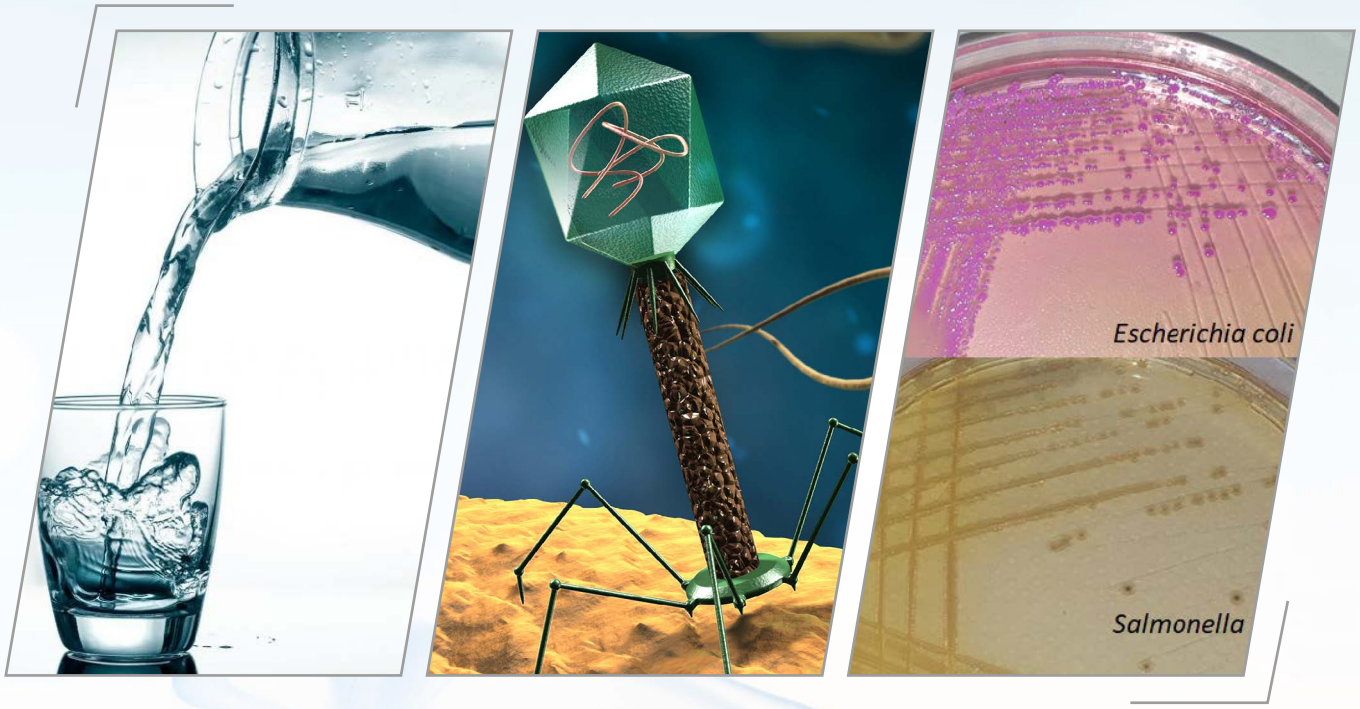
U.S.-Pakistan
Centers for Advanced Studies in Water

Isolation and Characterization of Antimicrobial Resistant Water Contaminant and Bacteriophage Remedy to Improve Water Quality

Final Report 2019



Principal Investigator:

Dr. Ayaz Ahmed, Dr. Panjwani Center for Molecular Medicine and Drug Research,
International Center for Chemical and Biological Sciences
University of Karachi, Pakistan

Co-Principal Investigator:

Dr. Adnan Khan, Department of Microbiology,
University of Karachi, Pakistan

MEHRAN UNIVERSITY
of Engineering & Technology
Jamshoro, Sindh, Pakistan

THE UNIVERSITY OF UTAH

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Authors

1. Dr. Ayaz Ahmed, Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Pakistan.
2. Dr. Adnan Khan, Department of Microbiology, University of Karachi, Pakistan.

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ACRONYMS AND ABBREVIATIONS

ATCC	American Type Culture Collection
ECP	Escherichia coli Phage
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended Spectrum Beta Lactamases
KWSB	Karachi Water and Sewerage Board
MDR	Multidrug Resistance
MOI	Multiplicity of Infection
NSDWQ	National Standards for Drinking Water Quality Quality for Pakistan
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
RAPD PCR	Random Amplified Polymorphic DNA Polymerase Chain Reaction
RO	Reverse Osmosis
SS Agar	Salmonella Shigella Agar
STP	Salmonella typhi Phage

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EXECUTIVE SUMMARY

Clean and safe water is among the basic rights of any community. Rapid urbanization, especially in Karachi city, poor sanitation system, and improper waste disposal are the key factors responsible for contaminated water related health issues. The use of unsafe water for drinking and cooking is responsible for more than 50,000 diarrheal related deaths annually. Among the diarrheal pathogens, multiple drug resistant *Escherichia coli*, *Salmonella typhi,* and other species are of prime importance. So, this situation demands appropriate measures, including easy and cheap methods to overcome this scenario. This research project was designed to evaluate the power of bacteriophage to treat diarrheal pathogens in vitro. In the first phase of this project, *E. coli* and *S. typhi* isolates were isolated from 18 main towns of Karachi. From these water sources, 100 bacterial isolates of multidrug-resistant (MDR) *E. coli* and 70 strains of *S. typhi* were isolated. These strains were characterized biochemically as well as genotypically using genus specific 16s rRNA, and also for the presence or absence of virulence factors. Of these, 4-5 highly virulent MDR strains of *E. coli* and *S. typhi* were used for phage isolation and characterization. Phages were isolated from the sewage samples collected from sewage water treatment plants. Out of 10 phages isolated for each bacterium, 3 were selected on the basis of their broad spectrum infectivity against selected 4 highly virulent species. Isolated phages for *E. coli* belonged to podoviridae and saphroviridae families, whereas, phages for *S. typhi* belonged to podoviridae and myoviridae families. These phages have great infectivity potential against highly virulent strains of respective pathogens with high plague forming units, reduced multiplicity of infection, pH, and thermal stability. Out of different combinations, cocktail of podoviridae (*E. coli*) and myoviridae (*S. typhi*) reduced the log colony forming units of bacteria in the presence of sludge suggesting that this cocktail might be active in natural scenario. So, this phage cocktail will be tested further in different animal model studies and activated sludge to further validate it efficacies.

1. INTRODUCTION

1.1 Background and Rationale

Clean and safe water is an essential constituent of life and an indicator for individual good health. Due to poor water governance and management, and sewage mixing in water bodies in Pakistan, the situation concerning the supply of hygienic and safe water is pathetic (Daud *et al*., 2017). The majority of the drinking water resources in urban and rural communities are fecally contaminated and have claimed millions of endemic deaths related to diarrhea alone (GBD, 2016; GBD, 2018). In Pakistan, the reasons for such outbreaks at the mass level are primarily due to unhygienic water and lack of public awareness. It is reported that contaminated water is responsible for 20-30% of all hospital cases and 60% infant death below the age of five (DD Report, 2016; WHO, 2007). As such, water is unsafe for drinking in Karachi, Sukker, Hyderabad, and Badin cities as per the World Health Organization (WHO) and National Standards for Drinking Water Quality for Pakistan (NSDWQ) (NSDWQ, 2008; WHO, 2011; Aziz *et al*., 2013). The World Wildlife Fund (WWF) also highlighted that water is responsible for substantial economic and human losses in Pakistan as 20-40% of hospital beds are occupied by patients suffering from water-related diseases such as typhoid, cholera, dysentery, and diarrhea which are responsible for one-third of all deaths (WWF, 2007).

Karachi, one of the metropolitan cities of Pakistan, is also facing contaminated water issues due to poor infrastructure and slum areas. The majority of the installed filtration plants are not enough to meet the criteria to make the water clean. About 30,000 individuals, mainly children, die annually in Karachi due to contaminated water (Yousuf *et al*., 2014; Eid *et al*., 2017). Groundwater supply is the preliminary source of water distribution in the majority of the cities of Pakistan, which contains an array of pathogenic microbes claiming a number of morbidity and mortality by consuming contaminated water. (Daud *et al*., 2017). Furthermore, water shortage is a major concern of Karachi city, both in developed or slum areas. People are forced to buy water from tanker mafia or other suppliers.

The majority of the population suffers from typhoid, dysentery, and diarrhoea due to contaminated water drinking. A study in the city of Khairpur of Sindh Province showed the presence of 73.83% total coliform and 45% fecal coliform among 768 collected drinking water samples (Shar *et al*., 2008; Shar *et al*., 2010). Another study showed the presence of 100% total and fecal coliform in the water samples of main reservoirs and tap waters (Shar *et al*., 2008). This scenario is more or less similar to all other major cities of Pakistan including Peshawar, Lahore and Karachi (Zahoorullah *et al*., 2003; Anwar *et al*., 2004; Sarwar *et al*., 2004; Hussain *et al*., 2007; Mumtaz *et al*.,

2011). Different other surveys also highlighted the presence of total fecal coliform, *Escherichia coli*, *Salmonella typhi,* etc. in the drinking water in Karachi and the other areas of Sindh due to cross-contamination between drinking water supply and sewage (Aamir *et al*., 2015; Syed *et al*., 2015; Mahar *et al*., 2018).

The Aga Khan University reported the prevalence of multidrug-resistant typhoid from the water samples taken from different areas of Karachi (http://www.coalitionagainsttyphoid.org/aga-khan-university-team-investigates-outbreak-of-antibiotic-resistant-typhoid-fever-in-hyderabad/). Beside Pakistan, these diarrheal pathogens are responsible for various outbreaks globally The water samples from Orangi Town, Karachi, showed the presence of sulfates far exceeding the WHO limits. Microbial examination revealed the presence of fecal coliform bacteria, especially *E. coli* and *S. typhi,* indicating sewage contaminations (Daud *et al*., 2017). Water samples from the Gulshan-e-Iqbal area of Karachi also showed sewage contamination in the majority of the water samples (Daud *et al*., 2017). During October 2004, a typhoid outbreak was reported near Karachi, which affected 300 people with three mortalities within one week. This outbreak was linked to contaminated water from a reservoir, the only source of drinking water in the village (Farooqui *et al*., 2009).

The lack of proper sewage treatment and related contamination of drinking water made different cities of Pakistan, including Karachi, vulnerable to mortalities and morbidities (Ahmed *et al*., 2006). Various other studies have also highlighted the presence of sewage contamination due to the presence of fecal coliform, *Escherichia coli, Salmonella typhi, Vibrio species,* etc. (Mahar *et al*., 2018; Qamar *et al*., 2018; Muhammad *et al*., 2019). So, this drastic scenario demands some strategies to overcome waterborne diarrheal infections.

