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PHAGE-BASED BIOCONTROL OF MULTI-DRUG-RESISTANT BACTERIA IN ISMAILIA CANAL WATER

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ABSTRACT

This study was aimed examined the antibiotic resistance profiles of wild *Aeromonas* strains isolated from the Ismailia Canal and wastewater treatment plants (WWTPs). Three presumptive *Aeromonas* strains were identified, with 68.4% verification using 16S-rDNA analysis. The strains included *Aeromonas caviae* (Ama1), *Aeromonas hydrophila* (Ama2), and *Aeromonas sp.* (Ama3), all showing significant drug resistance, indicating their potential as reservoirs for antibiotic resistance genes. Antibiotic sensitivity tests indicated that strains Ama1 and Ama2 showed 100% sensitivity to tetracycline (TE), amikacin (AK), ofloxacin (OFX), and vancomycin (VA), while strain Ama3 was more resistant. A novel aerophage, MK01, was isolated and shown to effectively lyse the *Aeromonas* strains, with high concentrations detected in the middle section of the Ismailia canal. Characterization of aerophage MK01 indicated that it belongs to the *Myoviridae* family and maintains stability across temperatures from 4°C to 72°C and pH levels from 4 to 10. Scanning electron microscopy showed that phage significantly reduced multi-drug-resistant (MDR) bacterial growth and impacted biofilm formation. Phage-mediated biocontrol was applied in a batch reactor with mixed cultures. Metagenomic analysis of treated batch reactor samples showed shifts in microbial community structures, with lytic aerophage MK01 decreasing MDR strains from 81.5% to 34.1% in 8 h. Aerophage MK01 effectively improved wastewater quality, resulting in an average increase of 56.45% in dissolved oxygen, alongside reductions in turbidity (80.01%) and biochemical oxygen demand (BOD) (46.15%) compared to batch reactors without the purified phage. The study concludes that phage-based biocontrol offers a promising approach for managing antibiotic-resistant bacteria in wastewater treatment, achieving effective results without producing chemical byproducts and ensuring safe reuse.

Key Words: Aeromonas, MDR, Aerophage, SEM, Metagenomics.

1. INTRODUCTION

Multidrug-resistant (MDR) bacteria pose a significant threat to global health and society **(Medina and Pieper, 2016)**. The World Health Organization (WHO) has noted rising levels of multidrug-resistant bacteria in the environment and has called for enhanced monitoring to combat antibiotic resistance **(Azzam *et al.*, 2017)**. Antibiotics are crucial for treating bacterial infections in both humans and animals; however, their misuse can lead to nutritional deficiencies and various side effects **(Rahman *et al.*, 2022)**. The unchecked use of antibiotics contributes to the proliferation of antimicrobial resistance, which is a growing concern worldwide. MDR bacterial strains present a serious risk to living organisms **(Fatima *et al.*, 2023; Almutairy, 2024)**. Key factors driving the emergence of these bacteria include excessive antibiotic use in livestock, wastewater discharge from the agricultural sector, natural selection pressures, and the presence of resistant plasmids **(Serwecińska, 2020; Wang *et al.*, 2023)**. Untreated mixed drainage water serves as a primary reservoir for multidrug-resistant bacteria and genes (MRB/MRG) **(Gomes et al., 2023; Azzam *et al.*, 2024)**. Ultimately, the presence of antimicrobials and multidrug-resistant bacteria in canal surface water is exacerbated by sewage pollution **(Canal *et al.*, 2016)**.

Aquatic environments, including drainage water, rivers, canals, and rayahs, play a significant role in the dissemination of antibiotic-resistant genes among bacterial species **(Azzam, 2015; Canal *et al.*, 2016; Fuentes *et al.*, 2019; Barathe *et al.*, 2024)**. Research indicates that sewage water serves as a hotspot for MDR, facilitating the spread of multidrug-resistant genes (MRG) into the ecosystem **(Elbahnasawy *et al.*, 2021)**. Canals, in particular, are prone to the proliferation of multidrug-resistant bacteria (MRB) due to the high levels of antibiotics discharged from human and industrial waste, which often contain elevated organic matter, ammonium, and phosphorus **(Azzam, 2010; Soliman *et al.*, 2022)**. This influx alters the carbon-to-nitrogen (C/N) ratio in surface water, promoting MDR bacterial growth. Horizontal gene transfer mechanisms such as conjugation, transformation, and transduction allow antibiotic-resistant genes to spread among different bacterial populations **(Qi *et al.*, 2023)**. Effluents carrying MRB or MRG can serve as reservoirs of antibiotic resistance in receiving water bodies **(Wu *et al.*, 2018)**. While chlorine is commonly used as a disinfectant in water treatment plants (WTPs) to manage antibiotic-resistant bacteria, its chemical disinfection process can also negatively impact beneficial microorganisms essential for nutrient removal **(Azzam et al., 2022; Flach *et al.*, 2024)**. Studies have shown that both ultraviolet (UV) and chlorine disinfection can inadvertently increase intracellular and extracellular antibiotic resistance genes in full-scale WTPs **(Calderón-Franco *et al.*, 2021; Azzam *et al.*, 2023; Lau et al., 2024)**. Despite various chlorine doses applied to inactivate antibiotic-resistant bacteria, approximately 38% of erythromycin-resistant genes and 76% of tetracycline-resistant genes remain unaffected **(Das, 2023)**. Thus,

there is a pressing need for alternative methods to target unwanted bacteria in WTPs.

Ismailia Canal, a vital waterway in Egypt, has been subjected to extensive pollution from various sources, including agricultural runoff and untreated wastewater **(El Gohary, 2015; Azzam and Ibrahim, 2021)**. This contamination not only disrupts the local ecosystem but also poses serious health risks to communities relying on the canal for irrigation, drinking water, and other human activities **(Elbana, *et al.*, 2017)**. The presence of MDR bacteria in this water source exacerbates the issue, highlighting an urgent need for effective biocontrol strategies **(El-Dougdoug *et al.*, 2020; Azzam *et al.*, 2024)**. Implementing phage therapy could provide a sustainable solution to mitigate the risks associated with these resistant strains **(Pirnay *et al.*, 2015)**. In recent years, a phage-based biocontrol strategy has been implemented in sewage water sanitation to mitigate issues such as biomass bulking, membrane biofouling, antibiotic-resistant bacteria, and other micropollutants **(Azzam and Faiesal, 2019; Soliman *et al.*, 2023)**.

Bacteriophages, or phages, are viruses that specifically infect and lyse both pathogenic and non-pathogenic bacteria **(Abdelsattar *et al.*, 2022)**. This approach, known as phage therapy, has been utilized across various fields, including medicine, industry, agriculture, and wastewater treatment, to target and diminish harmful bacterial strains **(Düzgüneş *et al.*, 2021)**. Most research in this area has focused on using pure cultures to manage bacterial hosts. While phage-mediated biocontrol has proven effective in reducing antibiotic-resistant bacteria and membrane biofouling during drainage water treatment processes, it appears that there is a lack of studies employing purified mixed cultures, such as activated sludge and sediments **(Shivaram *et al.*, 2023; Azzam *et al.*, 2024)**.

