 20 CC Lysogeny Broth (LB) and allowed to be incubated at 37 °C and shaken at 100 rpm for 180 min. The enriched product was then centrifuged at 4500 rpm at 4 °C for 5 min and the supernatant was passed through a 0.45 μm filter. Finally plaque assay method was performed using an overlay method and a culture of *P. aeruginosa* ATCC PAO was recruited for isolations (Fig. 1).

2.2. Phage amplification

Plaques were plucked by using a sterile pipette tip, dropped into 200 μL of Mg_2SO_4 and 100 μL of an overnight bacteria culture and incubated at room temperature for 30 min with gentle vortex every 5 min. Aliquots of the amplifications were added to an overlay containing 500 μL–1000 μL of top agar (semi-solid agar) [12].

2.3. RAPD-PCR

Phage DNA was extracted using a NORGEN DNA extraction kit (NORGEN, Canada). Genotyping was done through the use of four different primers: OPL5 (5′-ACGCAGGCAC-3′), RAPD5 (5′-AACGCGCAAC-3′), P1 (5′-CCGCAGCCAA-3′), P2 (5′-AACGGGCAGA-3′) based on the PCR cycle table (Table 1) [13].

2.4. Transmission electron microscopy (TEM)

Samples were prepared with uranyl acetate 2% for TEM. One drop of the sample was placed on a carbonic grid with an added drop of 2.5% glutaraldehyde. After waiting 1–1.5 min the grid was dried using filter paper and washed with deionized water. (TEM Ziess 900 recruited to take micrograph). One drop of uranyl acetate 2% was again added to sample allowed to settle for 2 min and was dried with filter paper and was ready for electron microscopy [14].

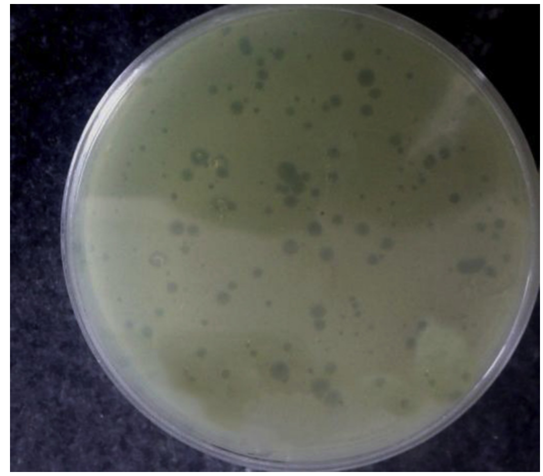

Fig. 1. Phage isolated against clinical strain from sewage.

3. Results

3.1. Isolation

Phage isolated from university sewage against clinical strain (tracheal secretion of a hospitalized male) (Fig. 1). Serial dilution done till 10^{-5} but all of plates had numerous uncountable plaques (data not shown).

Phage isolated against *P. aeruginosa* PAO strain (Fig. 2) lacked host ranges of relevant clinical strains unlike the phages that were isolated against clinical strains which were able to produce plaques on *P. aeruginosa* ATCC 15693 culture (data not shown).

Plaque purified by re-overlay method. Re-overlay plaques (re-O_1 in picture) were smaller than mother plaques (O_1 in picture) but they were too numerous to count (Fig. 3).

3.2. RAPD-PCR

Primer P2 and RAPD5 had more bands for typing this phage but there was just one band by primer OPL5 that showed this primer could not be useful for typing this phage (Fig. 4).

3.3. TEM

Electron microscopy indicated that the phage belonged to the *Siphoviridae* family and has head dimensions of a height of 120 nm

Table 1
RAPD-PCR cycle.

Time	Temperature
45 s	94 °C
120 s	30 °C
60 s	72 °C
4 cycles	
5 s	94 °C
30 s	30 °C
30 s	72 °C
25 cycles	
10 min	72 °C

Fig. 2. Plaques assay from a phage isolated against *P. aeruginosa* PAO.

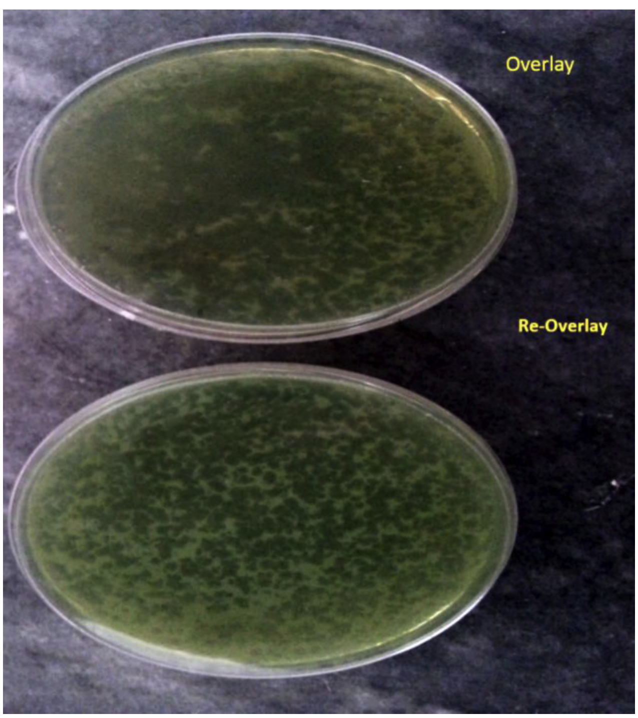

Fig. 3. Phage purification by re-overlay of plaques.

by a width of 50–60 nm. The morphology was similar to that of Pseudomonas phage PaMx72 (Fig. 5).