Considering the local emergence of *E. coli* and *Salmonella typhi* related waterborne illnesses, there is a need to find alternative measures to overcome these pathogens. Since the discovery of the first magic drug penicillin, Fleming warned the world that its misuse would ultimately lead to the development of resistance among bacterial species (Fleming, 1945). In the 21st century, the emerging resistance among bacterial species has outnumbered the discovery of new active antibiotics due to cost, lengthy clinical trials, and failure in clinical trials due to inefficacy and toxicity (Grenni *et al*., 2018). The majority of the active antimicrobials have failed in clinical trials. Hence, the bacteriophages due to their abundance in nature and selectivity provide the hope to treat the multidrug-resistant (MDR) water pathogens (Jeon *et al*., 2019).

Bacteriophage therapy has been used before, but after Fleming's discovery of penicillin, scientists shifted their research direction towards searching new pharmacophores (Lai *et al*., 2016). Bacteriophage lyses bacteria as a part of its life cycle. Phages are self-

replicating and only infect specific bacteria without hurting the normal microflora (Galtier *et al*., 2017; Domingo-Calap and Delgado-Martínez, 2018). Bacteriophages due to their high specificity can be used as special bullets and are nontoxic to eukaryotic cells. The emerging antimicrobial threats force researchers to revisit phage therapy. Different studies have shown the reduction of food and waterborne isolated *Escherichia coli* and *Salmonella typhi* by treating with phages individually or in cocktails (Bolocan *et al*., 2016; Jung *et al*., 2017; Nabil *et al*., 2018; Huang *et al*., 2018; Ramirez *et al*., 2018; Cieplak *et al*., 2018). So, this study was planned to isolate bacteriophages from sewage samples against fully characterized indigenous isolates of water contaminants and to prepare a phage cocktail effective against *E. coli* and *S. typhi* together.

2.1 Objectives

1. To isolate and characterize the diarrhea pathogens, i.e., *Escherichia coli, Salmonella typhi, and Shigella* species from contaminated water samples.
2. To isolate and characterize morphologically and genotypically the potential bacteriophage from the sewage water samples.
3. To determine their efficacy to reduce bacterial load in the artificial polluted and contaminated water in the lab facility.
4. To formulate a bacteriophage cocktail to destroy all the four diarrheal pathogens as one treatment.

2. MATERIALS AND METHODS

2.1 Phase-I: Isolation and Characterization of Microorganisms from Water Samples

2.1.1 Collection of water samples

For the isolation of potent bacteriophages, the bacterial contaminant of water sources needs to be isolated. Karachi Water and Sewerage Board (KSWB) is responsible for providing drinking water to the whole vicinity of Karachi through bulk conveyance system comprised of a complex network of canals, conduits, siphons, multistage pumping and filtration. In total Karachi is divided into 18 main towns, which further consist of union councils (n=178). So, a total of 10–15 samples were collected from each town of Karachi as recommended in WHO guidelines (Fig. 2.1). The water samples were treated within 6-24 hrs after collection. The properties of water samples such as turbidity, pH etc. were noted.

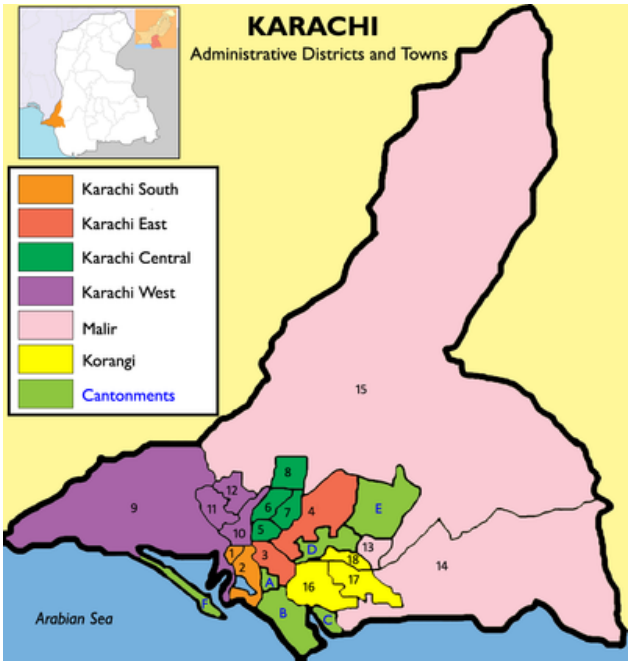

Fig. 2.1 Town-wise water sampling of Karachi city

2.1.2 Laboratory assessment of water samples

The microbial fecal contamination in drinking water supplies will be evaluated by determining bacterial load using standard methods as recommended in WHO guidelines. Standard plate count method was used to detect total aerobic count, followed by the detection of coliform and fecal coliform analysis by using the most probable number (MPN) and membrane filtration method. Bacteriological media used to detect total bacterial load comprised of Nutrient agar, MacConkey's agar, Salmonella Shigella (SS) agar, eosin methylene blue agar, and 5% sheep blood agar (Mahar *et al*., 2018).

2.1.3 Bacterial strains identification and characterization

Identification of the isolated microorganism is crucial to understand its prevalence at a particular drinking water site and for devising a proper treatment plan. The water samples were evaluated for the isolation and characterization of *Escherichia coli, Vibrio cholera, Salmonella typhi* and *Shigella* species biochemically as well genotypically by using 16s rRNA specific PCR. The bacterial isolates were cultured and maintained as recently reported by Ali *et al*. (2017) and Ahmed *et al*. (2016). The isolated species were further characterized on the following basis:

2.1.4 Antibiotic susceptibility testing and ESBL detection

The spread of antimicrobial resistance among microbes is a global concern. The prevalence of antimicrobial resistance among the isolated species of *Escherichia coli, Vibrio cholera,* and *Salmonella typhi,* was evaluated by Kirby Bauer Disk Diffusion method (National Committee for Clinical Laboratory Standards, 1997). The antibiogram was evaluated by using Mueller Hinton agar against ampicillin, ampicillin-sulbactam, amoxicillin-clavulanate, streptomycin and kanamycin, tetracycline, chloramphenicol, nalidixic acid, cefoperazone, cefotaxime, ceftazidime and aztreonam. These isolates were further characterized for extended-spectrum beta-lactamases (ESBL) production by using the double-disk synergy test as previously described (Ali *et al*., 2017).

2.1.5 Virulence typing

After complete genetic characterization and antimicrobial sensitivity pattern, the isolated microorganisms were screened for the degree of virulence based on biofilm formation, hemolysin production, relative quantification of total and reducing exopolymeric substances and motility (Yun *et al*., 2014; Ali *et al*., 2017; Liaquat *et al*., 2018). The strains were further genotypically characterized for various other virulence factors such as adhesion, biofilm formation, toxin and siderophore production, capsule, fimbriae and antimicrobial genes by using gene-specific primers (Table 2.1).

Table 2.1: List of primers

Bacteria	Gene	Sequence (5'----------> 3')
Escherichia coli	*papA*	F: ATGGCAGTGGTGTTTTGGTG R: CGTCCCACCATACGTGCTCTTC
	fimH	F: TGCAGAACGGATAAGCCGTGG R: GCAGTCACCTGCCCTCCGGTA
	iutA	F: GGCTGGACATCATGGGCCATGG R: CGTCGGGAACGGGTAGAATCG
	fyuA	F:TGATTAACCCCGCGACGGGAA R: CGCAGTAGGCACGATGTTGTA
	KpsMTII	F: GCGCATTTGCTGATACTGTTG R: CATCCAGACGATAAGCATGAGCA
	hlyA	F: AACAAGGATAAGCACTGTTCTGGCT R: ACCATATAAGCGGTCATTCCCGTCA
Salmonella typhi	*InvA*	F: CTGGCGGTGGGTTTTGTTGTCTTCTCTATT R: AGTTTCTCCCCCTCTTCATGCGTTACCC
	sitC	F: CAGTATATGCTCAACGCGATGTGGGTCTCC R: CGGGGCGAAAATAAAGGCTGTGATGAAC
	iron	F: ACTGGCACGGCTCGCTCTGTCGCTCTCT R: CGCTTTACCGCCGTTCTGCCACTGC
	tolC	F: TACCCAGGCGCAAAAAGAGGCTATC R: CCGCGTTATCCAGGTTGTTGC
	msgA	F: GCCAGGCGCACGCGAAATCATCC R: GCGACCAGCCACATATCAGCCTCTTCAAAC
	SopE	F: ACACACTTTCCACGAGGAAGCG R: GGATGCCTTCTGATGTTGACTGG
	prgH	F: GCCCGAGCAGCCTGAGAAGTTAGAAA R: TGAAATGAGCGCCCCTTGAGCCAGTC

2.2 Phase-II: Isolation and Characterization of Bacteriophage from Sewage Samples

2.2.1 Isolation, enrichment, and purification of bacteriophage

For isolation of bacteriophage, sewage samples were collected randomly from sewerage treatment plants of Karachi Water and Sewerage Board (KWSB: Site 1; SITE area: Site 2; Mehmoodabad: Site 3; Mauripur). Isolated *Escherichia coli, Vibrio cholera,* and *Salmonella typhi* were used to separate their respective lytic bacteriophages from sewage samples. Briefly, 20-100 ml of sewage sample were centrifuged to remove debris, and the resultant supernatant was supplemented with $CaCl_2$ (1 mmol L^{-1}). The supernatant was added to their bacterial host and incubated for 12h at 37°C with shaking (120 rpm). Then, the enrichment broths were centrifuged, pooled and passed through a 0.22 µm filter (Millipore, USA) to remove any bacterial contaminant. Bacteriophage suspension was stored in Luria-Bertani (LB) broth containing 50% glycerol at -80°C (Jamal *et al*., 2015; Huang *et al*., 2018).

2.2.2 Phage count or plaque forming unit (PFU)

For enumeration of the bacteriophage within a sample, the plaque-forming unit was determined by serial dilution of the bacteriophage suspension. Briefly, the phage suspension was serially diluted and incubated with their host bacterium (i.e. *Escherichia coli, Vibrio cholera, Salmonella typhi)* for 10-15 min. This suspension was mixed with soft agar and poured onto LB agar plate and incubated overnight at 37°C. Plaques were counted, and phage, which gave apparent clearance with a big ring of death, was scraped off from the top agar and added to phage buffer. This helped to isolate more specific and single phage, which was repeated further by using spot assay and PFU analysis to determine its infectious dosage (Huang *et al*., 2018).

2.2.3 Determination of latency time, burst size, bacterial reduction and multiplicity of infection

Selected bacteriophages were further evaluated for latency periods and burst sizes by one-step growth experiments using the protocol described earlier (Peng *et al*., 2018).

