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AN INNOVATIVE APPROACH USING LYTIC PHAGE MIX FOR WASTEWATER MANAGEMENT AND PATHOGEN CONTROL

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ABSTRACT

In the modern water management plans of nations that confront a severe lack of water resources, such as the Middle Eastern countries, the reuse of treated sewage water for agriculture is seen as an important alternative water supply. The goal of the current study was to use a lytic phage mixture to reduce both of *Escherichia coli* strains, coliforms, and other *Enterobacteriaceae* species in River Nile and drains outlets. The transmission electron microscope revealed morphological similarities between three novel phages (MCn4, MCn5, and MCn6) and those in the *Siphoviridae*, *Myoviridae*, and *Podoviridae* families. *Escherichia coli* ATCC® strain 11775 and *Escherichia coli* ATCC® strain 10536, the index coliform, were both lysed by these new phages. Different DNA polymerase (DP), tail protein (TP), and DNA polymerase accessory (DPA) gene sizes (2565 bp, 672 bp, and 951 bp, respectively) were reported by bioinformatics analyses, and all nucleotide sequences were recorded in the international GenBank. Phage-mediated bio-control in aquatic pathogenic bacteria relies on targeting various species and strains, with polyvalent MCn4, MCn5, and MCn6 phages exhibiting lytic effects on *Salmonella typhi*, *Proteus vulgaris*, and *Citrobacter freundii* strains. These phages had a significant lytic influence on the population of coliform bacteria after two hours of incubation. The study concluded that the use of this lytic coliphage mixtures for the decrease in coliform populations in sewage may be considered an efficient and cost-effective alternative to the expensive replacement of wastewater treatment plant

equipment and infrastructure, as well as for controlling various bacterial pathogens could be achieved throughout few hours.

Key Words: Bioinformatics; Microbiological; Innovation; Wastewater treatment; Water quality.

INTRODUCTION

Depending on the origin, the wastewater contain organic, inorganic, radioactive, and thermal pollutants and suspended solids **(Iwuozor, 2019 and Mushtaq *et al.,* 2020)**. An aerobic treatment process is used to treat the organic matter in municipal and industrial wastewater, called the activated sludge (AS) process. This process utilizes microorganisms such as heterotrophs, yeasts, algae, fungi, filamentous bacteria, and protozoa **(Gupta *et al.,* 2017 and Foong *et al.,* 2021)**. Although, the AS process has several functions, such as degradation or oxidation of carbonaceous & nitrogenous wastes , removal of fine solids and heavy metals. The microbes used in the process or system cause problems such as bulking, foam stabilization, and biofilm formation when their growth is not controlled **(Chu *et al.,* 2009; Zheng *et al.,* 2013; Verma *et al.,* 2019 and Shivaram *et al.,* 2023)**. It is observed that inconsistent and improper wastewater treatment can lead to the continued growth of microorganisms, resulting in environmental pollution in the receiving water and posing a risk to human health. Similarly, inadequate treatment of industrial wastewater can lead to biofilm formation and microbial induced corrosion (**Naidoo and Olaniran, 2014 and Kuppusamy *et al.,* 2016**).

Escherichia coli is among the most important , widespread waterborne pathogens and has been a global public health concern **(Odonkor and Ampofo, 2013)**. In recent years, the use of antibiotics has increased, leading to the emergence of some antibiotic-resistant bacteria. Few reports have found that 70% of the world's bacteria have developed resistance to antibiotics inadvertently sprayed on fields and livestock, resulting in polluted waterways **(Koch *et al.,* 2021)**. Antibiotics are also found in sewage treatment plants and can cause pathogenic microorganisms to develop antibiotic resistance. Antibiotic-resistant bacteria showed higher virulence. Such strains produce enzymes that degrade antibiotics. This phenomenon has increased with widespread antibiotic use **(Al-Gheethi *et al.,* 2015)**. With the emergence of antibiotic resistance, bacteriophage therapy has emerged as a candidate to inactivate antibiotic-resistant bacteria **(Torres-Barceló, 2018)**.

Bacteriophages are viruses that infect prokaryotes. They are the most abundant organisms on earth and are speculated to play an important role in bacterial population dynamics in the natural environment (**Mitarai *et al.*, 2016)**. Their potential to control bacterial populations has been confirmed in aquatic environments. Bacteriophages have also been identified as potential indicator organisms for wastewater treatment and the removal of viral pathogens **(McMinn *et al.*, 2017 and Dias *et al.*, 2018)**. Bacteriophages perform their antimicrobial activity by direct lysis of bacterial cells or by replicating their genome inside the host before lysis under stressful conditions **(Masuda *et al.*, 2021)**. Phages have the upper hand in suppressing the antibiotic-resistant strains of bacteria as the antibiotic resistance mechanism does not affect bacteriophage action. Phages are effective against planktonic bacteria and their biofilm structure with their ability to penetrate the biofilms and lyse the cells **(Azeredo *et al.*, 2021)**. *E.coli* forms bacterial biofilms in drinking water treatment plants and clogs the filters. These filters can be treated with expensive flushing and chlorination to remove biofilm formed **(Jassim *et al.*, 2016)**. A study was able to improve the efficiency of biofilm removal by combining chlorine treatment with bacteriophage treatment of the biofilm. The study successfully removed 92 % of the biofilm formed by *E.coli*, suggesting the possibility of combination treatment for biofilm control **(Ferriol-González, and Domingo-Calap, 2020)**.

The current study was aimed to discover and characterize new coliphages that could control *E.coli*, coliforms, and other *Enterobacteriaceae* species in the Nile and drainage water. The analysis included observing the morphology and host range of the bacteriophage, as well as characterization of its genetic materials through bioinformatics methods. Also, this study provides useful information for potential applications of the coliphage mixture towards biotechnology and for the safe reuse of treated wastewater in irrigation.

MATERIALS AND METHODS

Study area

The area in this study was chosen to represent two major water sectors in Egypt: River Nile and drainage water. It extended about 120 Km in the River Nile at the Rosetta branch. The branch was subdivided into five reaches based on locations of known waste inputs as illustrated in **Fig. 1**. Totally fifteen sites were chosen, three from each reach: five at drain outfalls (El-Rahway, Sabal, El-Tahreer, Zawiet El-Bahr, and Tala) and ten sites in Rosetta branch (five upstream and five downstream those drains outfalls). These are mixed drains from sewage, agricultural and industrial wastes **(Azzam et al., 2017)**.

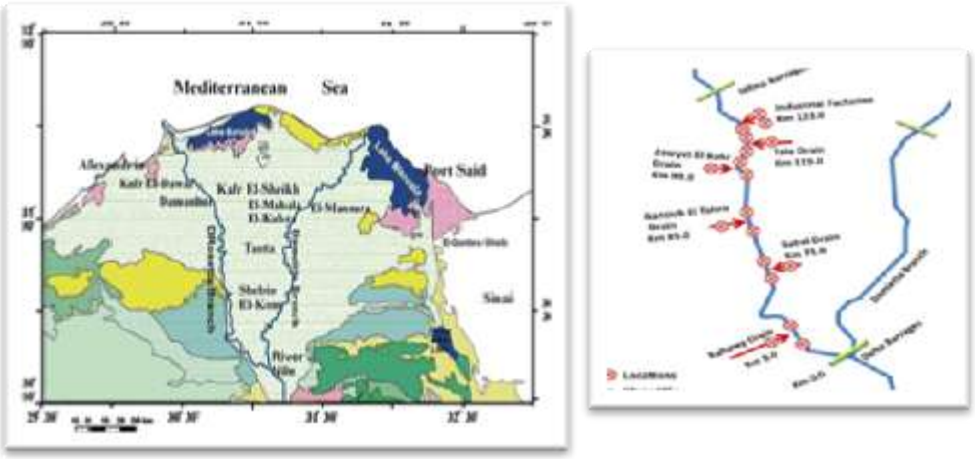

Fig (1): Map of sampling sites along the Rosetta branch.

Collection of water samples

The study started with samples collection in which 180 water samples were collected in duplicates, 120 from the Rosetta branch and 60 from drains outfalls as described above. Samples collection was processed in two different seasons (summer & winter, 2019-2020) according to Standard Methods for Examination of Water and Wastewater **(APHA, 2017)**. All collected samples were stored in an iced cooler box and delivered to Central Laboratory for Environmental Quality Monitoring (CLEQM), National Water Research Center (NWRC) where it has been analyzed.

Bacteriological characteristics

Water samples from different sources were assayed for bacterial indicators according to the Standard Methods **(APHA, 2017)**. For the enumeration of fecal coliforms (FC), the membrane filter technique was applied on modified fecal coliform (M-FC) agar using a filtration system completed with stainless steel autoclavable manifold and oil-free ‘‘Millipore’’ vacuum/pressure pump in which, water samples were filtered through a sterile surface girded ‘‘Sartorious’’ membrane of a pour size 0.45 µm and diameter 47 mm which retained aquatic bacteria. After filtration, the membrane was plated on m-FC agar with 1% rosolic acid (Difco, USA) and incubated at 44.5^{o}C/24h. Results were recorded as colony forming unit (CFU 100 ml^{-1}) using the following equation: Colonies / 100ml = Counted colonies / mL of sample filtered * 100.

These colonies were confirmed by streaking on eosin methylene blue (EMB) agar (Difco, USA) plates, a selective medium that inhibits bacterial growth except *E.coli* and enhances greenish metallic sheen colonies. Confirmation and verification were completed by microscopical and biochemical examinations (Gram staining, pigment production, and oxidase test) according to Bergey's Manual of Systematic Bacteriology **(Brenner *et al.*, 2005)** as well as by Analytical Profile Index (API 20 NE) assay (bioMérieux, France) according to **Juang and Morgan (2001)**. The whole assay lasts three to four days to complete.