In the present study, the developed and implemented new aerophage within a mixed system to combat MDR bacteria isolated from the Ismailia Canal and WWTPs were studied. This approach was carried out in a mixed-culture batch reactor, representing a promising strategy to tackle the challenges posed by antibiotic resistance. By harnessing the natural predatory capabilities of bacteriophages, it was aimed to enhance the safety and quality of this essential water resource. This ground breaking method not only seeks to protect human health but also strives to preserve the integrity of aquatic ecosystems, paving the way for more sustainable water management practices.

2. MATERIALS AND METHODS

2.1. Sampling and isolation of antibiotic-resistant bacteria

Water samples were collected from the Ismailia Canal and wastewater treatment plants (WWTPs) in Cairo, Egypt. Duplicate samples of 100 mL were filtered using a millipore nitrocellulose membrane with a pore size of 0.45 µm and then inoculated into 500 mL Erlenmeyer flasks containing 100 mL of Luria-Bertani (LB) broth supplemented with 60 µg/mL tetracycline (BD Difco, Thermo Fisher Scientific, Waltham, MA, USA). All flasks were incubated at 37

± 2°C for 24 hours under shaking conditions at 200 rpm. Following this, 10-fold serial dilutions were plated in duplicate on 20 µg/mL methicillin Bacto agar (BD Difco, Thermo Fisher Scientific, USA) and incubated at 35 ± 2°C for 24 - 48 hours. Well-isolated colonies were subcultured on the same medium. The identified single isolate underwent a BacLight bacterial viability test (Invitrogen, Thermo Fisher, USA) using an Olympus BX53 microscope. Based on the purity of the bacterial colonies and their morphological variability, well-isolated bacterial DNA genomes were selected for total DNA extraction using a commercial DNA genomics kit (Qiagen, Germany, Hilden). PCR amplification of the 16S rRNA gene was performed using universal primers 65F (TCCAGGTGTAGCGGTGAAAT) and 973R (TGAGTTTTAACCTTGCGGCC). The PCR reaction was conducted in 30 µL volume tubes, following the protocol outlined by **Azzam *et al.*, (2024)**, and included 2.5 mM dNTPs (3.00 µL), 25 mM $MgCl_2$ (3.00 µL), 10X PCR buffer (3.00 µL), 10 µM primers (2.00 µL), Taq DNA polymerase (3 U/mL, 0.20 µL), template DNA (25 ng, 2.00 µL), and dH_2O (16.80 µL). The thermal cycling conditions included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes, with a final extension at 72°C for 5 minutes. PCR products were visualized on a 0.8% agarose gel stained with ethidium bromide (1 µg/mL) and purified using a PureLink Quick Gel Extraction Kit according to the manufacturer's instructions. Sequencing of the purified bands was performed at MC Lab in South San Francisco, CA, USA, following the manufacturer's protocol with the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Version 3.1, Applied Biosystems) and analyzed on an ABI PRISM 3700 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA). The raw sequences were verified, filtered for quality, trimmed at the primers, and merged using Qiime2 v. 8 software (**Bolyen *et al.*, 2019**). The sequences were then compared with existing 16S rDNA gene sequences using the NCBI BLAST program. Phylogenetic analysis was conducted using a neighbor-joining approach with 1000 bootstrap replicates in MEGA X software (version 10.0).

2.2. Antibacterial activity

The tetracycline-resistant bacteria identified in this study were evaluated for their susceptibility to various antibiotics. The antibiotics tested included Amoxicillin/Clavulanate (AG) (a broad-spectrum β-lactamase inhibitor), Methicillin (MET) (which inhibits bacterial cell wall synthesis), Tetracycline (TE) (an inhibitor of cellular metabolic processes), Vancomycin (VA) (which alters bacterial cell membrane permeability and RNA synthesis), Ofloxacin (OFX) (which blocks bacterial DNA replication), and Chloramphenicol (C) (which inhibits microbial protein synthesis). These antibiotics were applied at different concentrations: 25, 60, 85, 250, and 500 μgmL^{-1}. The antibiotic sensitivity tests were conducted in triplicate using test tubes. Negative controls

(without antibiotics) and positive controls (with antibiotics) were incubated at 35 ± 2°C, shaking at 100 rpm for 24 hours. Bacterial growth in the test tubes was assessed by measuring the absorbance at 600 nm every 2 hours, and the resistance profiles for each antibiotic were represented as graphs showing the average absorbance values.

2.3. Isolation, propagation, and purification of bacteriophage

Specific phage was isolated from all water samples and propagated according to the method described by **Azzam *et al.*, (2024)**. In brief, 20 mL water samples from each site were centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatants were then filtered through a sterile 0.45 µm pore size nitrocellulose membrane. A mixture consisting of 50 mL of the filtered water sample, 50 mL of sterile tryptic-soy broth (TSB) medium, and 5.0 mL of overnight cultures of reference strains, including *Aeromonas hydrophila* ATCC 7966 and *Aeromonas hydrophila* ATCC 35654, was incubated at 37°C for 24 h. After standing at 37°C with shaking at 200 rpm for 2 h, the mixture was centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was then filtered through 0.22 µm nitrocellulose membranes to check for the presence of lytic aerophage using a spot test as described by **Azzam (2015)**. Specifically, 10 µL of the obtained supernatant was spotted on lawns of reference strains cultured on tryptic-soy agar (TSA) plates. The appearance of clear zones after overnight incubation indicated the presence of lytic aerophage specific to the host strain. The aerophage titer was quantitatively determined using the double agar overlay (plaque assay) method described by **Adams (1959)**. Phage suspensions from all water samples were serially diluted tenfold, and 100 µL from each dilution was combined with 100 µL of host bacteria, then mixed with 5.0 mL of sterile molten soft agar (0.7% w/v). This mixture was quickly poured onto TSA plates and allowed to harden for 30 minutes before being incubated at 37°C for 18 h. *A.hydrophila* ATCC 7966 and *A.hydrophila* ATCC 35654 were used as reference host bacteria at concentrations of 10^8 and 10^{11} $CFUmL^{-1}$. After 24 h, the plates were examined for plaque formation, and plaque-forming units were calculated according to **Stephenson (2010)** using the appropriate equation:

Plaque forming units (PFU)/mL = Numbers of plaques * Dilution factor / Phage volume plated (1 mL)

Phage obtained from the plaque assay was further purified following the method described by **Mohan Raj *et al.*, (2018)**. Single plaques exhibiting morphological similarity were selected and re-plated three times to ensure the production of pure phage stocks. The purified aerophage was then inoculated into 5.0 mL of TSB, mixed with 1% overnight culture of the host strain, and incubated at 37°C. Once complete lysis occurred, the mixture was centrifuged, filter sterilized, and treated with 1% (w/v) chloroform to eliminate any remaining bacterial cells. The purified aerophage was stored in 60% glycerol at -80°C for long-term preservation, while short-term stocks were kept at 4°C for future investigations.

2.4. Characterization of bacteriophage

2.4.1. Morphological characteristics (electron microscopy)

The purified aerophage was analyzed for morphological assessment using transmission electron microscopy (TEM) at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. Following the standard negative staining protocol described by **Ezzat and Azzam (2020)**. A phage suspension was prepared. Specifically, 3 µL of the aerophage solution was applied to carbon-coated copper grids, allowed to dry for 5 minutes, and then stained with a 2% uranyl acetate solution. All images were captured using a TEM (JEM1010, JEOL 1010, Tokyo, Japan) operating at a voltage of 100 kV.