2.2.4 Thermal and pH stability of the phage

As sewerage water is treated with different concentrations of chemicals and temperature, our potent lytic phages must be tested for thermal and pH stability. The phage suspensions were, therefore, treated at different temperatures (ranging from 30-80°C) and pH (ranging from 1, 3, 5, 7, 9 and 11). After treatment for one hour, the phage viability was determined by using the plaque-forming unit (Sambrook *et al*., 1989; Khan Mirzaei, 2015).

2.2.5 Microscopy and genetic characterization

Bacteriophages with the great infectious ability for the selected three strains were visualized for their morphology by using transmission electron microscopy. Phage genomic DNA was isolated by using the phage DNA isolation kit and characterized by using RAPD PCR or complete genome sequencing at the ICCBS Genome Center.

2.2.6 In *Vitro* eradication of susceptible host biofilm

In the sewage or a drinking water treatment plant, biofilm-forming bacteria (such as *Escherichia coli, Vibrio cholera, Salmonella typhi)* produce biomass on the membrane resulting in its chocking. Besides, it also provides a ground for transferring resistance genes among its residing microorganisms. Furthermore, in-vitro biofilms were made by using isolated bacterium mentioned above and challenged with the isolated highest virulent phages. It helped to determine biofilm inhibitory as well as eradication potential of isolated bacteriophages.

2.3 Phase-III: Bacteriophage Cocktail to Treat Multiple Species Contamination

2.3.1 Cocktail preparation and its efficacy

Bacteriophage cocktails were prepared by using different concentrations of each isolated potent phage to evaluate its effectiveness in a mixture to control multispecies level contamination. The efficacy of the phage cocktail was determined in vitro by testing on a mixed culture of the isolated pathogens. It was further tested in the presence of different ions, turbidity and pH to mimic the environmental niches.

2.3.2 Efficacy of bacteriophage cocktail to treat target bacteria in wastewater sludge

The effectiveness of the prepared bacteriophage cocktail was tested in wastewater sludge. Wastewater samples (2 liters) were collected from the KWSB sewerage treatment plant and placed into six 500 ml flasks, each containing 200 ml sample. These flasks were autoclaved to remove the bacterial contaminant. After autoclaving, the samples were cooled down to room temperature. Out of six flasks, 4 were inoculated with 10^8 cfu/ml of *Escherichia coli and Salmonella typhi (Mono specie);* whereas in remaining two flasks, both microorganisms were inoculated together at 10^8 cfu/ml (multispecies). Out of six, three flasks were inoculated with prepared bacteriophage cocktail and incubated in shaking incubator at 37^{o}C for 24 hrs. The samples were drawn from the flasks at 0, 12, 24, and 48 h and processed for microbiological evaluation. The calculated log cfu/ml was plotted against the respective time to observe a bacterial reduction in the presence of prepared bacteriophage cocktail.

2.3.3 Pharmacokinetic studies

Although bacteriophage are harmful to eukaryotes, yet the pharmacokinetics studies were performed in laboratory mice to rule out any toxicological effect of the prepared bacteriophage cocktail. The phages cocktail inoculums were administered intraperitoneally (i.p.) and intramuscular (i.m.) in mice and observed for 48 h for mortality. After 24 and 48 h, mice were dissected, and blood was collected in tubes containing sodium EDTA. Liver and spleen were fixed in 4% paraformaldehyde and observed further for any histopathological changes.

2.3.4 Statistical analysis

The statistical significance of the data was evaluated by using an appropriate statistical test such as t-test, one way ANOVA by using SPSS version 20 (IBM, USA).

3. RESULTS AND DISCUSSION

3.1 Isolation of *Escherichia coli* and *Salmonella typhi* from Water

Contaminated water poses a direct threat to the community. It is one of the crucial issues all over Pakistan due to waste disposals and mixing of sewage water with drinking water, besides overall negligence of authorities concerned with the supply of safe water. Estimates of different surveys suggest a high burden of diarrhea among children near Karachi or adjacent areas. Majority of childhood diarrheal diseases are attributed to unhygienic drinking water. Among various diarrheal pathogens, *Escherichia coli* and *Salmonella typhi* are more prominent. *Escherichia coli* is a Gram-negative pathogen, reportedly causing diarrhea and an indicator of fecal contamination in water (Shibata *et al*., 2004; Cabral *et al*., 2010; Gruber *et al*., 2014). On the other hand, *Salmonella typhi* is a human pathogen, responsible for causing systemic infections, especially fever and diarrhea. Various national and international studies highlighted the frequent isolation of these two pathogens from contaminated water sources.

The first phase of this study involved screening for the presence of *Escherichia coli*, *Salmonella typhi* and *Shigella* species in water samples collected from 18 different towns of Karachi. The isolation rates of *Escherichia coli, Salmonella typhi* and *Shigella* species were 96.7, 61.3, and 0.02%, respectively (Table 3.1). All the strains were identified phenotypically by pinpointed pink colonies (*E. coli*; MacConkeys Agar), black-centered colonies (*S. typhi*; SS agar) and colorless colonies (*Shigella*; SS agar). The strains were further characterized and confirmed using biochemical identification (RapID ONE panel; Thermo Scientific, USA) and genomic identification using 16s rRNA specific primers of three pathogens. As the isolation rate of *Shigella* species was too low, the remaining studies were centered towards *E. coli* and *S. typhi*.

3.2 Resistance Profile and Extended Spectrum *β* Lactamase Detection

The emerging resistance among bacterial isolates is a severe threat globally and reported to claim a major portion of mortality by 2050 (https://amr-review.org/). Antimicrobial resistance, especially in Pakistan, is on the rise due to self- and wrong medication. Fecal contamination of water due to improper wastewater treatment is the primary driver of resistance in microbes in the water (Shakoor *et al*., 2018). Out of 100 *E. coli* isolates, 60–70% were resistant to aminoglycosides (amikacin, streptomycin, and kanamycin), 70-90% to β lactam inhibitors (ampicillin and amoxycillin), and 20-60% cephalosporins (ceftriaxone, cefotaxime, ceftazidime). However, about 70% of strains were sensitive to nalidixic acid (quinolone) (Fig. 3.1A).

In case of *S. typhi,* out of 70 isolates, 70-80% were resistant to aminoglycosides (amikacin, streptomycin and kanamycin), 50-70% to β lactam inhibitors (ampicillin and amoxycillin), and 60-80% to cephalosporins (cefotaxime, ceftazidime). However, 80% of the isolates were sensitive to nalidixic acid (quinolone) and ceftriaxone (cephalosporins) (Fig. 3.1B).

Table 3.1: Town-wise water sample collection and number of target pathogens isolated from each of the samples

Towns of Karachi	No. of samples collected	Detection of target pathogens		
		Escherichia coli	***Salmonella typhi***	***Shigella* species**
Baldia	10	10	4	0
Bin Qasim	5	5	1	0
Gadap	5	5	1	1
Gulberg	10	10	5	0
Gulshan	10	9	3	0
Jamshed	10	8	7	0
Kimari	5	5	2	0
Korangi	10	10	9	0
Landhi	10	10	7	0
Liaquatabad	10	10	7	0
Lyari	5	5	3	1
Malir	5	5	4	0
New Karachi	10	10	6	0
North Nazimabad	10	8	5	0
Orangi	10	10	9	0
Saddar	10	10	8	0
Shah Faisal	10	10	6	0
SITE	5	5	5	1
Total number and (%)	**150**	**145 (96.7)**	**92 (61.3)**	**3 (0.02)**

These results are consistent with those of Shakoora *et al*. (2018) who also reported the high resistance to the third generation of cephalosporin among water isolates of *E. coli*. Similarly, Maryam *et al*. (2014) showed the high prevalence of ampicillin-resistant bacteria among the water samples from Lahore. Recently, Mahar and Mirani (2018) also showed the high burden of antimicrobial-resistant bacteria in the water samples collected from Hyderabad, Sindh. Other studies (Qamar *et al*., 2018; Muhammad *et al*., 2019) have reported the isolation of quinolone and cephalosporin-resistant *S. typhi* strains from Karachi and Hyderabad region, and the presence of multidrug-resistant

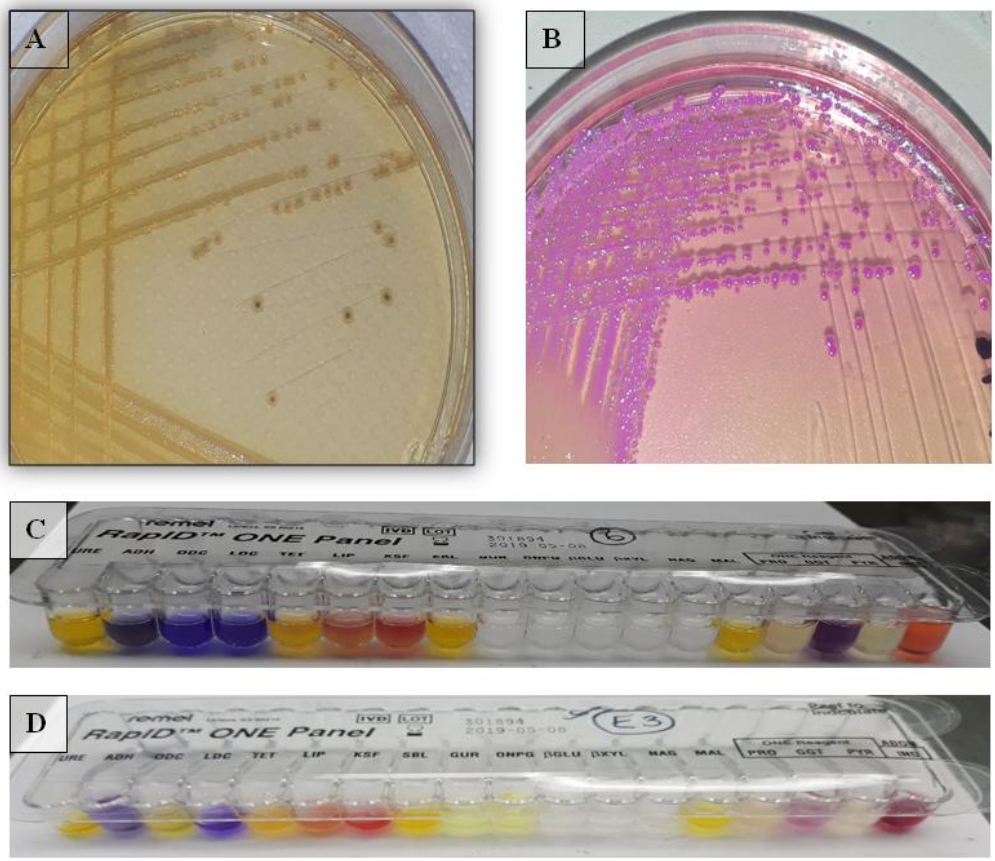

Fig. 3.1: Microbiological and biochemical identification of *Salmonella typhi* (A & C) and *Escherichia coli* (B & D)

(MDR) pathogens in water samples from different cities of Pakistan (Samra *et al*., 2009; Patoli *et al*., 2010; Shah and Zehra, 2014).