Bacterial DNA sequencing and phylogenetic analyses

Bacterial Genomic DNA of *E.coli* strains was purified using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and used as a PCR template. PCR amplification of 16S rRNA genes was conducted using the 16S Barcoding Kit (SQK-RAB204; Oxford Nanopore Technologies, Oxford, UK) containing 100µl of 2.5mM of dNTP, 10x PCR buffer, 3U of Taq DNA polymerase, 10ng template DNA, and 500ng of primer (F) 5'-TGCCTGATGGAGGGGGATAA-3', and primer (R) 5'-GGAGTTAGCCGGTGCTTCTT-3' which were designed for *E.coli* according to **Azzam *et al.*, (2022)**. The amplification program was set as an initial denaturation at 95 °C for 3 min, 25 cycles of 95 °C for 20 s, 55 °C for 30 s, and 65 °C for 2 min, followed by a final extension at 65 °C for 5 min. The sequencing reactions were performed using an Applied Biosystems Veriti™ Thermal Cycler (Thermo Fischer Scientific, Waltham, MA, USA). All nucleotide sequences were compared with recorded 16S rDNA gene sequences in the EMBL database using a BLAST server at the European Bio-Informatics Institute (EBI; http://www.ebi.ac.uk, Hinxton Hall, Cambridge, UK). DNA sequences were aligned using CLUSTAL W **(Thompson *et al.*, 1994)**. Phylogenetic analyses were performed using the neighbour-joining (NJ) method to test the support for the phylogeny with a bootstrap analysis based on 1000 replicates using MEGA ver. 7.0 **(Tamura *et al.*, 2013)**.

Enrichment and isolation of phages

The water samples (Drains and Rosetta branch) (50ml) were centrifuged at 8000 rpm for 10 minutes and the supernatant was filtered through 0.45-µm pore size polycarbonate membranes (Millipore, Bedford, MA, USA). The filtrate was added to each of the 50 mL sterile flasks containing tryptic soy broth (TSB) medium (Difco, USA) and inoculated with both of 100 µL fresh isolates of coliform, *E.coli* (ATCC® strain 11775) and *E.coli* (ATCC® strain 10536) then incubated at 37°C

for 5 h at 100 rpm, then centrifuged (10000 rpm, 10 min) (ThermoHeraeus Pico, Hanau, Germany) and filtered through a 0.2μm membrane filter (Millipore). Phage isolation was done by the double-layer method using tryptic soy agar (TSA) (Difco, USA). The plates were incubated at 37°C and examined for the presence of plaques after 12h. The plaque-forming units were calculated according to **Stephenson (2010)** using the following equation: Plaque forming units (PFU)/ml= (Number of plaques) x (Dilution Factor)/ Phage volume plated (ml). Two more successive single-plaque isolations were performed to obtain partially purified phage isolates. All picked plaque diameter was determined using a double-layer approach. All separated coliphage isolates were stored at 4°C with 1% chloroform **(Clokie *et al.*, 2018 and Olsen *et al.*, 2020)**.

Host range pattern and cross infectivity of the isolated phages

The isolated coliphages were investigated for host range specificity using three reference strains *E.coli* ATCC® strain 11775, *E.coli* ATCC® strain 10536, and wild isolates on TSA plates. While *Pseudomonas aeruginosa* ATCC® strain 15442 was used as negative control and lysis e-ciency (no lysis, clear plaque, and turbid plaque) was observed. Bacterial lawns of all di-erent bacterial species were propagated on TSA agar plates and 10 μL droplets of phages (1×10^{12} $PFUmL^{-1}$) were put on the lawns. The plates were incubated for 24 h and checked for the presence of plaques. The most e-cient phage was selected for further studies. The selection criteria included the lysis profiles, plaque clarity, and size. To determine the ability of the isolated phages to infect di-erent species other than *E.coli* and 20 μL of each phage were spotted over a lawn of di-erent bacterial species among the *Enterobacteriaceae*. After incubation at 37 °C for 24 h, the development of plaques in the plates was examined.

Morphological characteristics of phage cocktails

Three purified bacteriophage particles were stained with Na-phosphotungstate or uranyl acetate before observation in a Hitachi H600A electron microscope **(Azzam, 2015)**. A drop of each phage suspension (10^{12} PFU/mL) was placed on 200 mesh copper grids with carbon-coat formvar films and the excess was drawn o- with filter paper. A saturated Na-phospho-tungstate or uranyl acetate solution was then placed on the grids and the excess was drawn o- as before. Specimens were examined with an electron microscope in a Hitachi H600A electron microscope, Faculty of Agriculture, Mansoura University.

Structure genes sequencing and bioinformatics analyses

In this study, it was selected three highly purified *E.coli* phages for the determination, identification, molecular characterization of the structural genes including the tail protein (TP) gene, DNA polymerase (DP) gene, and DNA polymerase accessory (DPA) gene in viral particles. Total DNA was purified using a Norgen phage DNA kit (Norgen Biotek Corp., ON, Canada) according to the manufacturer's instruction, and the genomic DNA was visualized on 1% (w/v) agarose gel. The primers specific for *E.coli* phages were designed according to tail protein (TP), DNA polymerase (DP), DNA polymerase accessory (DPA) gene sequences recorded and available in the international databases of GenBank. The specific primers were selected from six primer sets based on temperature annealing (Tm) and % GC as shown in **Table 1**. The PCR reaction was performed in (30µl) volume tubes that contained the following: $dNTP_s$ (2.5 mM), 3µl; $MgCl_2$ (25mM), 3µl; PCR-buffer (10X), 3µl; Primer (10 P mol), 2µl; Taq DNA polymerase (5u/ml), 0.20µl; Template DNA (25ng), 2µl; $d.H_20$, 16.8µl using an automated thermal cycle (model techno 51z) programmed as follows:94ºC/1min, 60ºC/1min, and 72ºC/2min. The reaction was finally stored at 72ºC/10min. The PCR products were visualized on agarose gel (1% w/v) by ethidium bromide (1µg/ml). Bands were picked and purified by pure link quick gel extraction kit according to the manufacturer's instructions. Finally, purified bands were sequenced by Sanger DNA sequencing (MCLAB, South San Francisco, CA, USA).

All partial nucleotide sequences were submitted in the National Center for Biotechnology Information (NCBI) GenBank database, USA, and assigned their accession numbers. Then, sequences were compared with international databases using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov). The identified nucleic acid sequences were then translated to the corresponding peptide sequences using Transeq EMBOSS programs (http://www.ebi.ac.uk/Tools/st). Multiple alignments of sequences were performed using DNAMAN 5.2.9 software and CLUSTALW software version 1.74. The genetic distances were determined considering alignment gaps using Juckes and Cantor's method for correction of superimposed substitutions with MEGA X version 10.0 software. Phylogenetic relationships among identified *E.coli* phage isolates were measured by unweighting pair group method with arithmetic mean (UPGMA) through DNAMAN software and Neighbor Joining implemented through MEGA X version 10.0 software. Bootstrap

analysis (1000 replicates) was performed to assess the reliability of the constructed phylogenetic tree.

Table (1): Primer sets for structural genes (TP/DP/DPA) designed for purified *E.coli* phages.

Primer code	Sequence (5'->3')	Tm °C*	% GC**	Products
ECP01-Forward	5' GCGGAAGTGTATTATGCGGC 3'	59.77	55.00	2565bp
ECP02-Reverse	5' CCGCTTCGCTATCGCTATCA 3'	59.77	55.00	
ECP03-Forward	5' GCAACATGCAGCAGGTGTTT 3'	60.25	50.00	672bp
ECP04-Reverse	5' ATCAATGCACGCAATGCTCG 3'	60.25	50.00	
ECP05-Forward	5' ATGCTGGTGCATGATAGCGT 3'	60.18	50.00	951bp
ECP06-Reverse	5' CGCCTTTCACATAGCGGGTA 3'	60.18	55.00	

***Tm = Melting temperature; ** %GC = Percentage of guanine and cytosine content.**

Bacterial and viral accession numbers:

Both of bacterial 16S rRNA gene sequences for aquatic *E.coli* strains (MCn1, MCn2, and MCn3) and viral genes including tail protein (TP) gene, DNA polymerase (DP) gene and DNA polymerase accessory (DPA) for coliphage isolates MCn4, MCn5, and MCn6 were deposited at the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) with the accession number OP727307.1, OP727288.1, OP727806.1, OP745094.1, OP745095.1, and OP745096.1, respectively.

Effect of newly phage mix on water quality:

All field parameters were measured in the field and rechecked in the laboratory to ensure data accuracy; temperature, pH, and dissolved oxygen (DO) were measured in water samples using the multi-probe system, model Hydralab– Surveyor, Germany; Biochemical oxygen demand (BOD) was determined by ORION BOD fast respiratory system, model 890. Chemical Oxygen Demand (COD) was tested using the potassium permanganate method. The effect of coliphages on natural water quality was determined using the same procedure.

Bacterial growth curve by lytic phage mix:

The growth curve of *E.coli* ATCC® strain 11775 and *E.coli* ATCC® strain 10536 was determined as follows: 500µL of each of the overnight cultures were added to 50 mL of TSB (Difco, USA) and incubated at 37ºC with aeration speed of 120 rpm for 30 hours. The optical density (OD) of the medium was measured at 600 nm at three-hour intervals. For determining the growth curve of bacteria in the presence of lytic phages, 500µL of overnight culture was added to 50 mL of TSB. Then 500µL of purified phage, with titration of 16×10^8 $PFUml^{-1}$ was added to TSB and

incubated with the same conditions, the optical density (OD) of the medium was measured and recorded.

