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Isolation and characterization of lytic bacteriophage against common pathogenic bacteria

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Abstract

Regarding their specificity, bacteriophages have been widely investigated to combat bacterial infections. Phage therapy is proposed as a promising alternative antibacterial agent. The present study was conducted to isolate and characterize bacteriophages against clinical bacterial isolates from sewage water. Recovery of phage was high from the processed sewage water (66%) against tested bacterial hosts. Plaque assay revealed four different plaques morphology against *Escherichia coli* with high lytic activity. In contrast, one small morphology plaques were appeared against *Staphylococcus aureus* with low lytic activity. The mean phage titer of phage isolates was $7.7x10^{9}$ and $2.9x10^{11}$ plaque forming unit/ml for *S. aureus* and *E. coli* respectively. The isolated phages showed a narrow host range when tested against 19 different bacterial isolates. The electron microscopy revealed that EC1 Phage has the typical morphology of the family Podoviridae, order Caudovirales. The isolated bacteriophages need to be further characterized at the molecular level and tested in vivo to be used in one of the bacteriophage applications.

Keywords: Bacteriophages, Sewage Water, *Escherichia* Coli, *Staphylococcus Aureus*, Antibacterial Agent.

INTRODUCTION

The emergence of multi-drug resistance (MDR) bacteria strains, which are posing a global health threat, has developed the interest of scientists to use alternative strategies to control pathogenic bacteria (Ullah et al., 2021). Lytic bacteriophages or phage therapy, were suggested to be an attractive alternative to antibiotics for the biological control of bacterial disease (Yu et al., 2013).

Phages are widely distributed in soil, sewage, animal wastes and their secretions. Determination of their host range is very important for their use in phage therapy, while there are two types of host range, some phages can only infect one or a few bacterial strains so they have a narrow host range, while other phages can infect many species from different genera and this called broad host rang (Ross et al., 2016).

Unlike antibiotics, phages kill target bacteria specifically and do not destroy the normal flora of the host, so the application of phage therapy could be a natural and non-toxic method to reduce and

control the growth of human pathogenic bacteria (Masoud et al., 2020); The efficacy of phages to control bacterial growth had been extensively investigated, Shende and his colleagues isolated and characterized phages from dairy farm waste disposal using *E. coli* and *Bacillus subtilis* as the host system. The recovered phage had a broad host range (Shende et al., 2017). Other investigations succeeded in the isolation highly virulent phages with high specificity to their host even MDR strains like methicillin resistant *S. aureus*, *Acinetobacter baumanni*, and *E. coli* 0157:H7 (Esmael et al., 2021; Rasool et al., 2016; Sjahriani et al., 2021).

There is considerable attention on using phages to treat bacterial infections (Al-Anany et al., 2023); Le and his colleagues employed intravenous administration of phage in place of antibiotics to successfully treat a recurrent UTI caused by *Klepsiella pneumoniae* (Le et al., 2023). Some identified and isolated phages, however, have a very limited host range and are unable to infect distinct bacterial strains within the same species. This restriction might be overcome by preparing phage libraries, which would enable researchers to choose the best phage cocktail for a given infection (Duarte et al., 2024).

The present study aims to isolate and characterize bacteriophage from sewage water against common pathogenic bacteria, and this will be the first report on the isolation of a bacteriophage in Misurata city.

MATERIALS AND METHODS

Sample collection

Sewage water sample from the Wastewater Treatment Station in Misurata was collected in January 2022 in sterile container, brought to the lab and processed by centrifugation at 4000 r.p.m for 10 min to remove any large debris. Then the supernatant was filtered using 0.22µm syringe filter unit in an attempt to obtain phage containing water sample (Rasool et al., 2016).

Bacterial primary host

In this study, four-gram negative bacteria (2 *Acinetobacter sp*., *Pseudomonas aeruginosa*, and *Escherichia coli*) and two *Staphylococcus aureus* isolates were used as host strains for bacteriophage isolation. These clinical isolates were obtained from Misurata Reference Laboratory and Misurata Central Laboratory. Most of these isolates revealed a remarkable elevation of resistance to antibiotics.

Amplification and preliminary screening of bacteriophage

Phage contents in filtrated sewage water against each bacterial isolate were propagated by culture-enrichment method according to the Sambrook et al. protocol with minor modifications (Sambrook et al., 1989). Briefly: 15ml filtrate sewage sample and 25ml sterile nutrient broth were mixed with 10ml overnight culture of host stain and incubated at 37 C^0 for 48h. After that bacteria were removed by centrifugation at 4000 r.p.m for 10min then filtration of the supernatant via 0.22µm syringe filter unit.