Among the isolated MDR *E. coli* and *S. typhi* isolates, 20% and 7%, respectively, were extended spectrum β lactamases (Fig. 3.2). The reason behind the microbial load in drinking water is improper storage, the opening of various water supply shops without observing any guidelines and quality protocols, water shortages, and improper treatment of water.

3.3 Virulence Typing

Bacterial pathogenic islands, usually acquired *via* horizontal transfer of genes among bacterial species, are responsible for the display of a variety of symptoms after the onset of disease. The genetic variation is an essential factor for virulence, and it is linked to disease-causing abilities of particular pathogens. Virulence profiling is a valuable tool to characterize pathogens by using gene-specific PCR. For *E. coli,* six genes, responsible for adhesion (*fimH* and *papA*), toxin (hlyA), iron acquisition (*fyuA* and *iutA*) and capsular protein (kpsMTII) were selected. Almost all the strains carried the *fimH* gene, whereas, only 20% of strains were positive for the *papA* gene. Out of 100, 34,

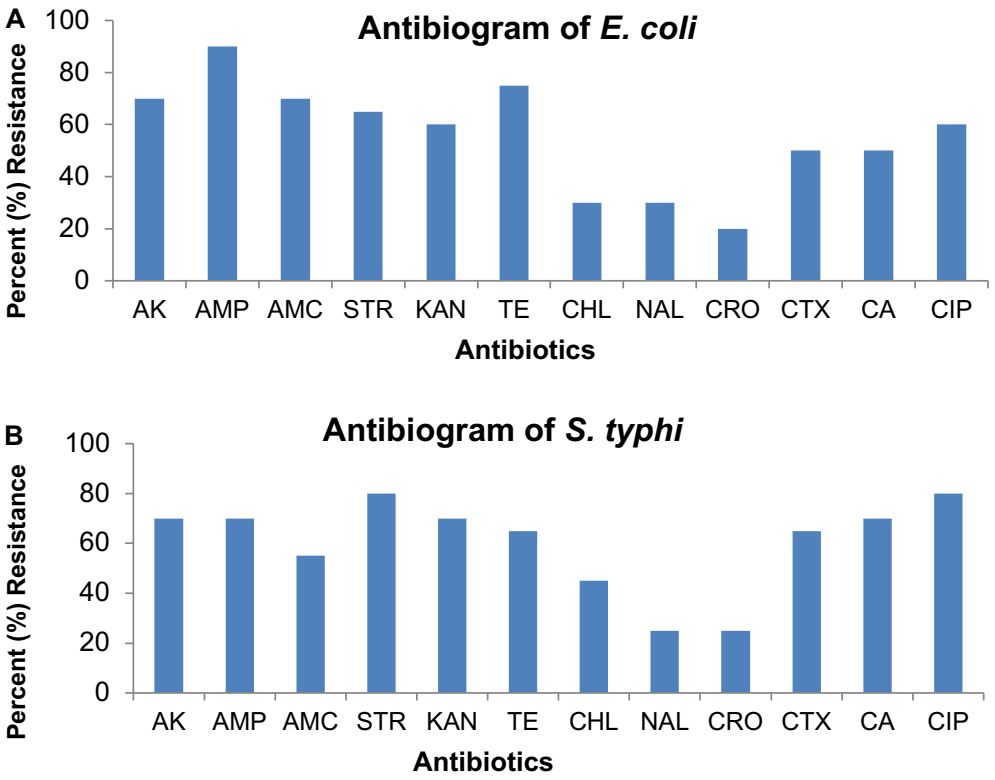

Fig. 3.2: Percent resistance profile of selected isolates: (A) Antibiogram of *Escherichia coli;* n=100 (B) Antibiogram of *Salmonella typhi;* n = 70. Antibiotics used: Amikacin (AK), Ampicillin (AMP), Amoxycillin – clavulanate (AMC), Streptomycin (STR), Kanamycin (KAN), Tetracyclin (TE), Chloramphenicol (CHL), Nalidixic Acid (NAL), Ceftriaxone (CRO), Cefotaxime (CTX), Ceftazidime (CA) and Ciprofloxacin (CIP)

52, 19 and 8% strains harbored *hlyA*, *fyuA, iutA* and *kpsMTII* genes, respectively (Fig. 3.3). Overall, 25% of the isolates carried a majority of the genes, suggesting that they might be highly virulent as they possess adherence property to host epithelium, toxin production to cause diarrhea, siderophore production genes to establish in the host, and capsular protein to avoid host immune response.

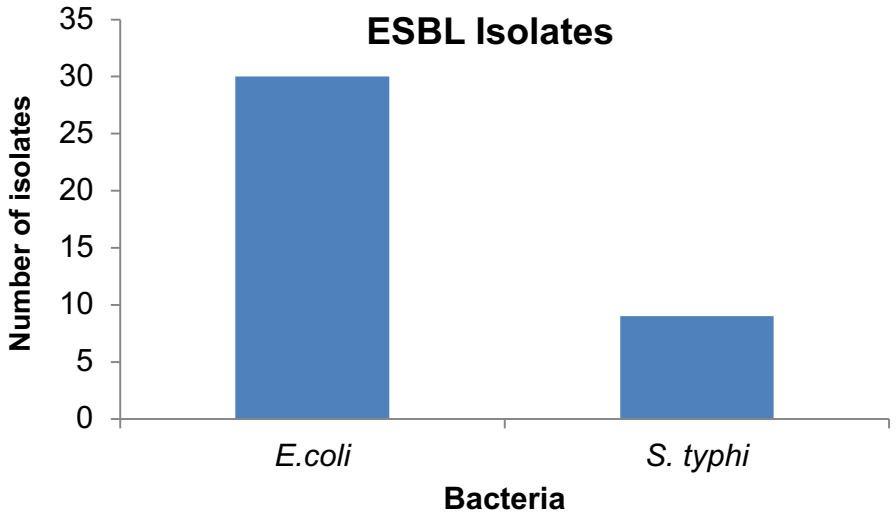

Fig. 3.3: Number of extended spectrum β lactamases water isolates of *Escherichia coli* and *Salmonella typhi*

On the other hand, for virulence typing of *Salmonella typhi* strains, seven genes were selected that were responsible for invasion (*invA* and *prgH*), toxin production (*sopE*), iron acquisition (*sitC* and *iroN*), and intracellular survival (*tolC* and *msgA*). Almost all the strains were positive for *invA,* whereas, only 40% of the isolates were positive for *prgH* (Fig. 3.4). Out of 70, 41, 51, 30, 43, and 23% were positive, respectively for *sopE, sitC, iroN, tolC* and *msgA*. Almost 40% of the isolates harbored majority of the genes and might be highly virulent to cause diarrhea among people who consume such water.

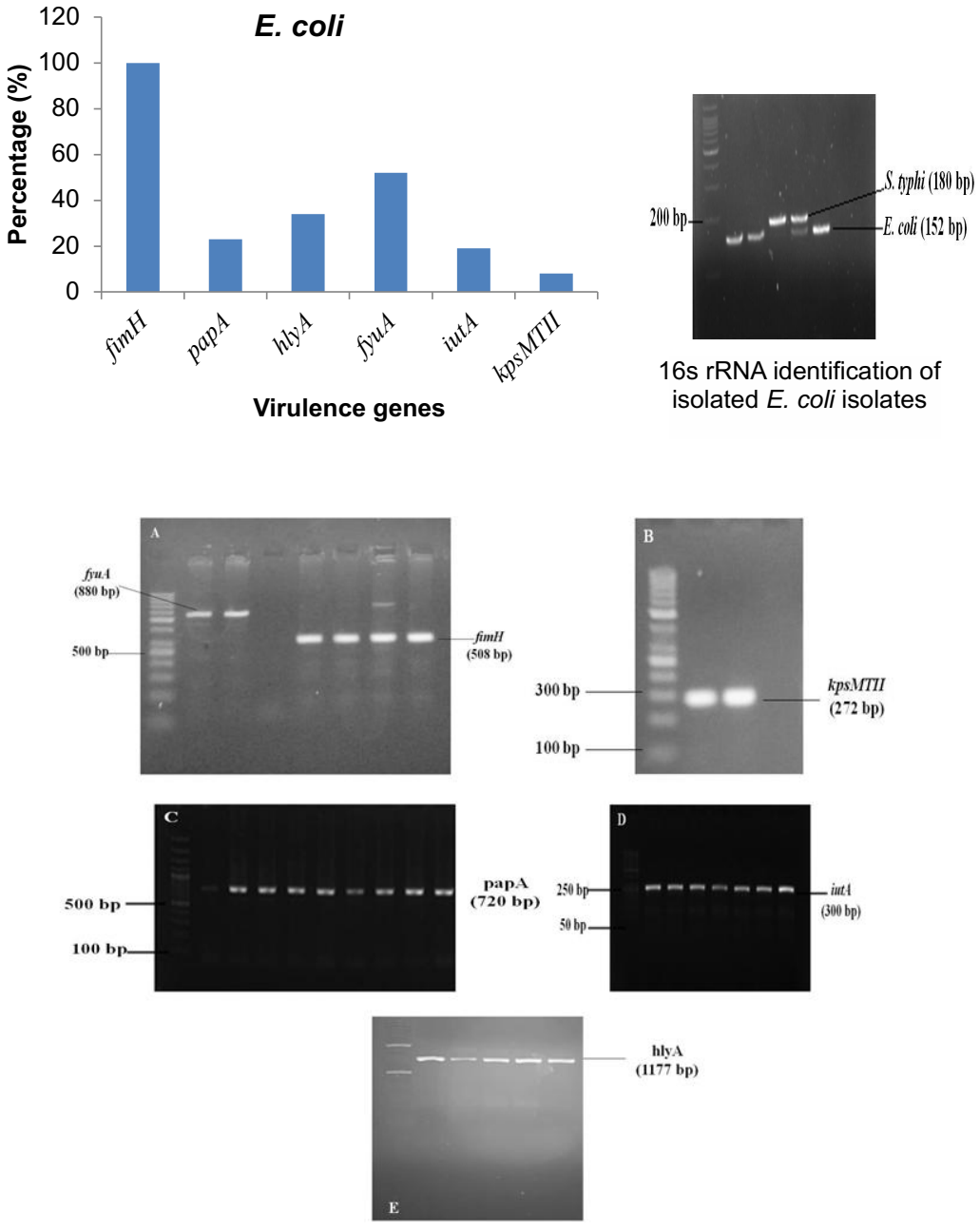

Fig. 3.4: Virulence genes profile of isolated *Escherichia coli* strains. Bar Graph showed the distribution of selected virulence marker. Gel electrophoresis images of virulence genes (A) *fimH* and *fyuA* (B) *kpsMTII* (C) *papA* (D) *intA* (E) *hlyA.*

3.4 Isolation of Bacteriophage to Treat *Escherichia coli* and *Salmonella typhi*

The second phase of the project involved the separation of bacteriophage to treat water isolates of *E. coli* and *S. typhi*. Bacteriophages are the pathogens which specifically kill bacterial cells. As a part of the lytic life cycle, these bacteriophages take over the charge of infected bacteria replication and translational machinery to produce new viral particles, thus killing the infected bacteria by bursting. Bacteriophages are the treatment of choice before the antibiotic era to cure an infection. They are so specific and do not affect eukaryotic or human cells or their processes (Woolsten *et al*., 2013).