Effect of novel phage mix on coliform counts:

The membrane filter technique (MF) of 100 mL of wastewater was measured in triplicate after 120 minutes of incubation at 37ºC. The phages for coliform removal and bacterial colonies reduction were assayed as follows: 1 mL of purified phage mix with titration of 10×10^{12} $PFUml^{-1}$ was added to wastewater and incubated at 37ºC/2h. Then the MF of phage-treated wastewater was measured using the same method. For obtaining a more realistic result, in the next step, 100mL of wastewater was mixed with 1 mL of purified phage at titration of 10×10^{12} $PFUml^{-1}$ and placed in a room with fluctuating temperatures of 30ºC ± 5ºC without any aeration for two to six hours. The MF of phage-treated wastewater was measured after two, four and six hours of treatment using the same method.

Statistical analysis:

The results were analyzed using SPSS software version 12.0.0.1 to calculate the min, max, and mean values of measured parameters. As well as MS EXCEL software version 2019 was used to calculate percentages, log-transformed data, and Pearson's correlation coefficient (r).

RESULTS AND DISCUSSIONS

Levels of bacteriological indicators in water samples

Results, using the membrane filter approach, showed that both of total coliform, fecal coliform, and fecal *streptococci* bacteria were detected in all examined water samples. Levels of prevalence were evaluated through the recorded counts in $CFU100ml^{-1}$. For better results illustration, the counts were expressed as 10 log $CFU100ml^{-1}$ and demonstrated as given in **Table (2)** and **Fig (2)**.

Total coliform levels

Mean values of TC densities varied between $392X10^5$ - $76X10^2$ $CFU100ml^{-1}$ at drains outfalls and $35X10^5$ - $28X10^2$ $CFU100ml^{-1}$ at the Rosetta branch in winter and summer seasons, respectively. More than 90% of all sites along the Rosetta branch are out of the international standard limits recommended by **Tebbutt (1998)** (TC should not exceed 5000 $CFU100ml^{-1}$). Much more restricted limits have been reported by **Cabelli (1978)** who recommended a maximum total coliform count of 1000 $CFU100ml^{-1}$, particularly in surface water that are going to be used as drinking water supply.

Table (2): Mean values of bacteriological indicators of some representative water samples.

Parameter	River Nile				Drains			
	Winter		Summer		Winter		Summer	
	Count	Log_{10}	Count	Log_{10}	Count	Log_{10}	Count	Log_{10}
TC*	$35X10^5$	6.5	$44X10^2$	3.6	$392X10^5$	7.6	$76X10^2$	3.9
FC**	$17X10^5$	6.2	860	2.9	$210X10^5$	7.3	$10X10^2$	3.0
FS***	$80X10^3$	4.9	20	1.3	$250X10^3$	5.4	49	1.7

* TC, Total coliforms; ** FC, Fecal coliforms; *** FS, Fecal *streptococci*

Fecal coliform levels

Mean values of FC count at the drain outfalls fluctuated around a maximum of $210X10^5$ $CFU100ml^{-1}$ in the winter season and a minimum of $10X10^2$ $CFU100ml^{-1}$ in the summer season. On the other hand, FC counts in the Rosetta branch ranged between $17X10^5$ and 860 $CFU100ml^{-1}$. Meanwhile, about 70% of the sites along the Rosetta branch throughout this study didn't comply with the standard levels. Restricted limits for surface water intended for use as drinking water supply (200 $CFU100ml^{-1}$) indicate unsafe water from a bacteriological point of view **(Cabelli, 1978)**.

Fecal *streptococci* levels

Mean values of FS counts fluctuated between a maximum of $250X10^3$ $CFU100ml^{-1}$ at the El-Rahawy drain and a minimum of 49 $CFU100ml^{-1}$ at the El-Tahreer drain. On the other hand, FS counts in the Rosetta branch ranged between 20 and $80x10^3$ $CFU100ml^{-1}$. Generally, sites in drain outfalls and the Rosetta branch exceeding 1000 CFU/100 ml^{-1} were reported out of international standard limits **Tebbutt (1998)**. The results revealed that there was a gradual increase in bacterial indicators counts (TC, FC& FS) from upstream to downstream, which might be attributed to the drains discharge into the branch, this agree with the results of **Azzam and Faiesal, (2019)**.

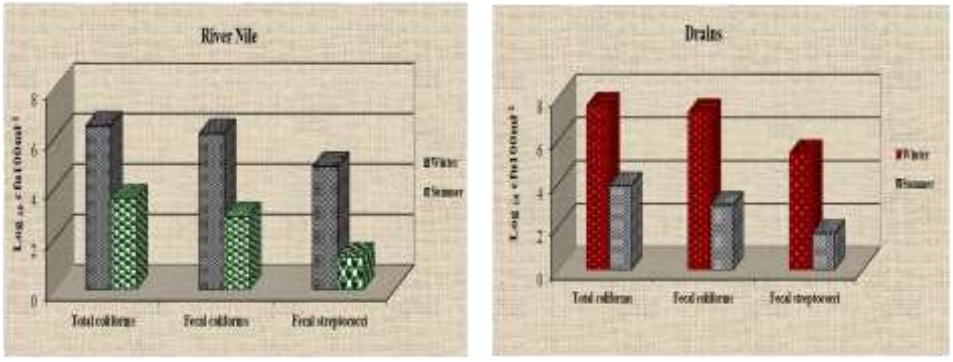

Fig (2): Prevalence degree of total coliform, fecal coliform, and fecal streptococci values in the Rosetta branch and drains.

The results revealed that there was a gradual increase in bacterial indicators counts (TC, FC& FS) from upstream to downstream, which might be attributed to the drains discharge into the branch. These results agree with those of **Azzam (2015) and El-Meihy (2018)**. Statistical analysis indicated a highly positive significant correlation (r = +0.99) between different bacteriological parameters (TC, FC, and FS). The same results were concluded by **El-Sayed *et al.*, (2020).** They used microbiological indicators and physicochemical analysis to evaluate water quality. Rosetta branch has poor quality water, while the Damietta branch has fair to good quality water. Regarding the correlation between bacteriological and physicochemical parameters showed positive strong correlation (r > 0.90) was observed with DO and BOD. It is worth mentioning that, there was a strong negative significant relationship between different bacterial indicators and DO (r = -0.72). This indicated that depletion in DO was strong evidence for bacterial water deterioration.

Levels of *E.coli* in the aquatic environment

In this investigation, out of 88 water samples processed in duplicates during two different seasons, 175 presumptive *E.coli* colonies were isolated from membrane filter assay. 98 (56%) from drains outlet, 47 (27%), and 30 (17%) respectively from drains downstream and upstream in the Rosetta branch as shown in **Table (3)** and **Fig (3)**. These colonies were typically 0.8 to 2.2mm in diameter and flat in appearance with grayish, white, moist, smooth, and opaque. Streaked colonies on MacConkey agar showed enhanced flat and pink colour, while microscopic examination revealed Gram-negative, non-spore-forming bacilli.

Table (3): Levels of *E.coli* strains detected in some representative water samples

Type	Locations			
	River Nile		Drains	
	Count	Percentage	Count	Percentage
Identified	**50**	**65**	**78**	**80**
Non-Identified	**27**	**35**	**20**	**20**
Total	**77**	**100**	**98**	**100**

Phenotypic identification and verification were further processed using API 20NE assay. Only 128 isolates representing about 73% of total isolates were confirmed as typical *E.coli*. Meanwhile, 47 (27%) were classified as being atypical colonies not belonging to *E.coli*. The Pearson's correlation coefficient (r) used to correlate true and false positive results of detected bacteria indicated a highly significant difference (P < 0.01) at 0.99998. Indeed, it seems possible to address

that, results misidentification reported through this study which constituted about 20% is considered a non-ignorable bias, particularly when we are dealing with bacteria associated with human infections **(Gould and STEC 2012)**. Results also matched those recorded by **Ezzat & Azzam, (2020) and Olowe *et al.*, (2017)** who discovered 25% misidentification using classical methods. The identification by traditional techniques could not overwhelm the problems of marked phenotypic variability demonstrated by *E.coli* and other closely related species (**Subbiah *et al.*, 2020 and Soliman *et al.*, 2023**).

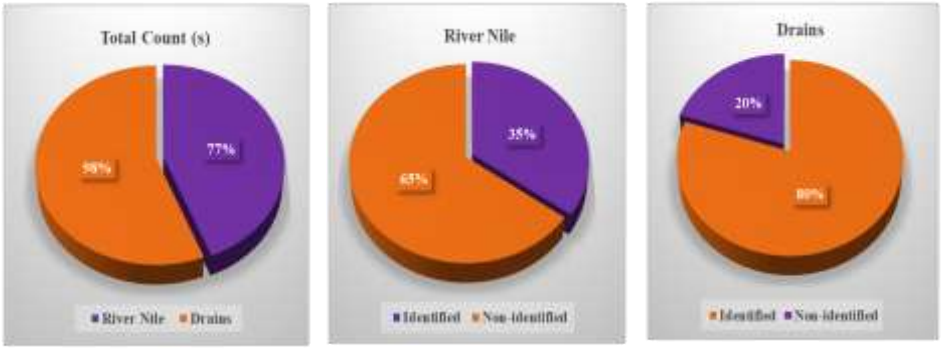

Fig (3): Counts of *E.coli* isolates and its prevalence percentages in drains and Rosetta branch.

Also, they usually fail to detect bacteria that become non culturable due to environmental stress. Lag time needed (2-3 days) for test completion could certainly postpone identification of contamination source and implementation of effective control measures (**Elbahnasawy *et al.*, 2021 and Hui *et al.*, 2022)**.