To test the presence of lytic phages in filtrates, spot assay and turbidity reduction assay were used. In the spot method; 100µl of filtrate was spot inoculated on the surface of counterpart log phase bacterial lawn on Mullar Hinton Agar plates. After 24h incubation period, plates were examined for the presence of lytic zones on bacterial lawns (Masoud et al., 2020; Rasool et al., 2016).

The test was repeated twice and two high lytic positive filtrates were selected for further experiments. Whereas in the turbidity reduction technique, 3ml log phase bacterial culture was inoculated

with 0.5ml from filtrate, OD of enriched culture is measured at 600nm wavelength with respect to control (bacterial culture only) via spectrophotometer at zero time, after 24h and after 48h, incubation period at 37 C^0 (Qamar et al. 2019). The two methods were repeated thrice before judgment the results.

Bacteriophage morphology

In order to determine the plaque morphology, double agar layer (DAL) method was conducted (Shende et al., 2017). Briefly: a mixture of serially diluted phage filtrates with their log phase host culture was added to 3ml molten soft agar and poured quickly onto solidified Mullar Hinton agar plates. The plague morphology was noted and counted after incubating the plates overnight at 37 C^0. The phage titer was expressed as plaque forming unit per 1 milliliter (PFU/ml) and determined by the following formula: (PFU/ml = (number of plaques X dilution factor) / volume of phage plated in ml) (Qamar et al., 2019).

E. coli Phage morphology was determined using a transmition electron microscope (TEM) with the same protocol described by Chen et al. (Chen et al., 2018). The phage filtrate was negatively stained with 2% unaryl acetate on carbon coated grids and examined under TEM (Zeiss EM10CR, Germany). The size and morphology of the phage were determined from three identical phage particles.

Host range

Spot assay was carried out as described previously to determine the host range of the two isolated phages. The primary bacterial host along with 19 different MDR bacteria stains were tested (2 *Acinetobacter sp.*, *Pseudomonas aeruginosa*, *2 Staphylococcus aureus*, *Enterococcus sp*., *6 Escherichia coli*, *3 Klebsiella sp*., *2 Citrobacter koseri*).

RESULTS AND DISCUSSION

Efficacy of sewage water for bacteriophage isolation:

Bacteriophages are obligate bacterial parasites that infect and kill bacteria in a specific manner. Considering of emergence of bacterial resistance to broad-spectrum antibiotics; phage therapy is proposed as a promising alternative antibacterial agent. Besides this, they are ubiquitous in nature and they have been estimated to be more abundant in aquatic systems especially, in sewage water (10^8-10^{10} / ml), compared to seawater and soil (Ullah et al., 2021).

In the present study, an untreated sewage water sample was processed for phage isolation against six different clinical pathogens. The final filtrate obtained after the enrichment step with each host was separately assessed against the coordinate host through spot assay.

Recovery of phage was high (66%); The tested sewage sample showed positive lytic spots against all the tested hosts except for two (figure1), which is in accordance with previous studies reporting the high recovery (72% - 93%) of bacteriophage in sewage water against *S. aureus, E. coli, B. subtilis and A. baumannii* [5, 6, 13](Alsaffar,2019; Rasool et al., 2016; Shende et al., 2017).

Lytic activity of processed sewage filtrates

In order to confirm the presence of bacteriophages in *E. coli* and *S. aureus* filtrates which revealed clear lytic zones in spot assay; each filtrate was added to both cultures of counterpart bacteria and monitored for cell lysis as indicated by reduction of culture turbidity comparing with control.

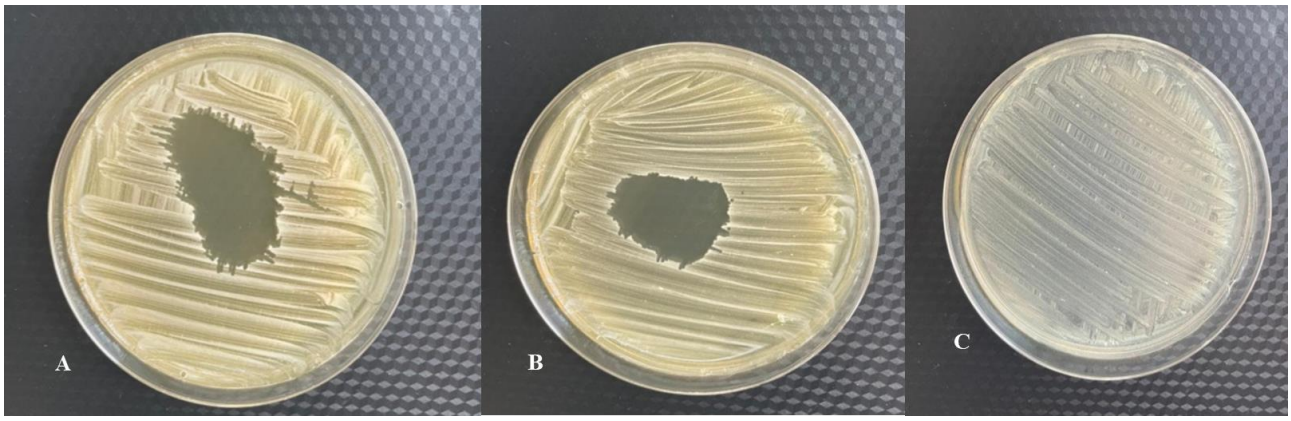

Figure (1). Spot assay for lytic bacteriophage screening is sewage water filtrates. A: The lytic activity of filtrates *on E. coli S1* (clear zone), B: Lytic activity of filtrates on *S. aureus* (clear zone). C: No lytic activity of filtrates on *Acineto-bacter sp* .