For the isolation of bacteriophages, sewage samples were collected from three sewage treatment plants of Karachi. Standard isolates (ATCC isolates of *E. coli, S. typhi*) and environmental isolates mentioned in Fig. 3.5 were used as a host to isolate

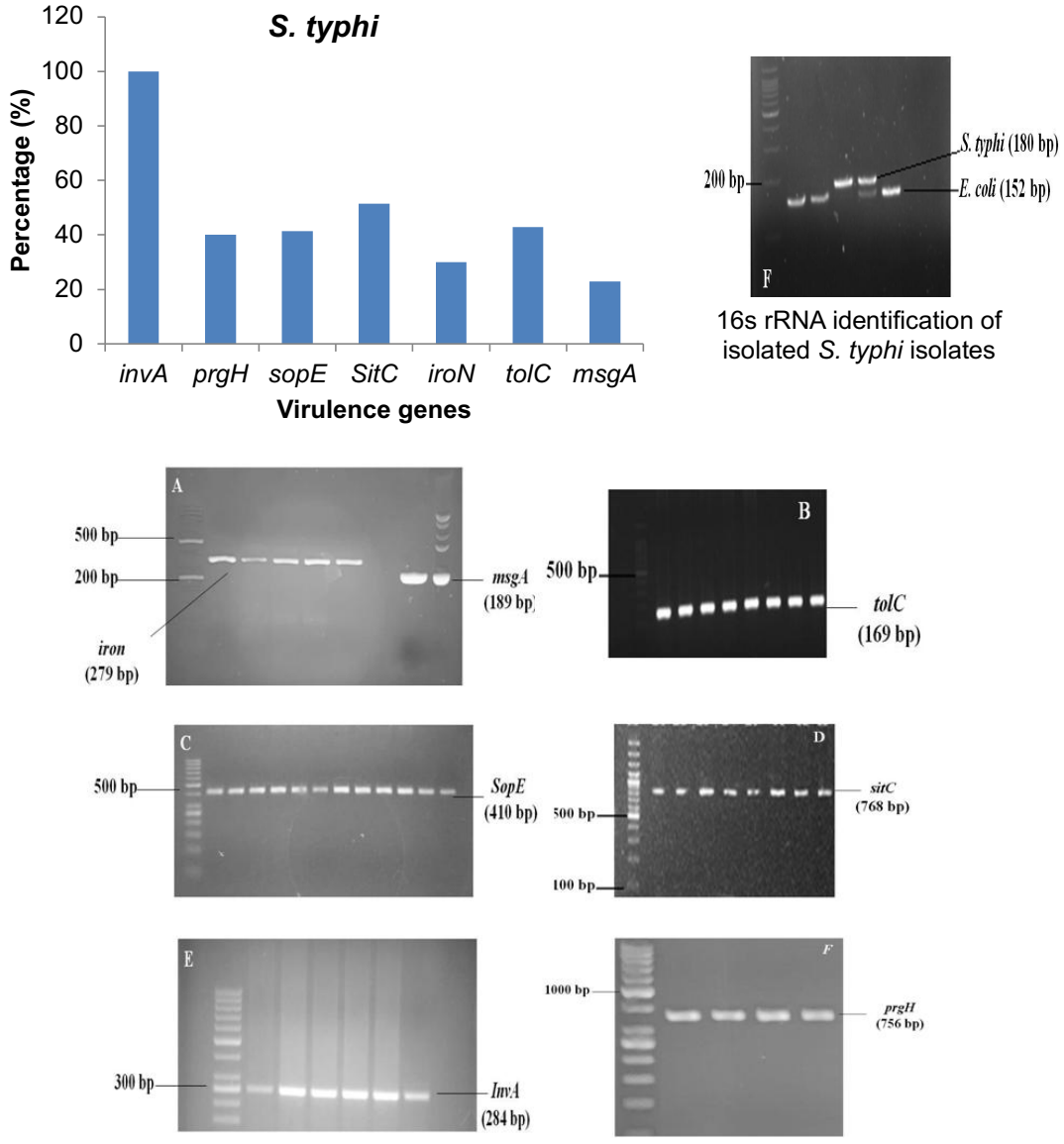

Fig. 3.5: Virulence genes profile of isolated *Salmonella typhi* strains. Bar Graph showed the distribution of selected virulence marker. Gel electrophoresis images of virulence genes (A) *msgA* and *iroN* (B) *tolC* (C) *spoE* (D) *sitC* (E) *invA* (F) *prgH.*

bacteriophage from sewage samples. From 30 different samples, ten bacteriophages each of *E. coli* and *S. typhi* were isolated and represented as *E. coli* (ECP) and *S. typhi* (STP) pages. Among the isolated phages, ECP 1, 5, 7, 8, and 9 were found to be potent with log plaque-forming unit (PFU) value more than 3 against the tested strains of *E. coli* (Fig. 3.6A). Whereas, STP 1, 4, and 5 were found to be more virulent against *S. typhi* strains, with log PFU value more than 3 (Fig. 3.6B). Considering the results, ECP 1, 7, and 9 for *E. coli* and STP 1, 4, and 5 for *S. typhi* were selected for further studies.

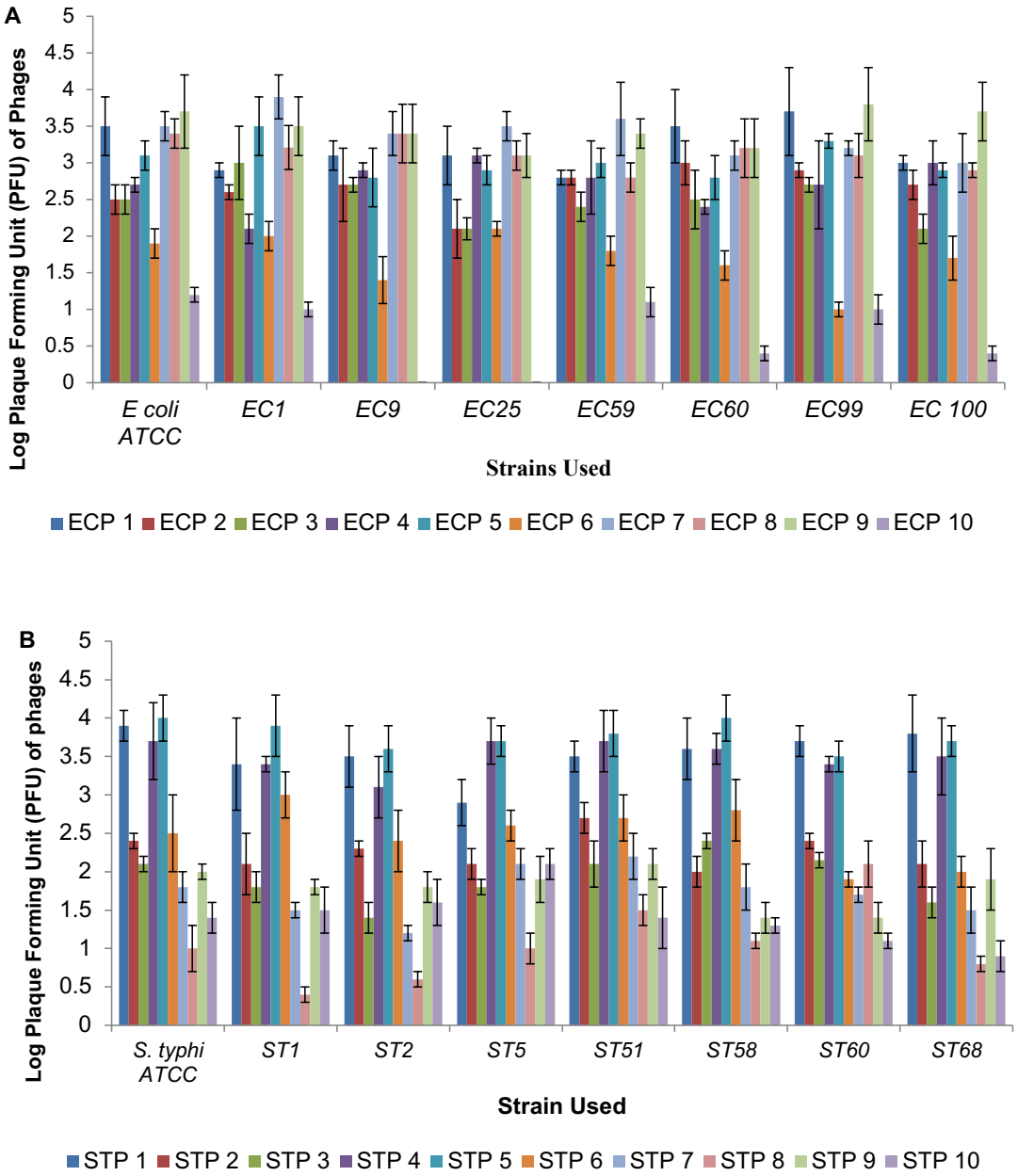

Fig. 3.6: Log Plaque Forming Units (PFU) of the isolated bacteriophages from sewage against (A) *Escherichia coli* and (B) *Salmonella typhi*

The time-kill assay was used to decipher the time point where these bacteriophages started to infect bacterial cells, thus reducing optical density related to bacterial growth. Isolated ECP1 started to infect some of the bacterial isolates even after 2h, whereas, it began to significantly reduce bacterial growth after 4h against all the tested *E. coli* isolates (Fig. 3.7A). Similarly, bacteriophage ECP7 started to inhibit bacterial growth significantly after 4h of incubation (Fig. 3.7B). On the other hand, the infectivity of ECP9 took more time, and it started to reduce significant bacterial growth after 6h (Fig. 3.7C).

In case of *S. typhi*, isolated bacteriophages STP 1, 4, and 5 were found to be equally virulent and started to significantly decrease the bacterial growth after 4h (Fig. 3.7 A, B, C). The phage resistance among the surviving bacteria was evaluated by spot analysis. It was noted that none of the bacteria had developed phage resistance during the time course of the experiment, as evident by the clear zone of inhibition. The multiplicity of infections is a term in phage therapy that refers to the number of phages added to bacteria, which significantly enhance adsorption and lysis of bacteria. It is essential to calculate the dosing of respective bacteriophages as it varies with the bacteriophage species and bacterial count.