2.4.2. One-step growth curve and stability of bacteriophage

The evolution of purified aerophage was assessed through a single-step growth experiment, as previously described by **Azzam (2015)**. In summary, the specific bacterial host, *A.hydrophila* ATCC 7966, was propagated and centrifuged at 10,000 rpm for 20 min at 4°C. The resulting pellets were then resuspended in 20 mL of fresh tryptic soy broth (TSB) medium, achieving an approximate concentration of 1×10^{10} colony-forming units ($CFUmL^{-1}$). All cells were harvested in 50 mL of TSB medium containing 1 mM $CaCl_2$ and mixed with partially purified phage at a multiplicity of infection (MOI) of 0.1 for 10 mins at 30°C. The cocultures were subsequently centrifuged at 20,000 rpm for 5 minutes and re-suspended in the original volume of TSB medium with 1 mM $CaCl_2$, followed by incubation in a water bath at 28°C with shaking at 200 rpm. Samples were collected at 5 min intervals over 40 min, and the aerophage titer was determined using the double-layer plaque assay technique, repeated three times. On the other hand, both thermal and equilibrium pH conditions were assessed using purified aerophage stocks with titers ranging from 45 to 93 x 10^{10} PFU/mL. These stocks were incubated at temperatures of 4, 25, 35, 55, 65, and 75°C for 1 h. The pH of the TSB medium, inoculated with purified aerophage isolate (36-80 x 10^9 PFU/mL), was adjusted to various values (4-12) using 1 M hydrochloric acid (HCl) and 1 M sodium hydroxide (NaOH), followed by incubation at 37°C for 1 h. The phage titer was then determined using the double-layer agar plaque assay technique.

2.4.3. Molecular verification of aerophage

The purified aerophage was identified through coat protein (CP) nucleotide gene sequencing. Total genomic DNA from the highly purified aerophage was extracted using a Mini kit (Qiagen, Germany, Hilden) and visualized on a 0.8% (w/v) agarose gel with ethidium bromide (1 µg/mL). Primers were designed based on CP-gene nucleotide sequences available in the International NCBI databases (http://www.ncbi.nlm.nih.gov). PCR amplification of the CP-gene was conducted using specific primers, 82F (CTGATTATTCCGGTGCAGCC) and 104R (ACATAGCCATAAATCGCCGC). The PCR reaction was performed in a total volume of 30 µL, which included 25 ng of template DNA, a mixture of

dNTPs (2.5 mM), primers (10 pmol), $MgCl_2$ (25 mM), buffer (1X), Taq polymerase enzyme (5 U/mL), and sterilized nuclease-free dH_2O. Gene amplification was carried out in an automated thermal cycler (model Techno 51z) with the following program: initial denaturation at 96°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min, concluding with a final extension at 72°C for 10 min. All PCR products were visualized on a 1% (w/v) agarose gel using ethidium bromide (1 µg/mL). The bands were excised and purified using a PureLink Quick Gel Extraction Kit according to the manufacturer's instructions. The phage was sequenced at MC Lab in South San Francisco, CA, USA (https://www.mclab.com), utilizing the dideoxyribonucleoside chain termination method developed by **Sanger *et al.*, (1977)**. The nucleotide sequence was automatically determined through electrophoresis of the cycle sequencing reaction products on a 310 Genetic Analyzer. Data were provided as fluorometric scans, from which the sequences were assembled using sequence analysis software. Partial nucleotide sequence was compared with international databases using the NCBI BLAST program. Multiple sequence alignments were conducted using DNAMAN 5.2.9 and CLUSTALW software version 1.74. Genetic distances were calculated using Jukes and Cantor's method to correct for superimposed variants, with MEGA X version 10.0 software. Phylogenetic distances among the identified aerophage and other aerophages published in international databases were constructed using the neighbor-joining method and the unweighted pair group method with arithmetic mean (UPGMA) in MEGA X version 10.0. The reliability of the constructed phylogenetic tree was assessed through bootstrap analysis with 1000 replicates **(Kumar *et al*., 2016)**.

2.4.4. Phage-host cells ratio of bacteriolytic activity in vitro

The purified plaque from the overlayer was picked and diluted in SM buffer (salt of magnesium: 100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-Cl (pH 7.5), and 0.01% gelatin). Subsequently, 1 mL of chloroform was added to remove any remaining bacteria from the buffer. The phage lysate was then centrifuged, and the supernatant was used to test the lytic activity of *Aeromonas* strains (Ama1/Ama2/Ama3) at various concentrations of phage-host ratio (0.01:100, 0.1:100, 1:100, 10:100, and 100:100), with the blank containing only bacteria (10^{10} $CFUmL^{-1}$). To evaluate the *in vitro* lytic efficacy of the phage, 1 mL of *Aeromonas* strains (Ama1/Ama2/Ama3) were inoculated into two flasks containing 100 mL of nutrient broth. Then, 100 µL of the phage filtrate with a multiplicity of infection (MOI) ranging from 0.01 to 100 was transferred into a test flask containing the *Aeromonas* strains (Ama1/Ama2/Ama3) in nutrient broth. The test and control flasks were incubated in a shaking incubator at 37°C and 100 rpm for 24 hours. After the incubation period, the optical density (O.D.) at 600 nm was recorded using a UV spectrophotometer (L9, Shanghai INESA Analytical Instrument Co., Ltd, Shanghai, China). This measurement

indicated the in vitro lytic efficacy of the phage against the *Aeromonas* strains (Ama1/Ama2/Ama3) under the tested conditions. The detailed protocol for isolating and characterizing the aerophage, including the use of SM buffer, chloroform treatment, and the various phage-host ratios, was crucial for evaluating the lytic potential of the phage against the target *Aeromonas* strains.

2.4.5. Assessment of phage lytic activity on *Aeromonas* biofilms

The lytic activity of the purified aerophage on the biofilm of *Aeromonas* strains (Ama1/Ama2/Ama3) was demonstrated using a scanning electron microscope (SEM). For this experiment, a negative control was set up where only the coverslip was placed in 1 mL of phosphate-buffered saline (PBS). Other sets were treated with and without the purified aerophage to examine the efficiency of the aerophage lytic activity on *Aeromonas* strains (Ama1/Ama2/Ama3) (10^{10} $CFUmL^{-1}$). *Aeromonas* strains (Ama1/Ama2/Ama3) were grown on borosilicate glass coverslips for 8 h, followed by the addition of 100 µL of the purified aerophage at a multiplicity of infection (MOI) of 10. The treated samples were placed in a sterilized glass petri plate for 2 h. After the treatment, the coverslips were washed twice with PBS and dried for 24 h at 37°C. The biofilms coated on the glass slides were then fixed with glutaraldehyde (2.5%) and dehydrated using a series of graded ethanol (30-100%) for 5 min. The glass slides were sputtered with gold after critical point drying, and the aggregated biofilms were examined using a scanning electron microscope (JEOL JSM5500 LV) at the Regional Center of Mycology and Biotechnology, El-Azhar University, Cairo, Egypt.