Molecular characteristics of *E.coli* strains

Because it avoids the problem of phenotype variability and overcomes the barrier of species misidentification, molecular characterization using genetic techniques is becoming the method of choice in environmental microbiology (**Van Rossum *et al.*, 2020)**. In the present investigation, three *E.coli* strains (MCn1/ MCn2 / MCn3) were selected for 16S-rDNA gene sequence analysis, based on their recognizable positive results in plaque assay with phage stock as well as purity and quantity of DNA yield. Multiple sequence alignment (MSA) was displayed to compare the nucleotide sequences of the three Egyptian strains; MCn1 (1475 bp), MCn2 (1464 bp) and MCn3 (818 bp) with other international strains. The nucleotide sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank database, USA. They were assigned the accession numbers OP727307.1, OP727288.1 and OP727806.1, respectively.

Aquatic *E.coli* strains detected by the proposed coliphage stock were 100% confirmed by a 16SrDNA-based PCR approach. These results agree with those reported by **Storto *et al.*, (2021)** who mentioned that, the potential for misidentification of wild *E.coli* strains in the ecosystem using molecular methods was nearly negligible. Meanwhile, conventional cultural methods could hamper the identification of contamination sources and the implementation of effective control measures. Molecular methods mediated superior specificity and sensitivity than conventional phenotypic diagnostic tests with percentages reaching 90-100% accuracy in similar studies **(El-Dougdoug *et al.*, 2020; Storto *et al.*, 2021 and Koh *et al.*, 2022)**.

On the other hand, the phylogenetic tree was constructed and showed the genetic relationship between novel *E.coli* (MCn1 / MCn2 / MCn3) strains and other submitted international strains from GenBank according to sequence similarity values. Nine clusters are clearly demonstrated in **Fig 4** in which, strains MCn1, MCn2 and MCn3 showed 98% homology with each other and 88% homology with *E.coli* strains 25922 and JCM. According to given accession numbers, the three strains were found to be highly homologous (76-98%) with strains from Iraq, Greece, Japan, and Thailand.

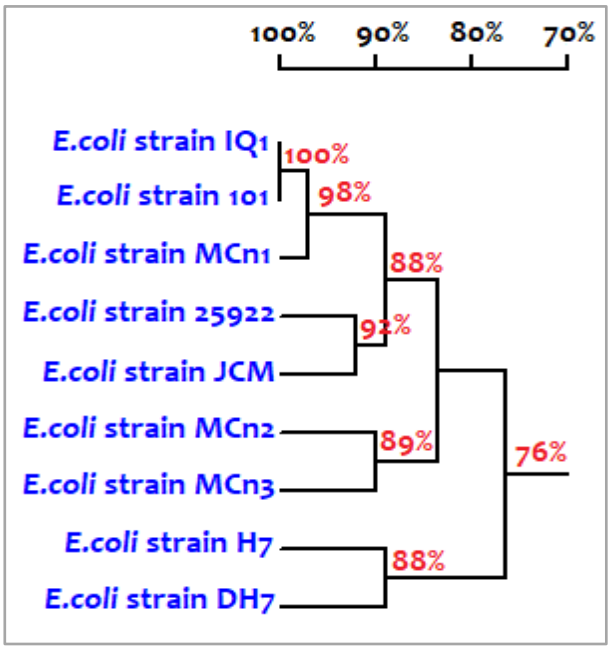

Fig (4): Phylogenetic tree of *E.coli* strains based on 16S rRNA gene sequence comparisons. Bootstrap values from 1000 replications are indicated at the branches.

Results indicate observable genetic variability among local *E.coli* strains detected by plaque assay. This definitely reflects the broad spectrum ability of coliphages employed in this study to target a wide array of environmental *E.coli* strains in water as much as possible and supports its high specificity (98%) concluded from earlier statistical analysis. Thus, it is more advantageous to get benefits from the synergistic effect of more than one phage rather than using them individually **(Elbahnasawy *et al.*, 2021 and Soliman *et al.*, 2023)**.

Spot and plaque assay of isolated coliphages

Bacteriophages are viruses that infect bacteria and replicate solely within their cells, making them identifiable anywhere their specific host (bacteria) occurs (**Chevallereau *et al.*, 2022**). In current study, the American Type Culture Collection *E.coli* ATCC® strain 11775 and *E.coli* ATCC® strain 10536 were employed as reference hosts to detect phages in water by spot test. Clearly, **Fig 5** demonstrates the incidence of *E.coli* phages in all tested water samples (n= 60), being maximum (10^7-10^{12} pfu ml^{-1}) at the drains outlet and minimum (10^5 pfu ml^{-1}) at drains upstream in the Rosetta branch. Concentrations were obviously higher in winter than in summer, most probably due to the sunlight effect which is known to be a pertinent factor governing the incidence of viruses and phages in the environment **(Stone *et al.*, 2019)**. Results confirmed that the number and behavior of phages were directly influenced by the densities of their specific host. As well as, because of the constant mobility in water, phages have a high chance of coming into contact with their host bacteria (**Abedon, 2023**). Many studies discussed and applied the detection of *E.coli* phages, particularly from sewage water and aquatic ecosystems subjected to sources of microbial pollution **(El-Dougdoug *et al.*, 2020; Ezzat and Azzam, 2020; Elbahnasawy *et al.*, 2021)**. The plaque characteristics were determined after the plaque assay technique was done using different bacterial strains of *E.coli* and appeared clear zones **(Fig 5)**. The plaque sizes ranged between 1-4mm in diameter, which were circular and regular in morphology. Similarly, both **Azzam *et al.*, 2014 and Soliman *et al.*, (2023)** detected and characterized *E.coli* phages of nearly comparable plaque sizes (1-5mm diameter) from surface and drainage water, respectively. Our study noted that, the dramatic decrease in *E.coli* cells on behalf of phage infection during reduction assay. The OD_{600} of bacterial titer (0.704nm) dropped to 0.508 nm upon mixing with phage after 24h of incubation.

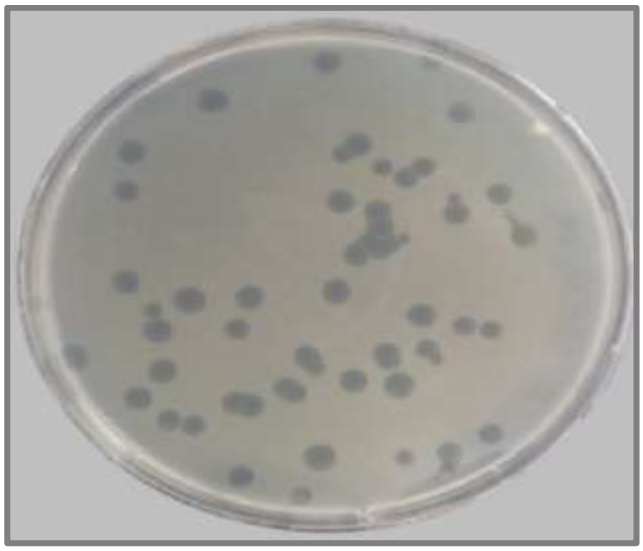

Fig (5): Plaque assay of *E.coli* phages after incubation at 37°C/24h.

Host range pattern and cross infectivity of the isolated phages

The ability of the phage to specifically target its host species and to infect as many strains as feasible are crucial for the plaque assay of novel bacteriophages. Plaques called "host clearing" are a sign of a susceptible host (**Azzam, 2015**). In this study, an attempt has been made to evaluate the specificity and host range pattern of three isolated phage stock (Mcn4 / Mcn5 / MCn6) and ensure the ability of these wild-type phages to target as many *E.coli* strains as possible in water samples. Among 180 typical *E.coli* isolates from various water sources, the *E.coli* phage stock was highly specific to 90% of tested strains compared to ATCC® strain 11775 and ATCC® strain 10536 as a positive control. Phage specificity reached about 96.1% in highly polluted samples (drains outfalls), followed by the Rosetta branch after drains discharge (82.9%) and before discharge (65.3%) as shown in **Table 4**. The susceptibility to phage infection was expected to increase in highly polluted regions, most likely because of a direct connection between phage and host concentration **(Azzam and Faiesal, 2019)**. On the other hand, results misleading didn't exceed 14.0% compared to those obtained by membrane filter assay (39.2%), and the time elapsed for test completion didn’t exceed 24h to get results. Additionally, the use of multiple lytic phages in the same sample ensured higher specificity. The same conclusions were reported by **Elbahnasawy *et al.*, 2021 and Soliman *et al.*, 2023**.

Table (4): Host range pattern of the three novel phages (MCn4/MCn5/ MCn6) against different bacterial strains.

Bacterial Host	River Nile		Drains	
	No. of tested strains	Percentage of (+) lysis	No. of tested strains	Percentage of (+) lysis
E.coli **(this study)**	30	80	50	92
E.coli **(this study)**	20	60	45	84
Salmonella typhimurium **(this study)**	0	0	4	100
Proteus vulgaris **(this study)**	5	100	11	100
Citrobacter freundii **(this study)**	0	0	3	100
E.coli **ATCC® strain 11775***	1	100	1	100
E.coli **ATCC® strain 10536***	1	100	1	100
P.aeruginosa **ATCC® strain 15442***	1	0	1	0

*** American Type Culture Collection (ATCC) (reference strains).**

The ability of the isolated phages (MCn4 / MCn5 / MCn6) to infect di-erent species other than *E.coli* and each phage were spotted over a lawn of di-erent bacterial species among the *Enterobacteriaceae*. Results confirmed that all isolated phages had a lytic effect on *S.typhi* and *P.vulgaris* suggesting that these phages are polyvalent. Moreover, only the two phages (MCn4, MCn6) had a lytic effect on *C.freundii*. All phages could not infect any Gram-positive bacteria **(Table 4)**.