Different MOIs have been used for experiment conducting, but in broth-based infection, assay ranges from 10 to 10^{-2} (Alves *et al*., 2016; Bett *et al*., 2016). So, we also used these concentrations to elucidate the MOIs of the isolated phages. The isolated bacteriophages for *E. coli,* ECP 1, 7, and 9, significantly reduced the growth of environmental isolates at 0.001, 0.01, 0.1, 1.0, and 10 MOIs, as evident by the reduced optical density of media as compared to control cells without phages (Fig. 3.8, 3.9 and 3.10). Similarly, for *S. typhi,* the isolated phages STP 1 and 4 significantly reduced the growth of environmental isolates at all the tested MOIs (Fig. 3.11 and 3.12). However, STP5 showed less reduction in bacterial growth as compared to STP 1 and 4 (Fig. 3.13).

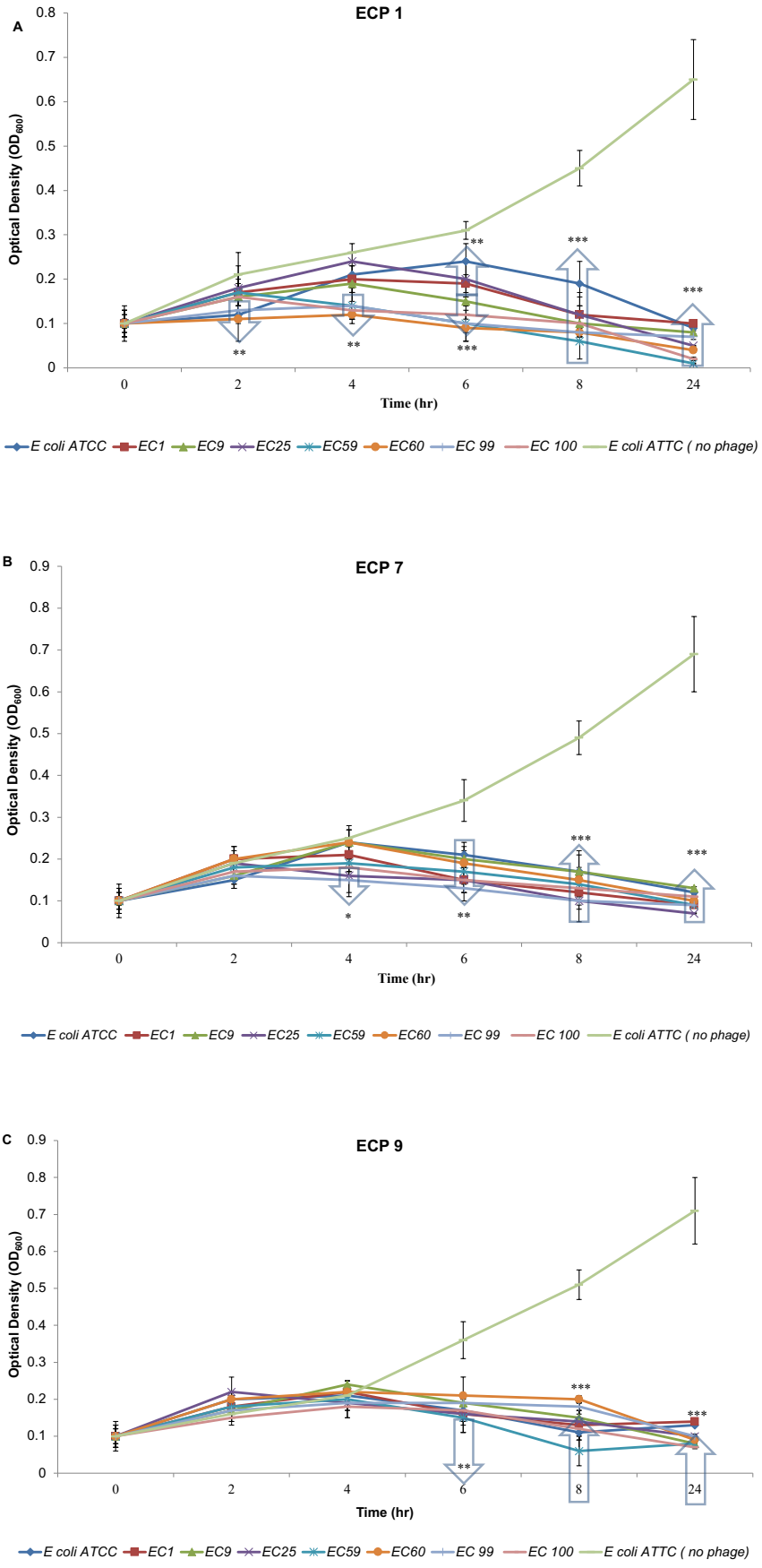

Fig. 3.7: Time Kill curve analysis of isolated *Escherichia coli* phages ECP1 (A), ECP7 (B) and ECP9 (C) against ATCC and environmental isolates of *E. coli.* Statistical significance of data was evaluated by using One-way ANOVA test and represented as **$p < 0.01$ and ***$p < 0.001$.

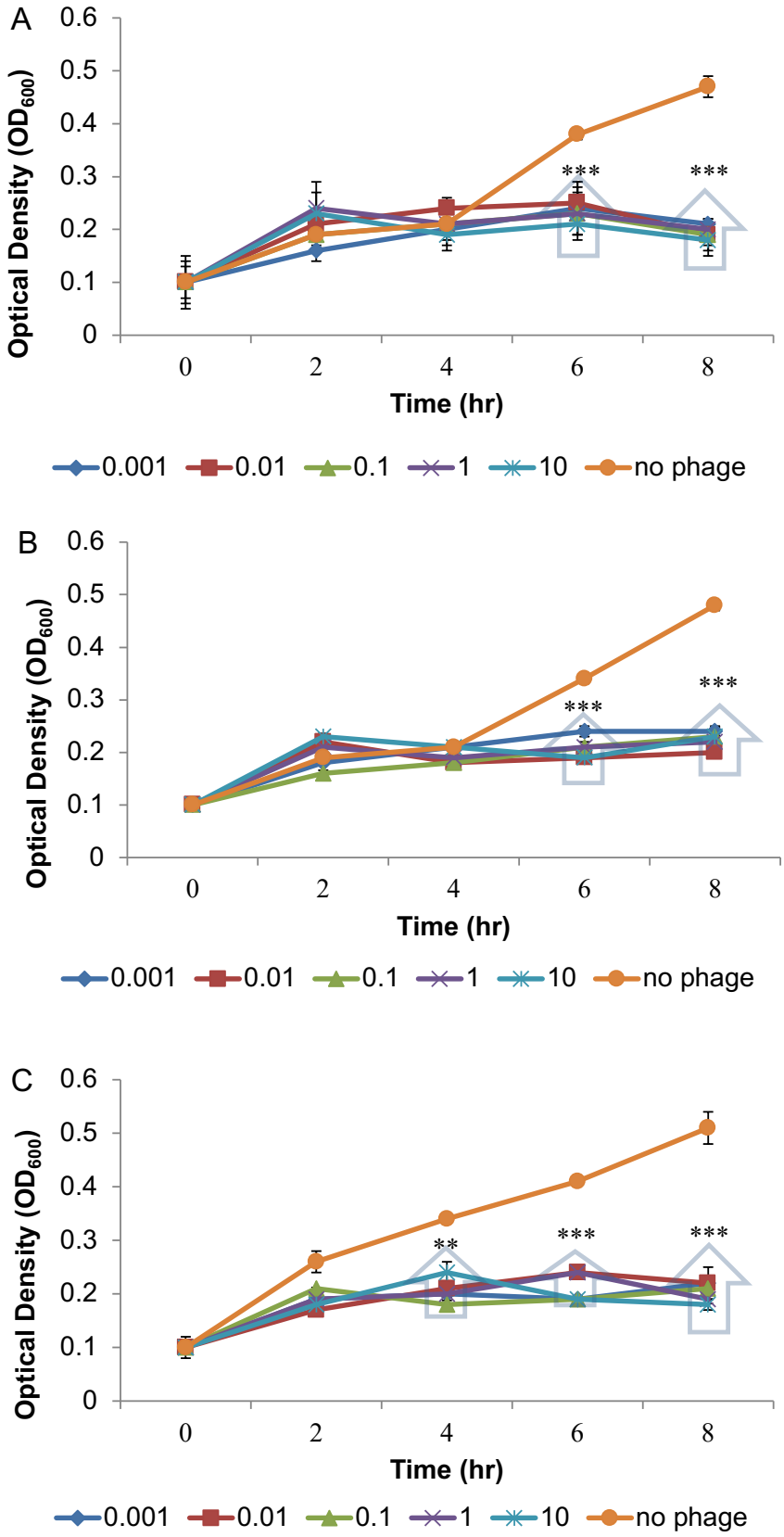

Fig. 3.8 **Effect of different Multiplicity of Infection (MOI) of isolated phage ECP1 for the reduction of water isolates of *Escherichia coli* (A) EC1 (B) EC59 (C) EC99. Statistical significance of data was evaluated by using One-way ANOVA test and represented as **$p < 0.01$ and ***$p < 0.001$.**