2.4.6. Phage-based biocontrol using metagenomic profile analysis

The experimental design using activated sludge (200 mL) for batch reactors was divided into two separate Erlenmeyer conical flasks (500 mL) with aeration. One set was added to 100 mL of *A.hydrophila* (10^{10} $CFUmL^{-1}$ grown with 0.25x nutrient broth) as a control. The other flask contained 100 mL of *A.hydrophila* (10^{10} $CFUmL^{-1}$) and 25 $PFUmL^{-1}$ of lytic aerophage (10^{12} $PFUmL^{-1}$) to determine the efficacy of lytic aerophage in mixed cultures and demonstrate the bacteriolytic activity of lytic aerophage on *A.hydrophila*. The experiment was performed in duplicate at different time points (6, 12, and 24 h). The samples were then performed for water quality tests, including turbidity, which was measured using the Nephelometric Turbidity Meter (Thermo Orion AQ4500 model), dissolved oxygen (DO), and biochemical oxygen demand (BOD) were conducted using the HYDROLAB multiparameter sonde WTW 350i, which was calibrated before use according to Water and Wastewater Examination Standards **(APHA, 2017)**. On the other hand, genomic DNA was extracted using a Mini kit (Qiagen, Germany, Hilden), Frontiers in analysis lab, Al Shorouk, Cairo, Egypt, and DNA yield was stored at -20°C. Library preparation was performed according to the standard instructions of the 16S Metagenomic Sequencing Library Preparation protocol (Illumina™, Inc., San Diego, CA, United States). One hypervariable region of

the bacterial 16S rRNA gene was amplified using aliquots of the isolated DNA from each sample. The V4-V5 regions were amplified using the 515F-Y (5′-GTGYCAGCMGCCGCGGTAA-3′) and the 926R (5′-CCGYCAATTYMTTTRAGTTT-3′) primers **(Parada *et al*., 2016)**. Sequences were obtained on the Illumina MiSeq™ platform in a 2 × 150 bp paired-end run (CeBiTec, Bielefeld, Germany), following the standard instructions of the 16S Metagenomic Sequencing Library Preparation protocol. Paired reads were merged and processed to remove short- or low-quality sequences. More than 70,327 filtered reads were obtained from each sample. Operational taxonomic units (OTUs) were defined at 97% sequence-identity cutoff using the VSEARCH algorithm. Taxonomic assignment was conducted using an online ribosomal database project (RDP) classifier (https://rdp.cme.msu.edu/classifier/).

3. RESULTS AND DISCUSSION

3.1. Isolation and verification of ARB from Ismailia Canal

Sewage and animal waste are considered major sources of the antibiotic tetracycline. This antibiotic is frequently associated with pathogenic bacteria in livestock operations and can readily seep into wastewater, significantly aiding its spread in the environment. Results of 16S rDNA gene sequencing were obtained for three bacterial strains. These strains exhibited distinct partial nucleotide sequences, subsequently submitted to international GenBank databases. Each strain was assigned an accession number PQ288982.1, PQ288983.1, and PQ288984.1, respectively for *A.caviae* strain Ama1, *A.hydrophila* strain Ama2, and *Aeromonas sp.* strain Ama3. The 16S rDNA gene was selected as the method of choice for verifying and genotypically identifying the purified bacterial isolates because it is a fundamental component in bacteria and contains a lot of nucleotides, which are considered to be conservative regions. A multiple sequence alignment (MSA) tool was used to compare the nucleotide sequences of the three bacterial strains with other 16S rDNA sequences of *Aeromonas* strains. The MSA analysis revealed significant genetic diversity among the bacterial strains. The phylogeny tree construction was planned by the comparable nature of sequence values and it showed eight clusters according to differences in genetic distances as shown in **Figure (1)**. Our findings revealed significant variability in genetic distance among all identified bacterial strains. *A.hydrophila* is a Gram-negative bacterium that poses significant challenges in aquatic environments. This microorganism is known for its remarkable resistance to most common antibiotics, which complicates treatment options for infections it may cause. Its ability to thrive in various habitats, including freshwater and brackish environments, further underscores its adaptability. *A.hydrophila* is a concern for public health, particularly in aquaculture and among individuals with compromised immune systems. The *A.caviae* strain Ama1, *A.hydrophila* strain Ama2, and *Aeromonas sp.* strain Ama3, showed optimum growth at approximately 35°C and a pH of

7-8.5. The widespread presence of tetracycline in the environment, likely originating from sewage and animal waste, has contributed to the development of antibiotic resistance in *Aeromonas* strains. This highlights the importance of addressing the sources of antibiotic contamination and implementing strategies to mitigate the spread of resistant bacteria in aquatic ecosystems.

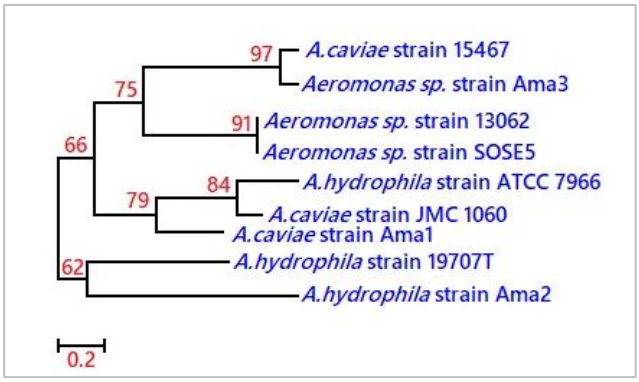

Figure (1). Neighbor-Joining tree of *Aeromonas* strains and other strains published in GenBank. Numbers represent bootstrap percentage values based on 1000.

This study found that the wild *Aeromonas* strains (Ama1, Ama2, and Ama3) isolated from the Ismailia Canal exhibited lower drug resistance compared to other reference *Aeromonas* strains. This observation suggests that the wild strains may act as reservoirs of antibiotic-resistance genes, capable of acquiring or transferring them to different microorganisms. In general, drug resistance is commonly observed in Proteobacteria, which is often associated with a rich source of multiple resistance plasmids. To investigate the antibiotic sensitivity of the *Aeromonas* strains, molecular analysis was conducted, and an antibiotic sensitivity test was performed using the following antibiotics at different concentrations: Amikacin (AG), Metronidazole (MET), Tetracycline (TE), Vancomycin (VA), Ofloxacin (OFX), and Chloramphenicol (C). The sensitivity was defined as the absence of bacterial growth on solid media containing any of the tested antibiotics. Conversely, the presence of bacterial growth indicated that the *Aeromonas* strains (Ama1, Ama2, and Ama3) were resistant to the treated antibiotics. Interestingly, the *Aeromonas* strains exhibited sensitivity under all TE/OFX/MET concentrations. However, the maximum extent of drug resistance was recorded for the antibiotics TE, AG, OFX, VA, MET, and C, respectively as shown in **Figure (2a, b, c, d, e, f)**. These findings suggest that the wild *Aeromonas* strains from the Ismailia Canal possess a relatively lower level of antibiotic resistance compared to other reference *Aeromonas* strains. This information is crucial for understanding the potential role of these strains as reservoirs of antibiotic-resistance genes and their ecological significance within the natural environment.