Transmission electron microscopy (TEM)

TEM imaging of partially purified phage particles revealed different structural characteristics and dimensions as demonstrated in **Table 5** and **Fig 6**.

Table (5): Morphological characterizations of environmental lytic coliphages as determined by TEM.

Phage	Families	Head Capsid (nm)		Tail	
		Length	Diameter	Length	Diameter
MCn4	***Siphoviridae***	**78±2**	**78±2**	**233±7**	**20±0**
MCn5	***Myoviridae***	**61±4**	**61±4**	**92±8**	**20±5**
MCn6	***Podoviridae***	**87±3**	**87±3**	**34±6**	**20±0**

The three phages are non-enveloped and have icosahedral capsids. MCn4 & MCn5 have a long non-contractile tail, while MCn6 has a short non-contractile tail. According to the International Committee on Taxonomy of Viruses (ICTV), the isolated phages belonged to the *Siphoviridae, Myoviridae,* and *Podoviridae* families. These two families

have been documented to include many phages which have the ability to infect members of *Enterobacteriaceae* **(Azzam, 2015 and Azzam and Faseail, 2019; El-Dougdoug *et al.*, 2020 and Soliman *et al.*, 2023)**.

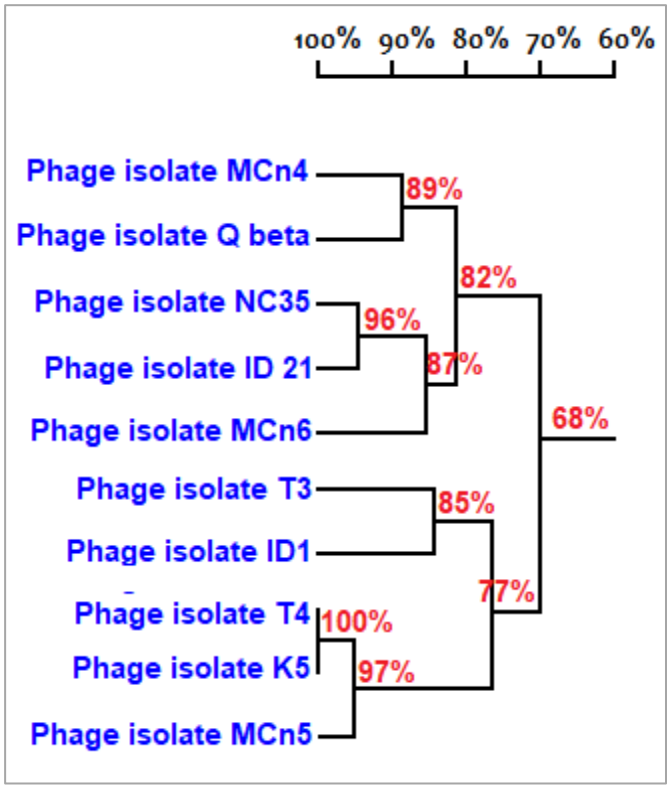

Fig (6): Neighbor-Joining tree of novel phages and other phages published in GenBank. Numbers represent bootstrap percentage values based on 1000.

Bioinformatics analysis of coliphages

Sequencing of DNA for the three coliphages (MCn4 / MCn5 / MCn6) showed different DP/TP/DPA gene sizes (2565bp, 672bp & 951bp), respectively. All nucleotide sequences were aligned with the same gene sequences of coliphages published in the international databases (NCBI, EMBL, and DDBJ). The multiple sequence alignment revealed diversity among novel Egyptian coliphage isolates (MCn4 / MCn5 / MCn6) compared with other isolates recorded in GenBank including both of: Phage Q beta, Phage NC35, Phage ID 21, Phage T3, Phage ID1, Phage T4 and Phage K5. The constructed phylogenetic nine showing the clustering relationship among coliphages produced seven major groups as shown in **Fig 7**. They were assigned their accession numbers; OP745094.1, OP745095.1 & OP745096.1, respectively.

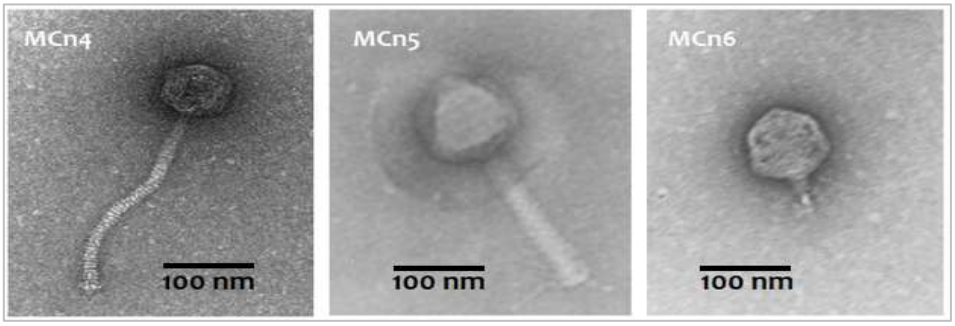

Fig (7): Electron micrographs of the three novel phage particles.

The bioinformatics analysis clearly provided a unique opportunity for a comparative illustration of nucleotide diversity among local new coliphages isolated from the Rosetta branch and drain outfalls in Egypt and other geographically distant phages submitted to GenBank, and it comprehensively reflects the novelty of the phage isolates used in the current study. As a result, our long-term goal is to increase the quantity of freshly isolated and characterized coliphages from aquatic habitats. Furthermore, our findings supported using these phages for directly detecting pathogens in water samples without the requirement to isolate pure bacterial cultures and the results agree with **Ezzat and Azzam, (2020); Soliman *et al.*, (2022) and Soliman *et al.*, (2023)**.

Effect of newly characterized phages on water quality

Results demonstrated in **Table (6)** showed that different physic-chemical characteristics and their concentrations varied with the environment begin investigating. It survived a temperature range (28-35°C), pH (6.95-8.5), dissolved oxygen (0.12-7.8 mgL^{-1}), and biochemical oxygen demand (2-115), chemical oxygen demand (16-231) in winter and summer season, respectively as shown in **Fig 8**. This highlights its biological tolerance to a wide scale of physic-chemical factors and underlines its capacity to adapt to environments with different trophic levels **(Azzam *et al.*, 2017)**. After being treated with the novel coliphage cocktails, the physicochemical characteristics of drain outputs and Rosetta branch downstream samples were gradually improved. Physiochemical indicators were evidently lowered in all treated water samples in the winter and summer seasons. According to Egyptian Law 48/1982 (Article 49, which deals with the water quality of surface water,

and Article 51, which is concerned with the water quality of drains before they are released into the surface water), all reported values are within acceptable ranges.

Table (6): Mean values of physicochemical analysis of representative sites.

Parameter	River Nile		Drains	
	Winter	Summer	Winter	Summer
Temperature (°C)	28	34	30	35
pH (unit)	8.5	7.2	8.1	6.95
DO* (mgL^{-1})	7.8	2.5	5.17	0.12
BOD** (mgL^{-1})	14	2.0	115	2.0
COD*** (mgL^{-1})	85	16	231	21

*** DO, dissolved oxygen; ** BOD, biological oxygen demand; ***COD, chemical oxygen demand.**

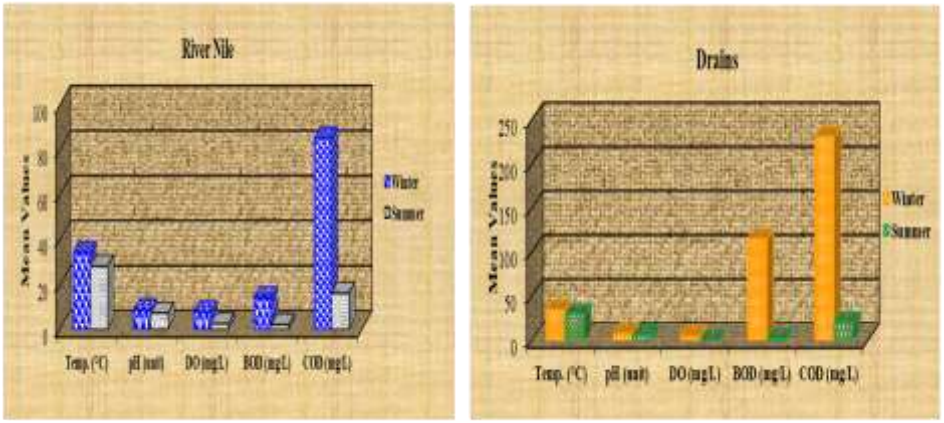

Fig (8): Comparison between physicochemical values in River Nile and drains outlets.

Growth curve and reduction of coliforms using novel phage mix:

The growth curve of *E.coli* ATCC® strain 11775 and *E.coli* ATCC® strain 10536 before and after treatment with specific lytic coliphage mix during incubation at 37°C/24h,demonstrated that the isolated phage mix could lysis and inhibits the normal growth of bacterial host **(Fig 9)**. As well as, the growth curve of the mix of wild *E.coli* strains MCn1, MCn2, and MCn3 after treatment with purified phages during incubation at 37°C/24h confirmed that all phages prevented the normal growth of wild strains. Also, results demonstrated that burst sizes and latent periods of siphovirus (MCn4), myovirus (MCn5), and podovirus (MCn6) phages were evaluated using a one-step growth curve experiment. The phages

tested had burst sizes of 77, 110, and 95 pfu per infected cell, with latent periods of 35, 50, and 60 minutes, respectively.