OD600nm of filtrate treated *E. coli* was recorded as 0.141 and 0.066 after 24h and 48h incubation period respectively. The reduction in the turbidity compared with the control indicates to presence of bacteriophages, which lyse the bacteria. The high reduction in the turbidity (0.066) compared with turbidity at zero time (0.559), suggests that phage in *E. coli* filtrate has high lytic activity. Whereas turbidity assay of *S. aureus* filtrate indicates to presence of phage with low lytic activity since it revealed a slight reduction in the turbidity between the zero time (0.315) and 48h (0.263) incubation period.

Characterization of phage plaque and determination of phage titer

Plaque assay by using the double agar layer (DAL) method was used to isolate and characterize the suspected phages in positive filtrates in this study. Under optimal conditions; plaque morphology can be a consistent feature of the bacteriophage. Except for T7, most phage plaques acquire a certain size, appearance and edges after a period of incubation. Therefore, it is assumed that a change in plaque morphology during the study may have resulted from a mutation in viral strain (Spanakis & Horne, 1987). Additionally, Shende suggests that variation in the plaque morphology may related to the differences in phage strain, agar strength and addition of cations (Shende et al., 2017).

Generally, more virulent phages have less lysis time and produce larger clear plaques with high plaque productivity (Galle et al., 2011). Depending on the morphology of plaques; four different lytic phage morph-type were observed against *E. coli* (figure2). In this study infection of *E. coli* S1 by these phages exhibited clear, circular plaques with infinitive edges and depending on differences in the size these morph-type recorded as: Large (3.5mm), medium (1.5-2mm), small (=1mm) and very small which referred to as phage EC1, phage EC2, phage EC3 and Phage EC4 respectively.

Electron microscopy of phage EC1 showed that the appearance of PhageEC1 was composed of an isometric polyhedral head approximately 43nm in diameter and a short tail approximately 12nm in length (figure 3). Based on these morphological characteristics and according to the International Committee on Taxonomy of Viruses (ICTV) classification; PhageEC1 was determined as a member of the family Podoviridae and the order Caudovirales (Chen et al., 2017). bacteriophage effective against *E. coli* belonging to the Podoviridae family was reported previously (Vera-Mansilla et al., 2022).

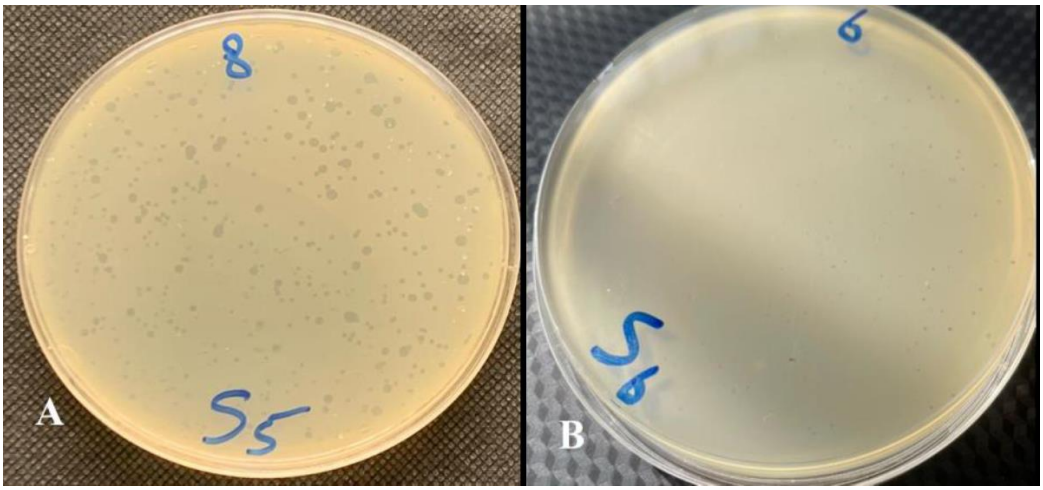

Figure (2). Plaques morphology. A: DLA plate of 10-8 dilution of *E. coli* S1 phages shows different morph-type plaques. B: DLA plate of 10-6 dilution of *S. aureus* S1 phages with one morph-type plaques.