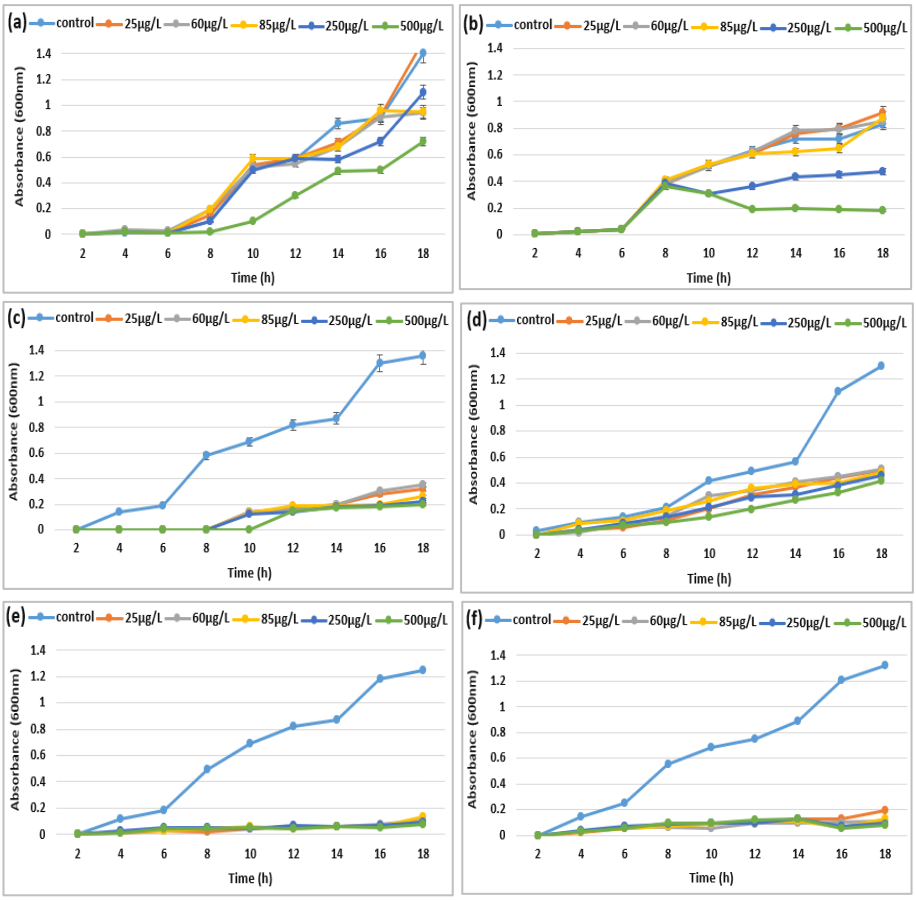

Figure (2). Antibiotic susceptibility pattern of *A.hydrophila* strain Ama2 at 25, 60, 85, 250, and 500 µgmL^{-1} concentrations, O.D. values at 600 nm. (a) Tetracycline (TE), (b) Amoxicillin/Clavulanate (AG), (c) Ofloxacin (OFX), (d) Vancomycin (VA), (e) Methicillin (MET), and (f) Chloramphenicol (C), respectively.

The study examined the antibiotic resistance and sensitivity of three *Aeromonas* strains: Ama1, Ama2, and Ama3. In the case of the Ama1 and Ama2 strains, resistance was reduced to 62% at a concentration of 500 µg/mL. Reduced resistance (93%) was observed with Methicillin (MET) and Chloramphenicol (C) at all concentrations tested. Interestingly, the *A.caviae* strain Ama1 and *A.hydrophila* strain Ama2 showed 100% sensitivity to Tetracycline (TE), Amikacin (AG), Ofloxacin (OFX), and Vancomycin (VA) at all used concentrations. In contrast, the growth of *Aeromonas sp.* strain Ama3 was inhibited by most of the antibiotics tested. Across all three *Aeromonas*

strains (Ama1/Ama2/Ama3), they showed resistance to tetracycline at all used concentrations. This finding is consistent with previous reports from Chungju, Korea, where 52% resistance was observed **(Lee *et al.*, 2018)**. However, this contradicts studies from the USA and Australia, where high sensitivity (94.3%) to tetracycline was reported for *Aeromonas* strains **(Le *et al.*, 2018)**. These results highlight the variable antibiotic resistance and sensitivity profiles of different *Aeromonas* strains, emphasizing the importance of local and regional surveillance to understand the evolving patterns of antimicrobial resistance in these bacterial species.

3.2. Isolation and morphological characterization of lytic phages in water

A qualitative and quantitative assay was conducted to evaluate the presence and abundance of *Aeromonas* phage in Ismailia Canal water samples. Two reference strains of *A.hydrophila* ATCC 7966 and *A.hydrophila* ATCC 35654 were used for the isolation, growth, and characterization of the phage. Our findings revealed that aerophage MK01 that infects *Aeromonas* strains (Ama1/Ama2/Ama3) was present in all sampled sites along the canal. The highest levels of aerophage were detected in the middle of the canal, ranging from 10^9 to 10^{12} plaque-forming units per milliliter ($PFUmL^{-1}$). The sites towards the end of the canal also exhibited relatively high aerophage levels, reaching up to 10^7 $PFUmL^{-1}$. Conversely, the lowest aerophage values were observed at the beginning of the canal, measuring around 10^5 $PFUmL^{-1}$. A single plaque isolation approach utilizing the reference ATCC strains was employed to characterize the distinct plaque pattern. The results demonstrated circular and regular plaque with a size of 1 mm, as depicted in **Figure (3a)**. A novel aerophage MK01 exhibiting high lytic activity against both *Aeromonas* strains was also identified. The isolation and characterization of bacteriophages from the natural environment is crucial to determine their evolutionary perspectives, ecological significance, and potential for therapeutic development (**Liu *et al.*, 2020; Ramos-Vivas *et al.*, 2021)**. This is particularly important given the continued emergence of multidrug-resistant bacteria and the challenges faced with traditional antibiotic treatments. Bacteriophages, as natural parasites of bacteria, are being reconsidered with antibiotics for treating super- or multidrug-resistant bacterial infections.

3.3. Structural features and classification of aerophage MK01

The structural characteristics of the isolated aerophage MK01, which effectively lyses *Aeromonas* strains (Ama1, Ama2, and Ama3), were examined using transmission electron microscopy (TEM). The micrograph of negatively stained aerophage MK01 revealed a distinct morphological type, as shown in **Figure (3b)**. The measurements indicated that aerophage MK01 has an apex diameter of 72 ± 4 nm and a length of 140 ± 4 nm. According to the International Committee on Taxonomy of Viruses (ICTV), aerophage MK01 is classified within the *Myoviridae* family and is characterized by its elongated capsid and contractile tail. Myoviruses are typically virulent bacteriophages that

operate in the lytic cycle, infecting their host bacteria without integrating their genetic material into the host cells. Previous studies have reported that other phages, such as N21, W3, G65, Y71, and Y81, could infect *Aeromonas* strains, also belong to the *Myoviridae* family and have a broad host range and high thermal and pH stability **(Azzam *et al.*, 2014; Liu et al., 2020; Ramos-Vivas *et al.*, 2021; Pereira et al., 2022)**. **Liu et al., (2020)** isolated two new phages that lysis *A.hydrophilia* belong to the *Myoviridae* family and show excellent bacteriolytic activity at MOI (1) with a wide host range.