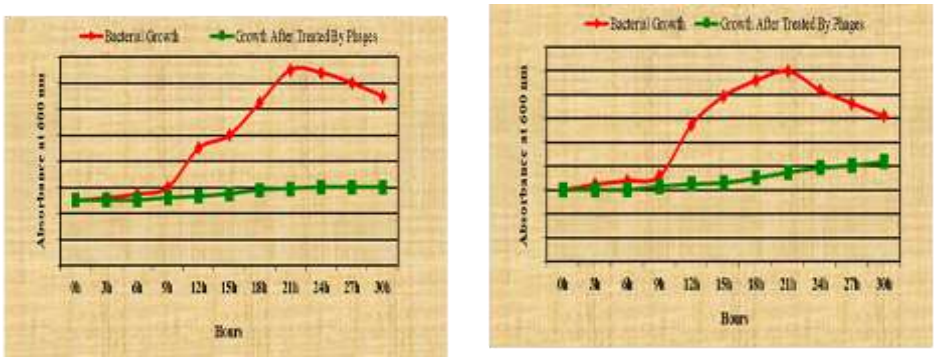

Fig (9): Bacterial growth curve for *E.coli* ATCC® strain 11775 and *E.coli* ATCC® strain 10536 after and before treatment by novel phage mix.

The coliforms membrane filter (MF) test of phage-treated wastewater was done in triplicate, and results after four hours of incubation at 37ºC, demonstrate that the counts both of total and fecal colonies resulted in a significant decline of MF as shown in **Table (7)**.

Table (7): Reduction of coliform count after treatment with novel phage mix in water.

	River Nile				Drains			
	Winter		Summer		Winter		Summer	
	Count	Log_{10}	Count	Log_{10}	Count	Log_{10}	Count	Log_{10}
Total coliforms ($CFU100ml^{-1}$)	$116X10^3$	5.1	146	2.1	$130X10^4$	6.1	250	2.3
Fecal coliforms ($CFU100ml^{-1}$)	$55X10^3$	4.7	29	1.4	$70X10^4$	5.8	50	1.6

The comparison of the MF test of wastewater coliforms before and after treatment with lytic phage mix showed that after four hours of incubation, MF at $35X10^5$ $CFU100^{-1}$ was reduced to $116X10^3$ $CFU100ml^{-1}$, i.e. there was a 28-fold reduction of coliform's load in wastewater **(Fig 10)**. The incubation of isolated coliphages with wastewater samples in a more realistic thermal condition (30°C ± 5°C), resulted in more promising outcomes. The MF of phage-treated wastewater after two, four, and six hours was measured in River Nile and drains outfalls, respectively. The examination showed that treating the wastewater sample with isolated coliphages resulted in a considerable reduction of coliforms so that the MF after two, four, and six hours of treatment declined 22, 60, and 89 times, respectively. Many authors have discussed several roles that bacteriophages play in the environment,

biofilm control, and wastewater treatment **(Wu *et al.*, 2017; Bolsan *et al.*, 2022 and Shivaram *et al.*, 2023)**.

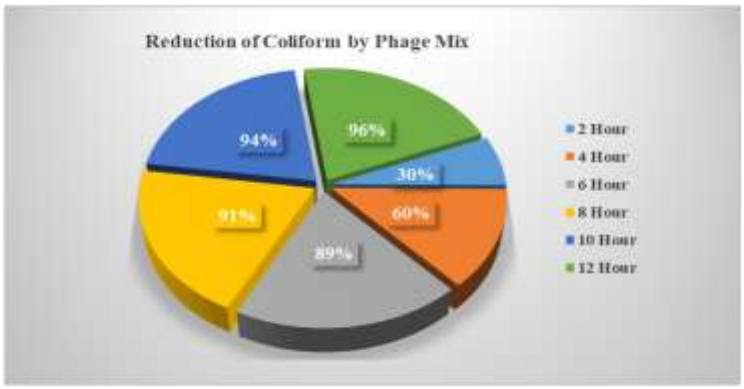

Fig (10): Percentage of coliform growth reduction by novel coliphage mix.

Waterborne pathogenic bacteria, including *E.coli* pose a major public health problem due to their potential for morbidity and mortality, harm to the environment, and the cost of removal using conventional wastewater treatment methods **(Jassim *et al.*, 2016)**. To address this issue, researchers have turned to programmable phages, which are highly efficient in eliminating target bacterial hosts without causing antibiotic resistance and can control biofilms and regulate nutrient cycles. Lytic phages have shown promise in wastewater treatment facilities, acting as antifoam agents and sludge biomass reducers by eliminating harmful bacteria (**Parmar *et al.*, 2017)**.

This study is the first to identify bacteriophages with lytic activity against various wild *E.coli* and coliforms from Egyptian waterways. The research was conducted using traditional methods that resulted in a low misidentification rate, which is important when dealing with bacteria that may cause human diseases **(Ezzat and Azzam, 2020)**. However, standard approaches for bacterial identification can be challenged by the phenotypic diversity of *E.coli* and similar species. Furthermore, non-culturable bacteria require specific unknown nutrients or environmental conditions that conventional methods may not detect. The delay in detecting the source of contamination and implementing control measures can also be a disadvantage of conventional procedures. In the environmental microbiology field, the bioinformatics approach has the potential to overcome issues with phenotypic variability and species misidentification. They also offer higher levels of specificity and

sensitivity, resulting in a lower likelihood of misidentifying *E.coli* in water as reported by **Hoosain *et al.*, (2023)**.

The behavior and abundance of coliphages are directly influenced by their densities of *E.coli* hosts (**Soliman *et al.*, 2023)**. The number of coliphages was higher due to the effect of sunlight on the incidence and dissemination of phages in aquatic environments. Coliphages in water can encounter their specific *E.coli* hosts due to their continuous movements (**Masuda *et al.*, 2021**). Similar to our findings, coliphages were discovered in surface and drainage water with titers between 10^5 and 10^{12} PFU mL-1 and a variety of plaque shapes and sizes. Our isolated plaques were spherical, regular in shape, and varied in size, in contrast to the phage hosts, *E.coli* ATCC® strain 11775 and *E.coli* ATCC® strain 10536. Many coliphages with plaques against *E.coli* ATCC® strain 13706 measuring 1–5 mm and 0.2-2 mm in diameter, respectively, were recovered from sewage water **(Azzam, 2015; McMinn *et al.*, 2017 and Elbahnasawy *et al.*, 2021)**. In response to *E.coli* (ATCC 13706), a distinct coliphage that was isolated *In Vitro* generated spherical plaques with a diameter of 2-5 mm (**Pereira *et al.*, 2017**).

TEM photos of coliphages revealed three physically distinct phages that differed in size and shape. According to the most recent ICTV classification of bacteriophages, the *Enterobacteriaceae* family has 982 different phages, 344 of which are associated with the *Myoviridae*, 297 with the *Siphoviridae*, and 265 with the *Podoviridae* **(Soliman *et al.*, 2023)**. *Siphoviridae* and *Myoviridae* phages are particularly relevant because of their lytic activity against human-originated non-O157 *E.coli* strains **(Elbahnasawy *et al.*, 2021)**. Similarly, coliphages from the *Siphoviridae* and *Myoviridae* families have been isolated from the Nile and drainage water collected in Egypt **(El-Dougdoug *et al.*, 2020)** and the United States **(Chevallereau *et al.*, 2022)**, as well as water samples in the United States. In our study, three novel coliphages (MCn4, MCn5, and MCn6) were isolated from the Nile and drainage water in Egypt and found to belong to the *Siphoviridae*, *Myoviridae*, and *Podoviridae* families, respectively. Genomic analysis of the TP/DP/DPA genes of coliphage isolates revealed their diversity and novelty after being compared to geographically distant phages from other regions recorded in international databases. The study demonstrated the ability of the phages to target and lysis a wide range of environmental *E.coli* strains, suggesting the potential for synergistic action when used together.

The success of phage-mediated bio-control for aquatic pathogenic bacteria depends on their host range pattern, which measures the maximum number of bacterial species and strains a phage can target. The appearance of varied plaque morphology during the plaque test indicates the host's vulnerability, and the study confirms the polyvalent nature of the MCn4, MCn5, and MCn6 phages. They all had a lytic effect on *S.typhi* & *P.vulgaris*, and MCn4 & MCn6 also infected one strain of *C.freundii*, making them polyvalent phages. The differences in bacteriophages host range may be associated with diminishing adsorption to an acceptable bacterial receptor **(Azzam, 2015)**. These findings are in line with those of **Yamaki, (2022)** who isolated and characterized aquatic coliphages that could infect strains of *E.coli* and *S.enterica* serovar Choleraesuis.

E.coli strains and other *Enterobacteriaceae* species isolated from mixed drains were shown to be more susceptible to phage infection than those recovered from the Nile. In water samples received from drain outlets, Rosetta branch locations, particularly those prior to drain discharge and downstream drain discharge, demonstrated strong coliphage specificity. Because of the direct affinity between phages and host densities, heavily polluted areas were considered to be more vulnerable to phage infection **(Ezzat and Azzam, 2020)**. As observed in earlier investigations, using a cocktail of different lytic phages on the same water sample resulted in greater specificity, as seen in previous studies **(Elbahnasawy *et al.*, 2021 and Soliman *et al.*, 2023)**.

Coliphages in water treatment can improve physicochemical characteristics by adhering organic molecules to weaker bacterial structures, which increases the surface area of the wall substrate and provides more space to adsorb particles. However, water turbidity can rise, and bacteria & viruses can be protected from disinfectants due to contamination with suspended solids such as clay, silt, airborne particulates, colloidal organic particles, plankton, and other tiny organisms derived from organic and inorganic substances. The health implications of water quality can be indirectly linked to turbidity because suspended particles can absorb unwanted inorganic and organic substances present in water. Environmental contaminants like turbidity, ammonia, and organic matter are toxic and require serious attention. Ammonia is an important form of nitrogen ion, but its high concentration in a water solution can cause toxicity to aquatic life and have severe effects on dissolved oxygen levels. Municipal sewage, agricultural waste,

and fertilizer factory outflow are the main sources of pollution. According to **Elbahnasawy *et al.*, (2021),** the most dangerous impacts occur when greater levels of ammonia cause a drastic decrease in dissolved oxygen levels and clearly apparent toxicity to aquatic life.