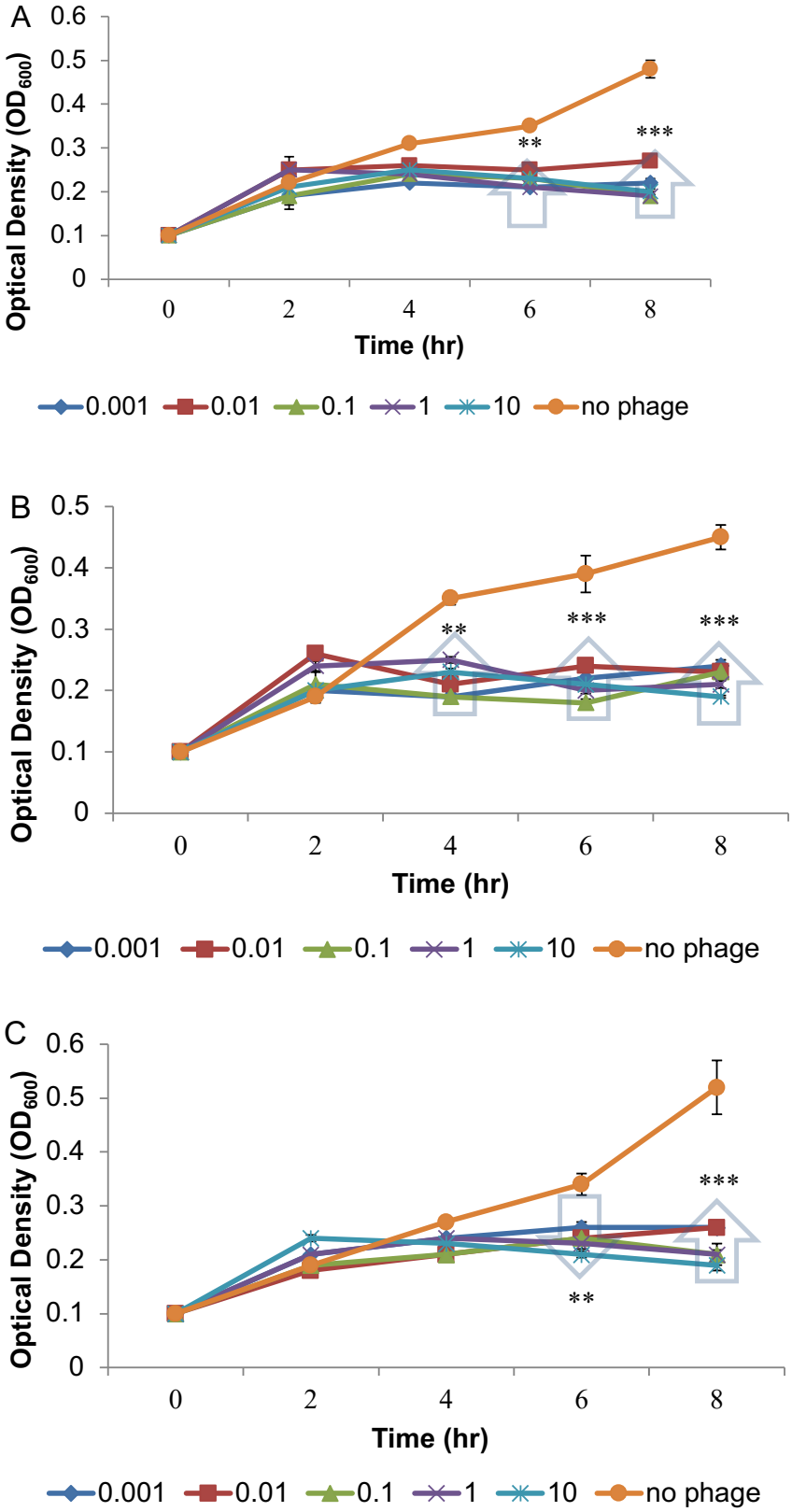

Fig. 3.9: **Effect of different Multiplicity of Infection (MOI) of isolated phage ECP7 for the reduction of water isolates of *Escherichia coli* (A) EC1 (B) EC59 (C) EC99. Statistical significance of data was evaluated by using One-way ANOVA test and represented as **$p < 0.01$ and ***$p < 0.001$.**

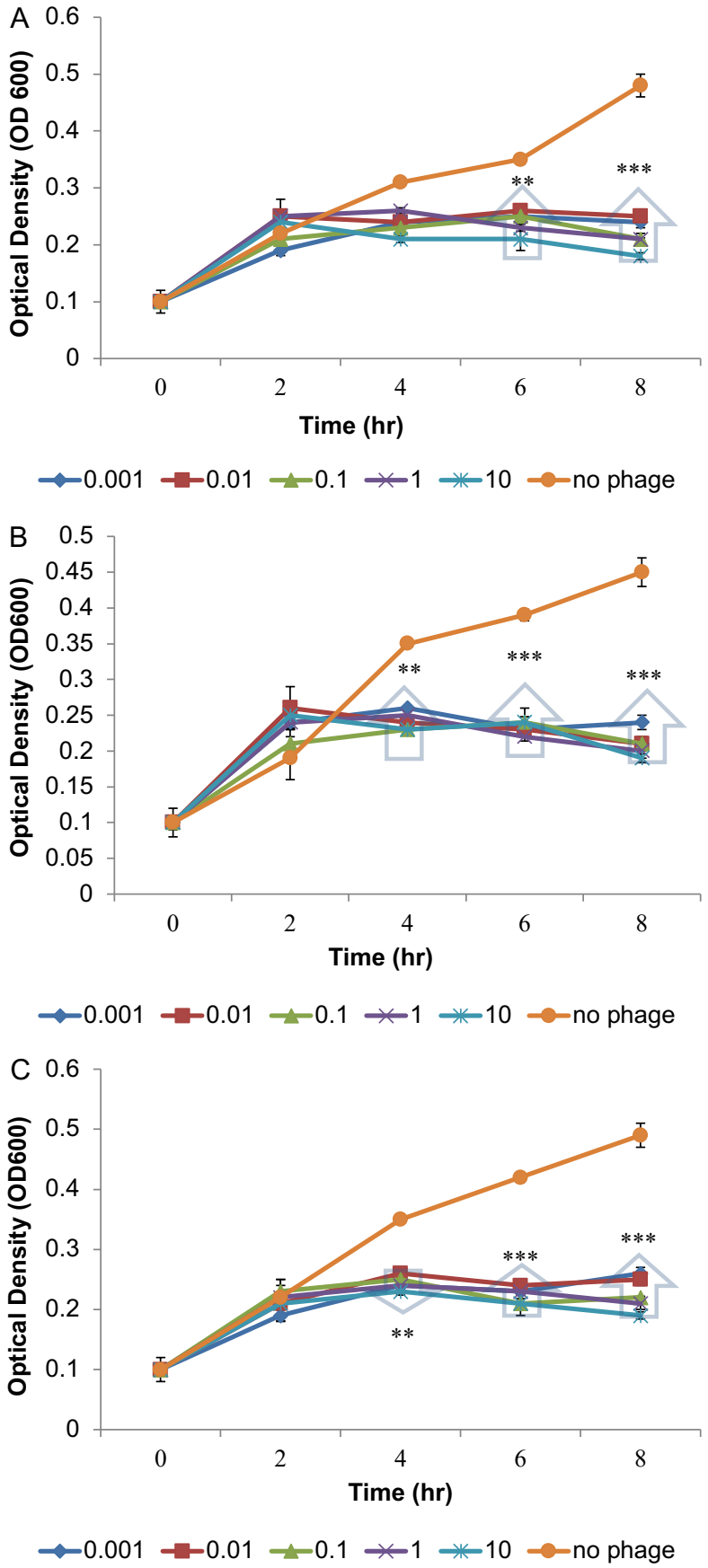

Fig. 3.10: Effect of different Multiplicity of Infection (MOI) of isolated phage ECP9 for the reduction of water isolates of *Escherichia coli* (A) EC1 (B) EC59 (C) EC99. Statistical significance of data was evaluated by using One-way ANOVA test and represented as **$p < 0.01$ and ***$p < 0.001$.

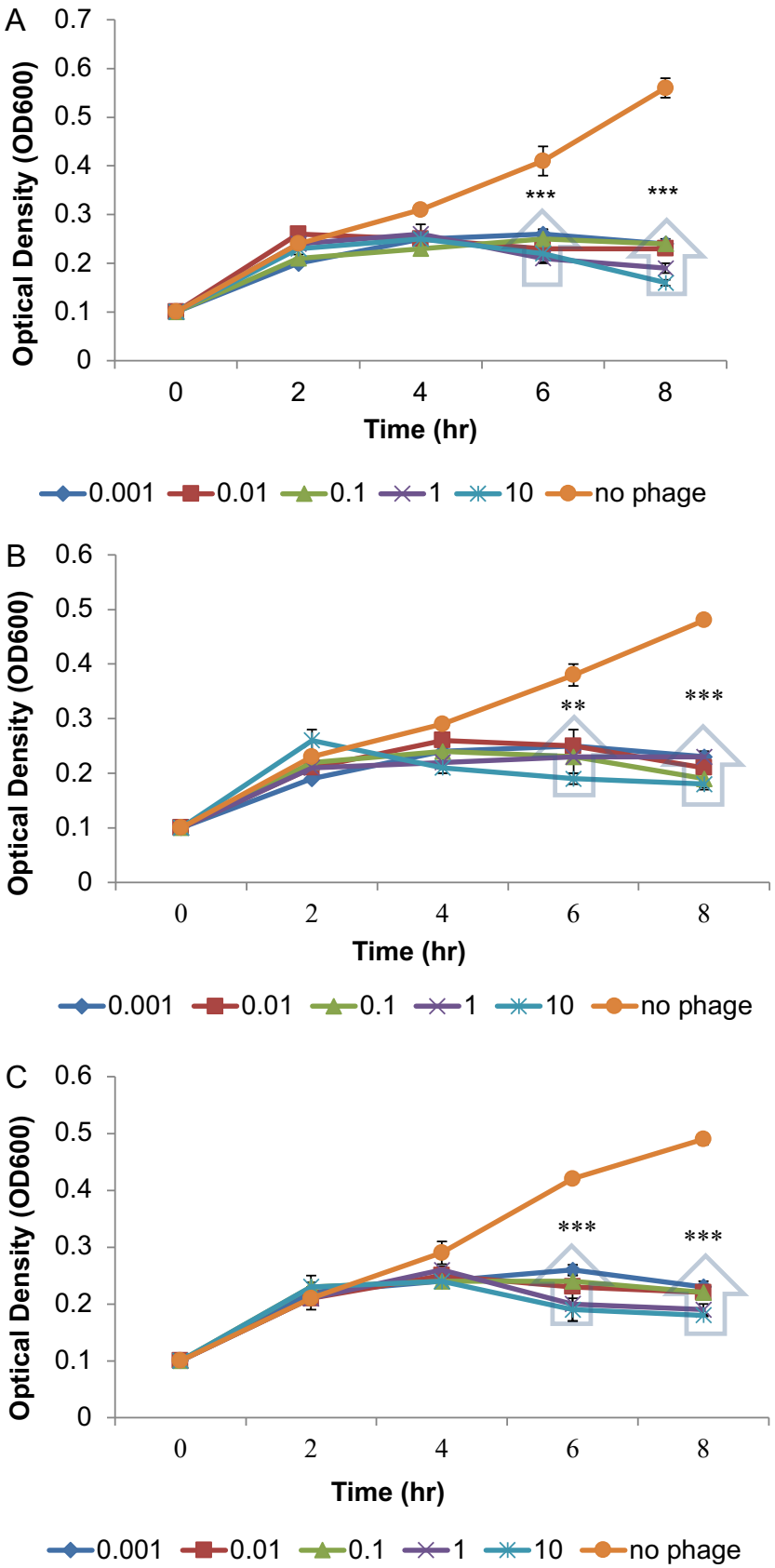

Fig. 3.11: Effect of different Multiplicity of Infection (MOI) of isolated phage STP1 for the reduction of water isolates of *Salmonella typhi* (A) ST5 (B) ST60 (C) ST68. Statistical significance of data was evaluated by using One-way ANOVA test and represented as **$p < 0.01$ and ***$p < 0.001$.