3.4. Lytic cycle and stability of aerophage MK01

To investigate the lifecycle of the aerophage MK01, we conducted a single-step growth experiment. The results determined that the *aero*phage MK01 exhibited a burst size of 3 PFU (plaque-forming units) per lysed bacterial cell colony. *aero*phage MK01 had latent periods of 145 minutes as shown in **Figure (3c)**. Such a low burst size was not unexpected since *Aeromonas* virus 31, which is genetically close is known to have a burst size of 7 new virions per infected cell **(Vincent *et al.*, 2017)**. Furthermore, we evaluated the thermal and pH equilibrium conditions of the newly isolated aerophage MK01 at different temperatures and pH values. Our findings indicated that the aerophage MK01 maintained stable titers within the temperature range of 4°C to 65°C. However, their infectivity decreased significantly at 79°C, and they lost their infectivity at 86°C. Regarding pH stability, the newly isolated aerophage MK01 demonstrated stability within a pH of 4 to 10. However, the titer of aerophage MK01 declined when subjected to extreme pH conditions of 2 and 12. Our novel isolated and characterized aerophage MK01 was assigned the accession number PQ351405.1 and showed almost 49% similarity with other published aerophages as shown in **Figure (3d)**. These findings demonstrate the lytic cycle kinetics and stability of the aerophage MK01, highlighting their potential uses in various temperature and pH environments.

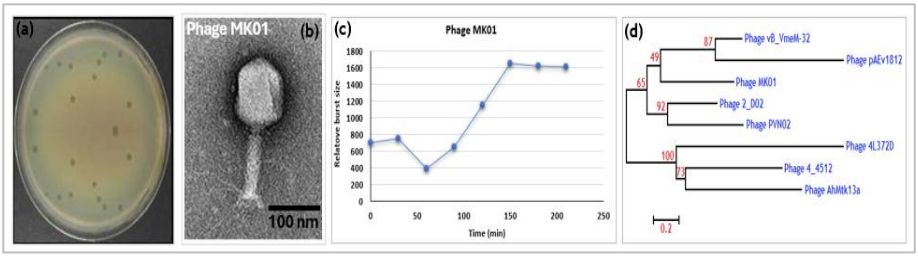

Figure (3). Isolation, characterization, and identification of aerophage MK01 from Ismailia Canal. (a) epresentative plaque assay of aerophage MK01 after incubation against *A.hydrophila* ATCC 35654 at 37^0 C for 24 h. (b) Electron micrographs of the newly isolated aerophage MK01 particle. (c) Single-step growth curve for aerophage MK01. (d) Phylogenetic tree of aerophage MK01 and related aerophages published in GenBank.

3.5. Phage-host cells ratio of bacteriolytic activity in vitro

The multiplicity of infection (MOI) refers to the ratio of virus particles to host cells **(Azzam, 2010)**. The bacteriolytic efficacy of aerophage MK01 was assessed using *A.hydrophila* strain ATCC 35654 at various MOI ratios. As illustrated in **Table (1)**, the absorbance of ATCC 35654 cultures without phages increased steadily over 24 h. In contrast, cultures with phages exhibited a gradual increase in absorbance during the first 2 h, followed by a significant decrease that was dependent on the MOI from 2 to 6 h. By the 6 h mark, absorbance levels had dropped to their lowest at all MOI ratios. Initially, the bacterial live cell count was recorded at 98.1% ± 0.2 at 0 h; however, over time, with phage-host ratios of 10:100 and 100:100, the levels of *A.hydrophila* strain ATCC 35654 host cells decreased significantly to 0.5% and 0.2%, with optical density (O.D.) values declining from 0.01 to 0.004. Thus, in vitro studies demonstrate that a single virion can effectively lyse 100 bacteria. MOI plays a crucial role in utilizing phages for targeted host control, as it quantifies the number of bacteriophage particles infecting the host cell **(Dennehy and Abedon, 2021)**. Furthermore, the number of plaques increased with MOI, indicating that aerophage MK01 effectively eliminates multi-drug resistant (MDR) bacteria under laboratory conditions. Even at a 1:100 ratio, the phage infection significantly reduced O.D. values, reducing 7.4% ± 0.1 compared to the control.

Table (1). Bactericidal effect of aerophage MK01 *in vitro*.

MOIs*	% Live cells	Absorbance O.D. (600 nm) **
Control	98.1±0.2	0.38±0.01
0.01:100	81.5±0.1	0.42±0.01
0.1:100	46.2±0.2	0.25±0.01
1:100	7.4±0.1	0.11±0.01
10:100	0.5±0.1	0.01±0.01
100:100	0.2±0.1	0.004±0.01

* **MOIs**: The multiplicity of infections. ** **O.D.**: Optical Density.

3.6. Reduction of cells controlled by MK01 aerophage

Scanning electron microscope (SEM) images illustrate the biofilm behavior on a glass slide, including the negative control, the biofilm formed by *A.hydrophila* strain ATCC 35654, and the aerophage MK01-treated biofilm of *A.hydrophila* strain ATCC 35654, as depicted in **Figure (4a, b, c)**. The negative control displayed a clear field, indicating the absence of bacterial contamination. Our findings reveal the impact of aerophage MK01 on the morphology and architecture of specific host biofilms before and after treatment. After 2 h of therapy with phage MK01 at a concentration of 10^{10} $PFUmL^{-1}$, minimal growth was observed on the glass slide. Previous research has demonstrated a reduction in bacterial cells and biofilm formation of multi-drug resistant (MDR) *Escherichia coli* following treatment with virulent phages

on modified nanocomposite membranes, as analyzed by SEM **(Yu *et al.*, 2019)**. Additionally, phages isolated from surface water within the *Podoviridae* family exhibited strong bacteriolytic activity against *Staphylococcus aureus* biofilms **(Dafale *et al.*, 2015; Cha *et al.*, 2019)**.

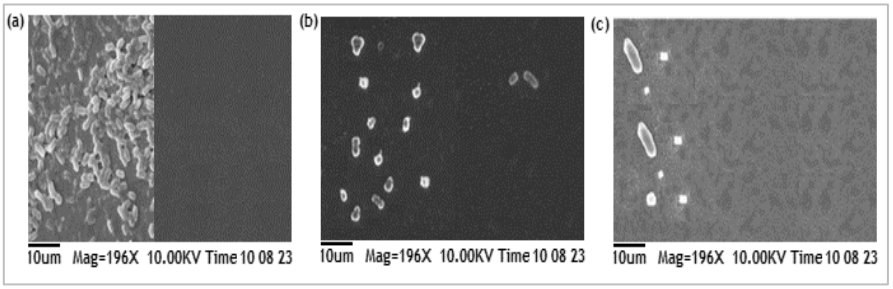

Figure (4). Electron micrographs of biofilm on a glass slide before and after aerophage MK01 treatment. (a) depicts the coverslip, (b) bacterial biofilm, and (c) Surface post-phage MK01-treatment.

3.7. Profile of metagenomics in batch reactors managed by aerophage MK01

The application of lytic phage in biological sewage treatment has primarily been restricted to pure culture systems, where specific bacterial strains are isolated to study the phage's efficacy in targeting and lysing pathogenic microorganisms **(Vela and Al-Faliti, 2023)**. This approach allows for a controlled environment where the interactions between phages and their bacterial hosts can be meticulously observed, leading to significant reductions in bacterial populations **(Bhattacharjee *et al.*, 2015)**.