The increase in dissolved oxygen (DO) levels observed after treatment with a phage mix may be due to a decrease in bacterial hosts that degrade organic matter and consume oxygen during this process (measured as biochemical oxygen demand or BOD and chemical oxygen demand or COD). High COD levels can lead to a decrease in DO, which can be fatal for aquatic life. Bacteriophages have the ability to lyse and kill a large fraction of bacterial species in aquatic environments while leaving free oxygen particles unharmed (**Ferriol-González and Domingo-Calap, 2020)**.

To date, few studies have examined the use of coliphages as an efficient biological control for eliminating or reducing the coliform microbial burden in drainage water sanitation. The coliphage cocktail MCn4/MCn5/MCn6 has shown lytic action against *E.coli* and coliform populations in the Nile and drainage water. Similarly, after a 4-hour treatment interval, the coliphage cocktail PR01/PR02/PR03 outperformed mixed phages in suppressing *E.coli* **(Elbahnasawy *et al.*, 2021)**. The elimination effectiveness for coliforms was maximized after 12 hours of incubation with a coliphage cocktail at 37^0 C. Lytic bacteriophages were employed to combat *E.coli* in drainage water, reducing the total load of coliforms and fecal coliforms by 28 and 32 folds, respectively, after eight hours of incubation. Coliform reduction is a vital indicator of efficacy in wastewater treatment strategies, and coliphage treatment successfully achieved the objective **(Jassim *et al.*, 2016)**. Additionally, the effectiveness of coliphages in treating drainage water was further verified under customary temperature conditions (**Masuda *et al.*, 2021)**. In conclusion, coliphages exhibited promising potential in reducing the coliform population in drainage water and could serve as an efficient tool for bacterial control in wastewater treatment.

CONCLUSION

This study reveals the diversity of coliphages and their possible application in wastewater treatment. Results show that newly isolated local lytic coliphages were effective at reducing the number of *E.coli* strains, coliforms and other bacterial species among the *Enterobacteriaceae* in River Nile and drainage water. In turn, this lowers the physicochemical characteristics such as BOD, COD and raises the

levels of DO, which may ultimately improve the quality of the water. Our method was shown to be straightforward, quick, affordable, and targeted for the elimination of a variety of *E.coli* strains, which are significant water-borne opportunistic pathogens of public health concern. In order to commercialize immobilized phage-purified and immobilized products as bacterial controlling agents in various water resources, it is important to isolate, characterize, and identify more phages in the future.

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REFERENCES

Abedon, S.T. (2023). Bacteriophage adsorption: likelihood of virion encounter with bacteria and other factors affecting rates. Antibiotics, 12(4): 723.

Al-Gheethi, A.A.; J. Lalung; E.A. Noman; J.D. Bala and I. Norli (2015). Removal of heavy metals and antibiotics from treated sewage effluent by bacteria. Clean Technol. and Environ. Policy, 17:2101-2123.

American Public Health Association (APHA) (2017). Standard Methods for the Examination of Water and Wastewater, 23rd edition. American Public Health Association, Washington, DC.

Azeredo, J.; P. García and Z. Drulis-Kawa (2021). Targeting biofilms using phages and their enzymes. Current Opinion in Biotechnol., 68:251-261.

Azzam, M.I.; S.M. Ezzat; K.A. El-Dougdoug and B.A. Othman (2014). Rapid quantitative detection of Enteric viruses in River Nile and drainage water, Egypt. Egyptian J. Virol., 11 (2): 159-175.

Azzam, M.I. (2015). Eco-diversity of aquatic bacteria and viruses isolated from River Nile and drainage water in Egypt. Ph.D. Thesis Fac. Agric., Ain Shams Univ., Cairo, Egypt.

Azzam, M.I.; S.M. Ezzat; B.A. Othman and K.A. El-Dougdoug (2017). Antibiotics resistance phenomenon and virulence ability in bacteria from water environment. Water Sci., 31 (2):109-121.

Azzam, M.I. and A.A. Faiesal (2019). Novel "Superspreader" coliphages for detecting microbial water pollution. Int. J. Environ., 8(1): 57-70.

Azzam, M. I.; A.S. Korayem; S.A. Othman and F.A. Mohammed (2022). Assessment of some drinking water plants efficiency at El-Menofeya Governorate, Egypt. Environ. Nanotechnol. Monitoring & Management, 18: 100705.

Bolsan, A.C.; H.C. Rodrigues; H.C. Abilhôa; C.E. Hollas; B. Venturin; N.C. Gabiatti and M.C. De Prá (2022). Bacteriophages in wastewater treatment: Can they be an approach to optimize biological treatment processes. Environ. Sci. and Pollution Res., 1:10.

Brenner, D.J.; N.R. Krieg and J.T. Staley (2005). Bergy's Manual of Systematic Bacteriology, 2nd edition. V. (2), the Proteobacteria. Springer, New York.

Cabelli, V. (1978). New standards for enteric bacteria In: Water Pollution Microbiology. Mitchell, R. ed., John Wiley, New York, 2:233-271.

Chevallereau, A.; B.J. Pons; S. van Houte and E. R. Westra (2022). Interactions between bacterial and phage communities in natural environments. Nature Rev. Microbiol., 20(1): 49-62.

Chu, L.; S. Yan; X.H. Xing; X. Sun and B. Jurcik (2009). Progress and perspectives of sludge ozonation as a powerful pretreatment method for minimization of excess sludge production. Water Res., 43(7):1811-1822.

Clokie, M.R.; A.M. Kropinski and R. Lavigne (2018). Bacteriophages: Methods and protocols-Vol. III. Methods in Molecular Biology.

Dias, E.; J. Ebdon and H. Taylor (2018). The application of bacteriophages as novel indicators of viral pathogens in wastewater treatment systems. Water Res., 129: 172-179.

El-Sayed, S.M.; M.H. Hegab; H.R. Mola; N.M. Ahmed and M.E. Goher (2020). An integrated water quality assessment of Damietta and Rosetta branches (Nile River, Egypt) using chemical and biological indices. Environ. Monitoring and Assessment, 192:1-16.

Elbahnasawy, M.A.; E.E. ElSayed and M.I. Azzam (2021). Newly isolated coliphages for bio-controlling multidrug-resistant Escherichia coli strains. Environ. Nanotechnol. Monitoring & Management, 16: 100542.

El-Dougdoug, N.; M. Nasr-Eldin; M.I. Azzam; A. Mohamed and M. Hazaa (2020). Improving wastewater treatment using dried banana leaves and bacteriophage cocktail. Egyptian J. Botany, 60(1):199-212.

El-Meihy, R.M. (2018). Microbiological and physicochemical evaluation of River Nile (Rosetta branch). Annals of Agric. Sci. Moshtohor, 56: 217-226.

Ezzat, S.M. and M.I. Azzam (2020). An approach using a novel phage mix for detecting *Pseudomonas aeruginosa* in water. Water Environ. J. 34 (2):189-202.

Ferriol-González, C., and P. Domingo-Calap (2020). Phages for biofilm removal. Antibiotics, 9(5): 268.

Foong, L.C.; C.W. Loh; H.S. Ng and J.C. Lan (2021). Recent development in the production strategies of microbial carotenoids. World J. Microbiol. and Biotechnol., 37:1-11.

Gould, L. H. and STEC Clinical Laboratory Diagnostics Working Group. (2012). Update: Recommendations for diagnosis of Shiga toxin-producing *Escherichia coli* infections by clinical laboratories. Clinical Microbiol. Newsletter, 34(10):75-83.

Gupta, A.; R. Gupta and R.L. Singh (2017). Microbes and environment. Principles and Applications of Environ. Biotechnol. for a Sustainable Future, pp 43-84.

Hoosain, N.; J. Korsman; P.O. Kimathi; P. Kachambwa; R. Magoba and S.L. Murray (2023). AquaSens: Exploring the use of 16S rRNA next-generation sequencing to determine bacterial composition of various water matrices.Water SA, 49(2):117-125.

Hui, Y.; Z. Huang; M.E.E. Alahi; A. Nag; S. Feng and S.C. Mukhopadhyay (2022). Recent advancements in electrochemical biosensors for monitoring the water quality. Biosensors, 12(7):551.

Iwuozor, K.O. (2019). Prospects and challenges of using coagulation-flocculation method in the treatment of effluents. Adv. J. Chem. Section A, 2(2):105-127.

Jassim, S.A.; R.G. Limoges and H. El-Cheikh (2016). Bacteriophage biocontrol in wastewater treatment. World J. Microbiol. and Biotechnol., 32(4): 70.

Juang, D.F. and J.M. Morgan (2001). The applicability of the API 20E and API Rapid NFT systems for the identification of bacteria from activated sludge. Electronic J. Biotechnol., 4(1): 1-2.

Koch, N.; N.F. Islam; S. Sonowal; R. Prasad and H. Sarma (2021). Environmental antibiotics and resistance genes as emerging contaminants: methods of detection and bioremediation. Current Res. Microbial Sci., 2: 100027.

Koh, X.P.; Z. Shen; C.F. Woo; Y. Yu; H.I. Lun; S.W. Cheung and S.C.K. Lau (2022). Genetic and ecological diversity of *Escherichia coli* and cryptic *escherichia* clades in subtropical aquatic environments. Frontiers in Microbiol., 13:128.