4. Discussion

With regarding to National Nosocomial Infections Surveillance System, *P. aeruginosa* must be considered as the third most common agent of hospital acquired infection which isolated from various cases such as patients suffering from respiratory diseases, cancer, children and young adults with cystic fibrosis and burns. It is reported that 10.1% of *P. aeruginosa* hospital acquired infections is contributed to mortality. Various antibiotic resistance mechanisms in *P. aeruginosa* are major reasons of this phenomenon [15,16].

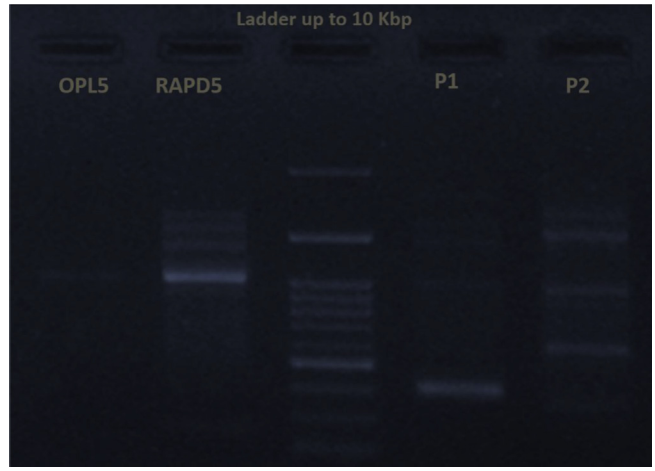

Fig. 4. RAPD-PCR for phage isolated against *P. aeruginosa* PAO.

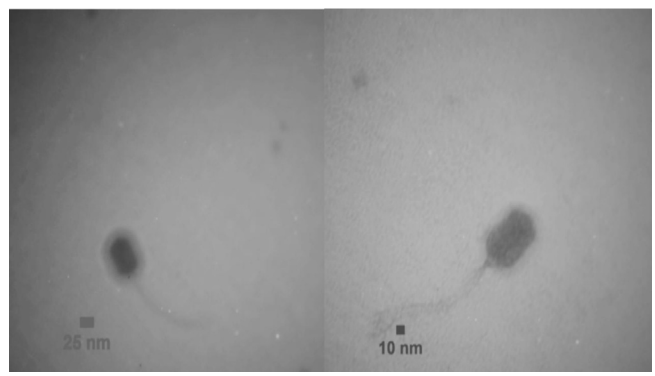

Fig. 5. TEM of phage isolated against *P. aeruginosa* PAO.

P. aeruginosa naturally has strong potency to adhere to solid surface and forms biofilm. In biofilm form *P. aeruginosa* could show resistance to various antibacterial elements such as chemical, physical and absolutely antibiotics. Biofilm formation is known as an essential strategy that utilized by bacteria, especially in human's body. Also, biofilm is responsible for numerous medical devices which are associated with infections. After failing of antibiotic efficiency in encountering with biofilm and antibiotic resistance bacteria, scientists reintroduced phage to world as effectiveness agent for encountering with this issue [5].

The previous study by Knezevic [17] reported that the investigation indicated that *Pseudomonas* phage could exploit LPS and pilus on bacteria as surface and therefore could enter to biofilm and eliminate bacteria. Another study by Azizian et al. [18], indicated phage potency to inhibit and remove biofilm formation so that, *Pseudomonas* phage in special concentration was more effective to inhibit or remove biofilm. On the other study by Sepúlveda-Robles et al. [19] on diversity of *Pseudomonas* phage, there was a various range of phage families for *Pseudomonas*. In this study, they showed *Pseudomonas* phages belonged to *Siphoviridae* by as 59% frequency and *Podoviridae*, *Myoviridae*, and *Leviviridae* by as 19%, 18% and 4% respectively. Also, they reported three new leviviruses for this bacterial specie.

Phage isolated against clinical strain in this study was capable of infecting ATCC 15693 while, phage isolated against a standard PAO strain had no efficacy towards any of the clinical strains. This phenomenon could be explained by a wide host range tendency among some phages. These phages are called broad host range which were first introduced by Jenson E.C., and also they are known as gene diversity carriers in nature. These phages could infect *Pseudomonas*, *Enterobacteriaceae*, Gram positive bacteria and *Bacillus* [20]. The present results show that isolated phage had high titer. Serial diluted phage suspensions (10^{-1}–10^{-5}) made numerous plaques on the bacteria plate. Generally, regards to phage application success in the Soviet Union and Eastern Europe, especially in 1940, there is hope to widespread phage application [21].

The United States Food and Drug Administration (USFDA) and European Medicines Agency (EMA) have programs to establish guide lines for clinical trials for phage applications. There is a need form standard protocols for evaluating the safety and efficacy of phages that could evaluate different aspects of a phage application [22]. Phage purification and sterilization of equipment is necessary for entry phage into clinical trials through Good Manufacturing Practices (GMP). Though, FDA recommends the use of phages for phage therapy, there is an opposite opinion about phage therapy in Eastern Europe that prefers to exploit cocktail of phage therapy [23,24].

5. Conclusion

To the best of our knowledge the current study was one of the first studies in this part of Iran that could evaluated the isolation and characterization of *P. aeruginosa* phage in this part of Iran. This research confirmed that sewage as a rich source of phage against *P. aeruginosa* PAO. The finding of the current research will increase optimism about the future researches to investigate on the phage therapy and also usage of proteolytic enzymes of phage which can be recruited instead of the whole viral particles. The usage of phage-derived proteolytic enzymes in combination with β-lactamses may have sufficient potency to eliminate or prevent the formation of bacterial biofilms [25].

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