However, the inherent complexity of sewage water, which contains a diverse array of microbial communities, poses challenges for implementing lytic phages in mixed culture settings. In these environments, the dynamic interactions among various bacterial species can hinder the effectiveness of phage therapy, as some bacteria may develop resistance or outcompete the targeted strains. As a result, while lytic phages show promise for enhancing wastewater treatment, their full potential remains largely untapped in real-world applications, necessitating further exploration into their use within more complex biological systems. **Shivaram *et al.*, (2023)** explored the use of a bacteriophage as a biosensor for detecting pathogenic microbes and for bacteriophage-mediated biocontrol of biomass bulking in a pure culture of *A.hydrophila*. The reduction of biofouling was examined through the application of lytic phages that infect isolated single bacteria on a modified nanocomposite membrane **(Anam *et al.*, 2020)**, but the effects in mixed cultures have been infrequently studied. Typically, single cultures are employed in phage therapy applications within medical, food, and environmental sciences, yet understanding phage efficacy in mixed cultures is crucial for utilizing

phage-based biocontrol in biological wastewater treatment processes. In mixed cultures, such as activated sludge and biomass, achieving effective phage therapy poses challenges, particularly with traditional methods like the double agar plate approach. It is essential to assess whether the addition of aerophage influences the removal efficiency of organic and inorganic nutrient compounds from the original bioreactor when applied in mixed cultures of sewage treatment systems.

Our results, presented in **Table (2)**, indicate improvements in water quality parameters (turbidity, DO, BOD) after treatment with purified lytic aerophage MK01 compared to the batch reactor without phage addition, as shown in the same table. Similar findings were reported in studies by **Azzam *et al.*, (2024)**, which documented gradual enhancements in water quality using a new polyvalent coliphage mixes. Conversely, some research highlighted that, despite investigating phage addition in the context of sludge bulking, no significant changes in COD and ammonia removal were observed in biomass bulking control.

Table 2. Water quality monitoring in batch reactors treated with and without aerophage MK01.

Treatment	Turbidity (NTU)			DO (mgL^{-1}) *			BOD (mgL^{-1}) **		
	0h	12h	24h	0h	12h	24h	0h	12h	24h
Batch reactor without lytic phage	75±10.1	21±0.1	10±0.5	0.3±0.0	1.02±0.0	5.09±0.0	120±0.2	79±0.0	13±0.0
Batch reactor with lytic phage	75±10.1	8±0.1	2±0.0	0.3±0.0	4.68±0.0	11.1±0.0	120±0.2	55±0.0	7±0.0

* DO: Dissolved oxygen, BOD: Biological oxygen demand.

Next-generation sequencing (NGS) and metagenomic analysis have transformed water quality assessment by comprehensively examining microbial communities in aquatic environments. NGS processes millions of DNA sequences simultaneously, revealing insights into microbial diversity, abundance, and functional capabilities, which are key for identifying pathogens and understanding environmental impacts. Additionally, metagenomic analysis can detect antibiotic resistance genes, informing better management strategies and public health outcomes. Our metagenomic analysis of treated bioreactor samples, both with and without the injection of aerophage MK01, revealed that 70,327 filtered reads were identified, with an impressive accuracy of 99.98%. At the same time, non-identified reads accounted for just 0.02%. As illustrated in **Figure (5)**, the classified microbial communities included Terrabacteria (32.78%), Pseudomonadota (30.78%), Betaproteobacteria (11.18%), Gammaproteobacteria (10.44%), Alphaproteobacteria

(5.30%), and Archaea (0.07%). The Terrabacteria group dominated during the initial phase of reactor operation, as shown in **Figure (6)**.

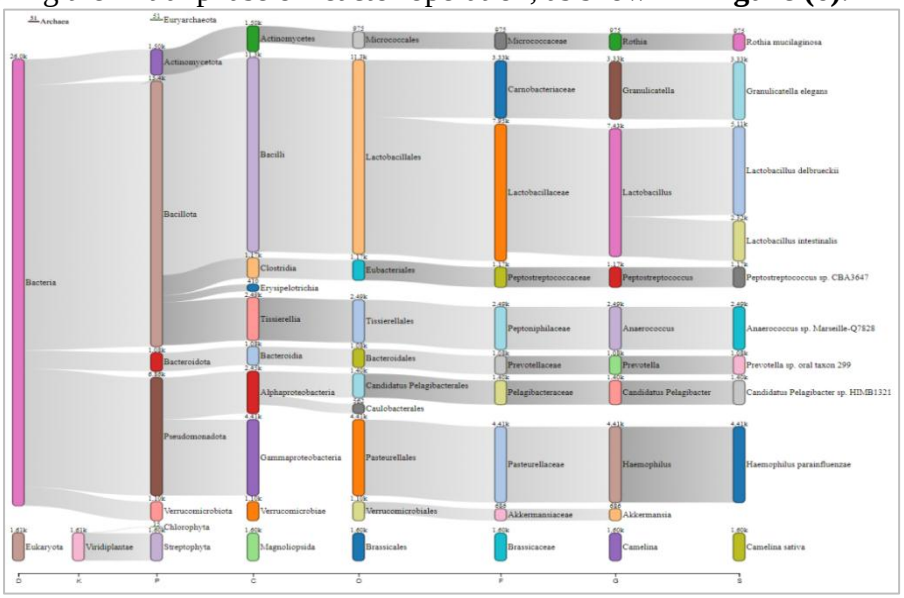

Figure (5). Significant microbial diversity detected through DNA metagenomics in a bioreactor sample treated with aerophage MK01.

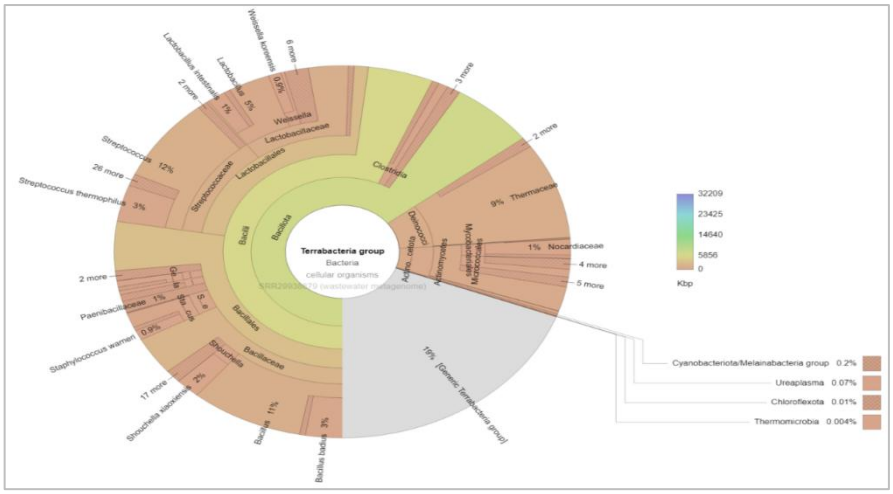

Figure (6). The interactive krona plots of the bacterial full taxonomy discovered in the bioreactor sample.

Notably, the distribution of bacterial communities remained stable in the absence of the lytic phage; however, the introduction of aerophage MK01 resulted in approximately a 45% reduction in the distribution of

Gammaproteobacteria between 8 - 12 h. All sequence data have been submitted to the National Center for Biotechnology Information (NCBI)/ European Molecular Biology Laboratory (EMBL)/ DNA Data Bank of Japan (DDBJ) and assigned the accession numbers as follows: Bioproject (PRJNA1136524), Biosample (SAMN42535108), and SRA FastQ reads (SRR29938879).