Kuppusamy, S.; T. Palanisami; M. Megharaj; K. Venkateswarlu and R. Naidu (2016). Ex-situ remediation technologies for

environmental pollutants: A critical perspective. reviews of Environment. Contamination and Toxicol., 236:117-192.

Masuda, Y.; S. Kawabata; T. Uedoi; K.I. Honjoh and T. Miyamoto (2021). Construction of leaderless-bacteriocin-producing bacteriophage targeting *E.coli* and neighboring gram-positive pathogens. Microbiol. Spectrum, 9(1): e00141-21.

McMinn, B.R.; N.J. Ashbolt and A. Korajkic (2017). Bacteriophages as indicators of faecal pollution and enteric virus removal. Letters in Appl. Microbiol., 65(1): 11-26.

Mitarai, N.; S. Brown and K. Sneppen (2016). Population dynamics of phage and bacteria in spatially structured habitats using phage λ and *Escherichia coli*. J. Bacteriol., 198(12):1783-1793.

Mushtaq, N.; D.V. Singh; R.A. Bhat; M.A. Dervash and O.B. Hameed (2020). Freshwater Contamination: Sources and Hazards to Aquatic Biota. Freshwater Pollution Dynamics and Remediation, pp 27-50. DOI:10.1007/978-981-13-8277-2_3

Naidoo, S. and A.O. Olaniran (2014). Treated wastewater effluent as a source of microbial pollution of surface water resources. Int. J. Environ. Res. and Public Health, 11(1): 249-270.

Odonkor, S.T. and J.K. Ampofo (2013). *Escherichia coli* as an indicator of bacteriological quality of water: An overview. Microbiol.Res., 4(1): e2.

Olowe, B.M.; J.O. Oluyege; O. Famurewa; A.O. Ogunniran and O. Adelegan (2017). Molecular identification of *Escherichia coli* and new emerging enteropathogen, *Escherichia fergusonii*, from drinking water sources in Ado-Ekiti, Ekiti State, Nigeria. J. Microbiol. Res., 7(3): 45-54.

Olsen, N.S. ; L. Forero-Junco ; W. Kot and L.H. Hansen (2020). Exploring the remarkable diversity of culturable *Escherichia coli* phages in the Danish wastewater environment. Viruses, 12(9):986.

Parmar, K.M.; Z.J. Hathi and N.A. Dafale (2017). Control of multidrug-resistant gene flow in the environment through bacteriophage intervention. Appl. Biochem. and Biotechnol., 181:1007-1029.

Pereira, C.; C. Moreirinha; M. Lewicka; P. Almeida; C. Clemente; J.L. Romalde and A. Almeida (2017). Characterization and *In Vitro* evaluation of new bacteriophages for the biocontrol of *Escherichia coli*. Virus Res., 227:171-182.

Shivaram, K.B.; P. Bhatt; B. Applegate and H. Simsek (2023). Bacteriophage-based biocontrol technology to enhance the efficiency of wastewater treatment and reduce targeted bacterial biofilms. Sci. Total Environ., 862, 160723.

Soliman, R.M.; B.A. Othman; S.A. Shoman, M.M. Gado and M.I. Azzam (2022). Assessment of Bahr El-Baqar drain and its environmental impact on manzala lake in Egypt. J. Ecology and Natural Resources. 6(3):1-14.

Soliman, R.M.; B.A. Othman; S.A. Shoman, M.I. Azzam and Gado, M.M. (2023). Biocontrol of multi-drug resistant pathogenic bacteria in drainage water by locally isolated bacteriophage. BMC Microbiol., 23(1):1-11.

Stephenson, F.H. (2010). Calculations for Molecular Biology and Biotechnology, 2nd ed., California, pp 83-98.

Stone, E.; K. Campbell; I. Grant and O. McAuliffe (2019). Understanding and exploiting phage-host interactions. Viruses, 11(6):567.

Storto, D., L.B.C. Nara; D.I. Kozusny-Andreani; L.S. Vanzela; C.F.M. Mansano; M. Bilal and J.H.P. Américo-Pinheir (2021). Seasonal dynamics of microbial contamination and antibiotic resistance in the water at the Tietê Ecological Park, Brazil. Water, Air, and Soil Pollution, 232(7):257.

Subbiah, M.; M.A. Caudell; C. Mair; M.A. Davis; L. Matthews; R.J. Quinlan and D.R. Call (2020). Antimicrobial resistant enteric bacteria are widely distributed amongst people, animals and the environment in Tanzania. Nature Communications, 11(1): 228.

Tamura, K.; G. Stecher; D. Peterson; A. Filipski and S. Kumar (2013). MEGA 7: molecular evolutionary genetic analysis (MEGA) software version 7.0 Mol. Biol. Evol. 30(12):2725-2729.

Tebbutt, T. (1998). Principles of Water Quality Control. (5th ed.), Hallam University.

Thompson, J.D.; D.G. Higgins and T.J. Gibson (1994). Clustalw. Improving the sensitivity of progressive multiple sequences alignment through sequencing weighting, positions specific gap penalties and weight matrix choice. Nucleic Acids Res., 22:4673-4680.

Torres-Barceló, C. (2018). The disparate effects of bacteriophages on antibiotic-resistant bacteria. Emerg. Microbes Infect., 7(1):168.

Van Rossum, T.; P. Ferretti; O.M. Maistrenko and P. Bork (2020). Diversity within species: Interpreting strains in microbiomes. Nature Rev. Microbiol., 18(9):491-506.

Verma, T.; S. Tiwari; M. Tripathi and P.W. Ramteke (2019). Treatment and recycling of wastewater from tannery. Advances in Biological Treatment of Industrial Waste Water and Their Recycling for a Sustainable Future. Water Sci. Sci. Topic., 51-90.

Wu, B.; R. Wang and A.G. Fane (2017). The roles of bacteriophages in membrane-based water and wastewater treatment processes: A review. Water Res., 110:120-132.

Yamaki, S.; K. Yamazaki and Y. Kawai (2022). Broad host range bacteriophage, EscoHU1, infecting *Escherichia coli* O157: H7 and *Salmonella enterica*: Characterization, comparative genomics, and applications in food safety. Int. J. Food Microbiol., 372: 109680

Zheng, C.; L. Zhao; X. Zhou; Z. Fu and A. Li (2013). Treatment technologies for organic wastewater. Water Treatment, 11:250-286.

طريقة مبتكرة بإستخدام خليط من الفاجات متعددة الفاعلية لإدارة مياه الصرف الصحي والتحكم في مسببات الأمراض البكتيرية

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تتمثل خطط إدارة المياه بالطرق الحديثة خاصة في الدول التي تواجه نقصًا حادًا في موارد المياه مثل دول الشرق الأوسط في إعادة استخدام مياه الصرف الصحي المعالج للزراعة على أنها مصدر بديل ومهم لمياه الري. لذا تهدف الدراسة الحالية إلي إستخدام خليط من الفاجات الصديقة للبيئة والتي يمكنها تحليل وتقليل أعداد السلالات المتنوعة من بكتريا الإيشيريشيا كولاي والكوليفورم (بكتريا القولون) وأنواع بكتيرية أخري تندرج ضمن عائلة البكتريا المعوية *Enterobacteriaceae* في مياه نهر النيل وبعض مصبات المصارف الصحية. وأوضحت نتائج الدراسة أنه بالفحص المورفولوجي للفاجات المعزولة بإستخدام الميكروسكوب الإليكتروني النافذ وجود تشابة موروفولوجي بين هذه الفاجات الجديدة والتي سميت بفاجات MCn4 و MCn5 و MCn6 بتلك الموجودة في عائلات *Siphoviridae* و *Myoviridae* و *Podoviridae*. كما أكدت نتائج الدراسة قدرة الفاجات المعزولة علي تحلل السلالات المرجعية *Escherichia coli* ATCC® 11775 و *Escherichia coli* ATCC® strain 10536 بالإضافة إلي بكتريا القولون وقلت تركيزاتهم بشكل ملحوظ. وأوضحت نتائج تحاليل التتابعات الجينية والمعلوماتية الحيوية أن هناك ثلاث جينات وراثية بأحجام مختلفة للفاجات الجديدة حيث وصل حجم جين د ن أ البوليميراز حوالي 2565 زوج قاعدة ، وجين بروتين الذيل حوالي 672 زوج قاعدة و جين د ن أ بوليميراز المشارك حوالي 951 زوج قاعدة وتم تسجيل جميع التتابعات النيوكليوتيدية بـ بنك الجينات الدولي بالولايات المتحدة الأمريكية. وإعتمد

التحكم الحيوي بإستخدام الفاجات الجديدة المتعددة الفاعلية MCn4 و MCn5 و MCn6 خلال الدراسة علي كيفية إستهداف وإصابة أنواع وسلالات بكتيرية متنوعة خاصة المسببة للأمراض وأظهرت نتائج الدراسة أن هذه الفاجات لها القدرة الفائقة علي تحلل والحد من سلالات بكتريا *Salmonella typhi* و *Proteus vulgaris* و *Citrobacter freundii* مما يؤكد المدي العوائلي الواسع لها في الأوساط المائية. كما أن لخليط الفاجات الجديدة القدرة علي تحلل مجموعة بكتريا القولون وتقليل تركيزاتها خلال ساعتين من الخلط بهذا المزيج. تتخلص الدراسة إلي أن تطبيق إستخدام خليط من هذه الفاجات الجديدة للحد من تركيزات بكتريا القولون في مياه الصرف الصحي يمكن إعتباره بديلاً فعالاً من حيث التكلفة مقارنة بالتكاليف الهائلة والبنية التحتية لمحظة معالجة مياه الصرف الصحي ، بالإضافة إلي قدرتها الفائقة في التحكم في مسببات الأمراض البكتيرية المختلفة خاصة الممرضة خلال ساعات قليلة.