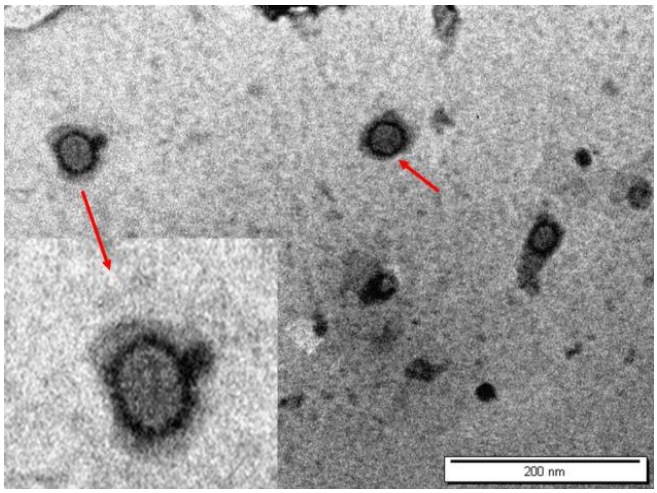

Figure (3). Transmission electron micrograph of phage EC1(marked with red arrows).

For the determination of phage titer, 10^{-8} and 10^{-9} dilution plates having 198 and 58 plaques were selected (falling in the recommended range of 30 - 300). The total *E. coli* plague titer was high in the used sewage water sample ($2.9x10^{11}$ PFU/ml.). the present findings are in accordance with earlier reports of Shende et al., who isolated three different phage morph-types against *E.coli* with mean phage titer ranging between $3x10^{10}$ and $5x10^{12}$ PFU/ml (Shende et al.,2017). Qamar and his colleagues found 5 different lytic coli-phage with (10^{2}-10^{7} PFU/ml) titer in sewage water (Qamar et al., 2019). However, other studied showed low coli-phage plaque assay results (21-86 PFU/ml) (Megha et al., 2017).

Infection of MDR S. aureus isolates by phage produced one morph-type clear tiny plaques on the surface of the double layer agar plate with a mean titer of $7.7x10^{9}$ PFU/ml (Figure 2), the isolated phage against *S. aureus* S1 strain designated as phage SA. The tiny clear plaques were previously reported for *S. aureus* phages (Kaur et al., 2012; Rasool et al., 2016).

Host range of isolated phages

The isolated phages were tested against a variety of multi drug resistance clinical bacterial isolates. Among the nineteen strains, none of the bacteria was found sensitive to phage SA except its prima-

ry hosts. Besides their respective hosts, phage ECs showed lytic activity against two MDR isolates: *Acinetobacter sp*. and *Staph. aureus*.

Likewise present study, various investigations revealed that host specific bacteriophages against human MDR pathogen are prevalent and can be readily isolated from sewage samples (Kaur et al., 2012; Rasool et al., 2016; Shende et al., 2017).

The narrow host range reported during this study conforms to the reports of Carey-Smith et al., Shende et al., Qamar *e*t al., and Othman et al., who had isolated narrow host range phages restricted to respected host only or to maximum of two bacterial species (Carey-Smith et al., 2006; Othman et al., 2015., Qamar et al., 2019; Shende et al., 2017). The Specificity of phages is due to the fact that the virion of the virus interacts with specific receptors on its host's surface and this interaction cannot be achieved if a slight change happens in the structure of the receptor. Therefore, highly specific phages have applications in phage typing methods for the identification of bacteria at the sub species level (Megha et al., 2017). In contrast, broad host range phages are more useful in some applications of bacteriophages, mainly, phage therapy since a broad host range phage would be equivalent to broad-spectrum antibiotics. Noteworthy, narrow host range phages against MDR bacteria are effective for personalized phage therapy (Mattila et al., 2015).

CONCLUSION

Specific bacteriophage against multidrug resistance bacteria could be isolated from sewage water that was positive for 66% of tested MDR bacteria hosts in the present study. High titers of five different morph-type phages were isolated; four against *E. coli* with high lytic activity and one against *S. aureus* isolate. The isolated phages showed a narrow host range mainly specific to the respective host or a maximum three different species. This preliminary study needs to be extended to include more sewage water samples and more different hosts. Additionally, the isolated bacteriophages need to be further purified and characterized to evaluate it is lytic efficacy at the vivo level.

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ETHICS

There are no ethical issues that may arise after the publication of this manuscript.

Duality of interest: The authors declare that they have no duality of interest associated with this manuscript.

Author contributions: Asma Alilesh and Marwa Al-wash designed the research work and carried out the major experiments. Asma Alilesh performed the scientific discussion and wrote the manuscript. Tasneem M. Alswehly1and Khawla A. Aween assissted in experiments.

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