3.8. Possibilities and restrictions for drainage water treatment approaches

In actual drainage water treatment approaches, phage-based biocontrol can be applied in multiple ways, including the reduction of biofilm formation, sludge expansion, and the proliferation of multi-drug resistant (MDR) bacteria **(Mathieu *et al*., 2019; Bayat *et al*., 2021; Reyneke *et al*., 2024)**. Although numerous studies have demonstrated the effectiveness of lytic phages in managing undesirable bacterial populations in water and drainage water treatment processes, most have focused on individual phage isolates **(Azzam *et al*., 2024)**. To improve the efficacy and consistency of phage applications for eliminating residual MDR in the effluent of drainage water treatment approaches (DWTs), future investigations should prioritize the development of phage mixes and address the challenges posed by lysogenic conversion in practical settings.

Our study will offer several recommendations for identifying the optimal stages at which super-specialized phages can be introduced to enhance the effectiveness and efficiency of pollutant removal, particularly biological contaminants. This approach aims to make phage applications both cost-effective and complementary to existing treatment processes. For possibilities implementation in real-world drainage water treatment systems (DWTs), it's essential to determine the optimal dosing location for the isolated phage to enhance the efficacy and stability of decentralized wastewater treatment systems (DWTs). In a conventional activated sludge process, phage should be administered before the secondary settling tank to prevent harming beneficial microorganisms responsible for removing organic matter and nutrients. In a membrane bioreactor, phage can be added directly to target bacteria that cause biofouling, as there is no settling tank present. In activated sludge systems, phage targeting MDR strains can be introduced at two different points: before and after the secondary settling tank. Additionally, incorporating a dedicated tank for phage therapy could improve MDR removal by ensuring adequate retention time. Despite the benefits of phage-based biocontrol, several limitations exist during drainage water treatment processes. Antibiotic resistance genes (ARGs) can be transferred to other bacteria through transformation and transduction. Furthermore, maintaining a stock of various phages, known as a phage cocktail, is necessary to effectively target specific antibiotic-resistant bacteria. However, this process can be both time-consuming and labor-intensive.

4. CONCLUSION

The presence of multidrug-resistant (MDR) bacteria in treated wastewater is unavoidable due to the lack of effluent discharge limits in water reclamation facilities. In Cairo, Egypt, *Aeromonas sp.* and its lytic phage were isolated from the Ismailia Canal and WWTPs, with *Aeromonas sp.* constituting about 82.4% of MDR bacteria in these facilities. Aerophage MK01 effectively reduced the bacterial population by 87% after 12 h and 15% after 24 h of incubation. The isolated *Aeromonas* strains (Ama1/Ama2/Ama3) exhibited resistance to various antibiotic concentrations. The application of aerophage MK01 in mixed cultures can diminish the population of target bacteria, which could otherwise transfer antibiotic resistance traits to wastewater treatment processes and surrounding water bodies. These findings suggest that lytic phages may serve as a viable alternative to reduce MDR in wastewater without generating chemical byproducts. Future research should explore the integration of phage-based approaches into real-world wastewater treatment processes to enhance their efficacy against MDR bacteria.

Research Involving Humans and Animals Statement

This research does not involve human or animal study.

Informed Consent

Not applicable.

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Availability of data and materials

The sequence reads of *A.caviae* strain Ama1, *A.hydrophila* strain Ama2, and *Aeromonas sp.* strain Ama3 strains, Aerophage MK01 isolate, Bioproject, Biosample, and SRA FastQ reads are available in GenBank under accession numbers PQ288982.1, PQ288983.1, PQ288984.1, PQ351405.1, PRJNA1136524, SAMN42535108, and SRR29938879.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

Conceptualization, Methodology, Bioinformatics analyses and Software, Data curation, Validation, Writing-original draft, and Visualization Mohamed Ibrahim Azzam. Material preparation, Methodology, Formal analysis, and

Investigation Kawthar A. Omran and Abeer A. Faiesal. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

Our manuscript does not involve any animal or human data or tissue and its "Not applicable".

Consent for publication

Our manuscript does not contain data from any individual person and its "Not applicable".

Availability of data and materials

All data generated or analysed during this study are included in this published article.

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التحكم الحيوي القائم على الفاجات المتخصصة للبكتيريا المتعددة المقاومة للمضادات الحيوية في مياه ترعة الإسماعيلية

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تهدف هذه الدراسة إلي إختبار مقاومة المضادات الحيوية لسلالات الإيروموناس البرية المعزولة من قناة الإسماعيلية ومحطات معالجة مياه الصرف الصحي. تم تحديد ثلاث سلالات احتماليه من سلالات الإيروموناس، والتي تم تأكيد تعريفها بدقة بنسبة 68.4% باستخدام تقنية 16S-rDNA . شملت السلالات *Aeromonas caviae* (Ama1)، و*Aeromonas hydrophila* (Ama2)، و*Aeromonas sp.* (Ama3)، وجميعها أظهرت مقاومة كبيرة للمضادات الحيوية المتنوعة، مما يشير إلى إمكانية إحتوائها علي الجينات المقاومة للمضادات الحيوية. أظهرت اختبارات حساسية المضادات الحيوية أن السلالات Ama1 و Ama2 أظهرت حساسية بنسبة 100% للتتراسيكلين(TE) ، والأميكاسين(AK) ، والأوفلوكساسين(OFX) ، والفانكومايسين(VA) ، بينما كانت السلالة Ama3 أكثرهم مقاومة. تم عزل إيروفاج جديد MK01 ، وأظهر قدرته الفعالة على إصابة وتحلل سلالات الإيروموناس الممرضة، مع اكتشاف تركيزات عالية منه في وسط ترعة الإسماعيلية. أظهرت نتائج الفحص بالميكروسكوب الإلكتروني النافذ أن الفاج الجديد MK01 ينتمي إلى عائلة *Myoviridae* ويظل محتفظا بحيويته وقدرته علي تحلل عوائل متعددة عند درجات حرارة من 4 إلى 72 درجة مئوية ومستويات pH من 4 إلى 10. كما أظهر الفاج معمليا قدرة كبيرة علي انخفاض نمو البكتيريا متعددة المقاومة (MDR) وأثر على تكوين الأغشية الحيوية بشكل ملحوظ. تم تطبيق التحكم البيولوجي المعتمد

على الفاج الجديد في مفاعل دفعي مع أنظمة بيئية متنوعة. أظهر التحليل الميتاجينومي لعينات المفاعل المعالج تغييرات في هيكل المجتمعات الميكروبية، حيث أن الفاج MK01 أمكنه تقليل تركيزات سلالات MDR بنسبة تتراوح من 34.1% إلي 81.5% خلال 8 ساعات فقط. كما ساهم الفاج MK01 في تحسين جودة مياه الصرف الصحي المعالج بشكل فعال، مما أدى إلى زيادة متوسطة بنسبة 56.45% في الأوكسجين المذاب، بالإضافة إلى تقليل العكارة (80.01%) وطلب الأوكسجين البيوكيميائي 46.15% (BOD) مقارنة بالمفاعلات الدفعية بدون الفاج المنقي. وتخلص الدراسة إلى أن التحكم البيولوجي المعتمد على الفاج الجديد يقدم نهجاً واعداً لإدارة والتحكم في البكتيريا المقاومة للمضادات الحيوية أثناء معالجة مياه ال

صرف الصحي، مع تحقيق نتائج فعالة دون إنتاج مركبات ثانوية أو سطية كيميائية وضمان إعادة الاستخدام الآمن لمياه الصرف المعالج كمصدر جديد لمياه الري.