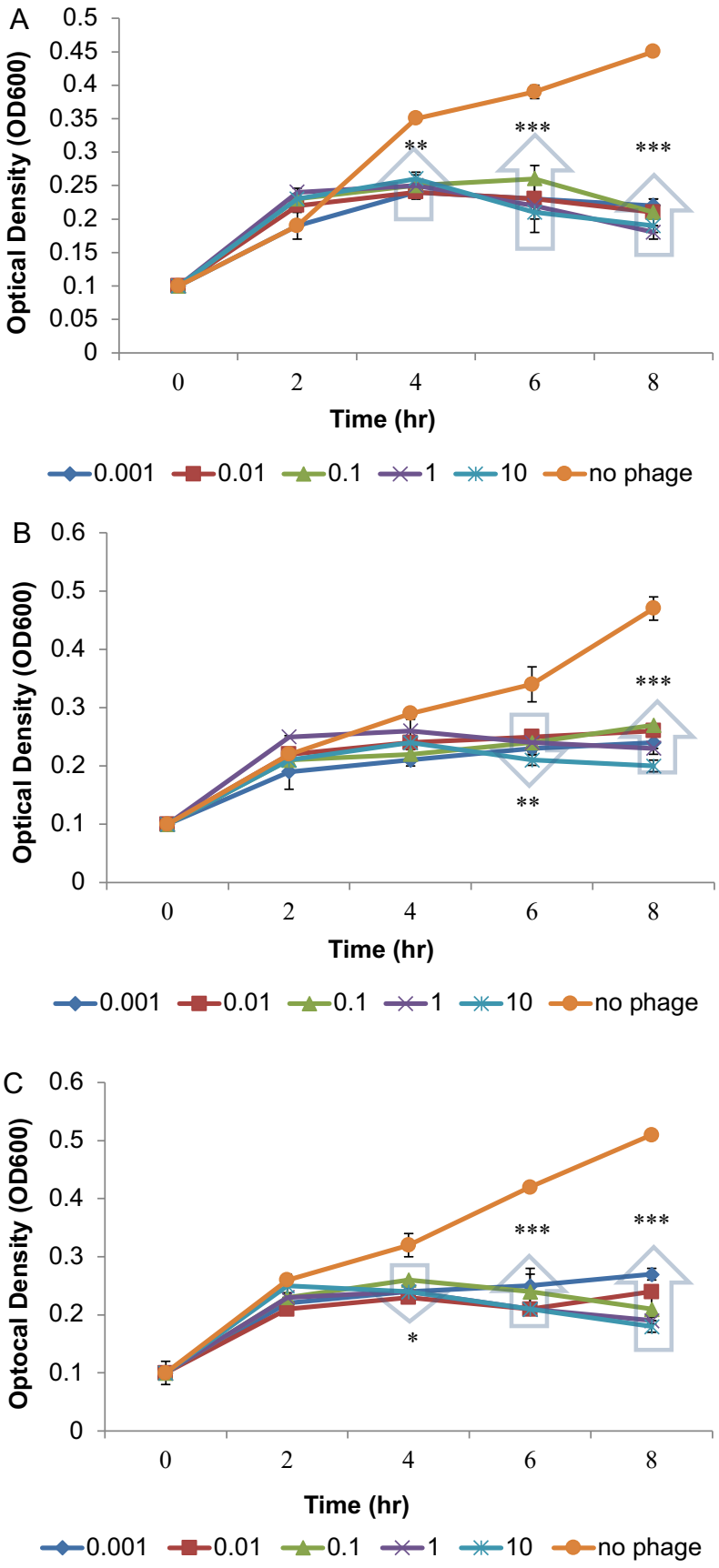

Fig. 3.12: Effect of different Multiplicity of Infection (MOI) of isolated phage STP4 for the reduction of water isolates of *Salmonella typhi* (A) ST5 (B) ST60 (C) ST68. Statistical significance of data was evaluated by using One-way ANOVA test and represented as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

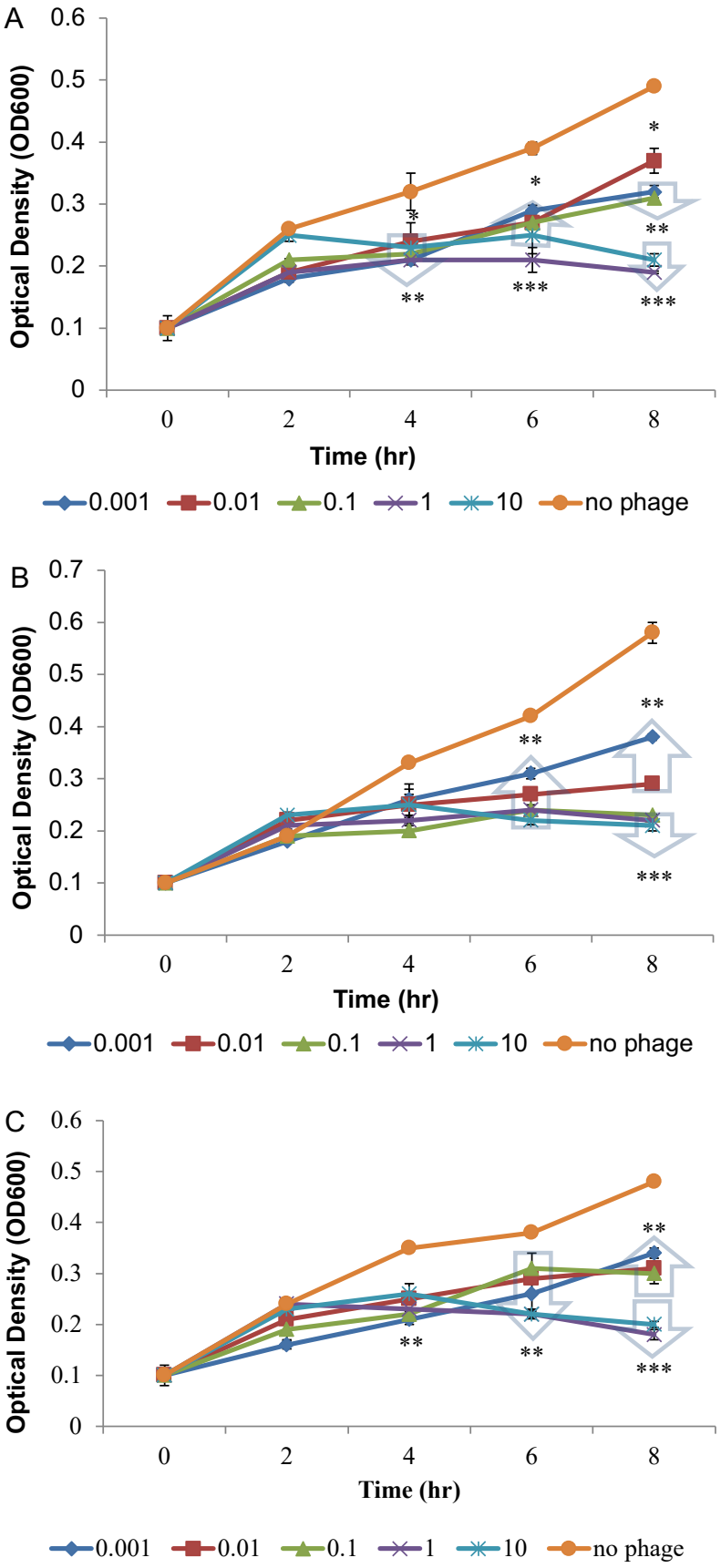

Fig. 3.13: Effect of different Multiplicity of Infection (MOI) of isolated phage STP5 for the reduction of water isolates of *Salmonella typhi* (A) ST5 (B) ST60 (C) ST68. Statistical significance of data was evaluated by using One-way ANOVA test and represented as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Different studies have shown that isolated phages give better results at MOI of 1 and 10 (Jung *et al*., 2017; Hunag *et al*., 2018; Peng *et al*., 2018). For phage preparation, two critical aspects need to be considered to calculate efficacy and dosage. The latent period is the time required for the phage to induce cell lysis. Second is the burst size which corresponds to the number of phages released after lysis of cells (Abedon *et al*., 2001). The latency time for the isolated phage ECP 1 and 7 was found to be 35 and 27 min, respectively, whereas their burst size was 212±15 and 189±11, respectively. The latency time for ECP9 was significantly higher as compared to ECP 1 and 7, with reduced burst size (Table 3.2).

Table 3.2: Latency time and burst size of selected bacteriophages for *E. coli* and *S. typhi*

Bacteriophages	Family	Latency time (min)	Burst size
Escherichia coli			
ECP1	Podoviridae	35	212 ± 15
ECP7	Saphoviridae	27	189 ± 11
ECP9	Podoviridae	55	136 ± 18
Salmonella typhi			
STP1	Podoviridae	25	195 ± 20
STP4	Myoviridae	34	175 ± 15
STP5	Myoviridae	70	147 ± 13

The *S. typhi* phages also have larger burst sizes (STP1 195±20; STP4 175±15) and reduced latency time (STP1 25 min; STP4 34 min) as compared to STP5 (latency time 70 min; burst size 147±13) (Table 3.2). Similarly, the isolated phages in another study showed reduced latency period, i.e., 45 min with high burst size (Svab *et al*., 2018). Petsong *et al*. (2019) also reported enhanced burst size with a reduced latent period for the phase isolated for *S. typhi*.

Genome sequencing study showed that three (ECP 1 and 9 and STP1) of the isolated phages belonged to *podoviridae,* two (STP4 and 5) to *myoviridae* and one (ECP7) to *saphroviridae* family. Unfortunately, due to the unavailability of a functional electron microscope, the actual picture of the bacteriophages could not be taken. Based on the literature, page belonging to *podoviridae* family possesses icosahedral capsid with a small non-contractile tail (Hamdi *et al*., 2016). Bacteriophage in the *myoviridae* family has elongated heads and tail with long fibers (Comeau *et al*., 2012). Whereas, phage belonging to *saphroviridae* family has icosahedral capsid with a long tail and short fibers (Kupczok *et al*., 2018).

3.5 Thermal and pH stability

During water treatment, different chemicals are used which are responsible for fluctuating pH as well as various temperatures are used. So, the thermal and pH stability of the isolated phages were also evaluated. The *E. coli* phages, ECP 1 and 7, were found to be more stable at pH ranges 3–11 (Fig. 3.14A). ECP9 was found to be stable at pH 3-9 but has significantly reduced percent plaque-forming units as compared to ECP 1 and 7 (Fig. 3.14A). Furthermore, ECP 1 and 7 were resistant to the temperature flux from 35 to 75°C with plaque-forming units >70% whereas ECP 9 was less flexible to heat than ECP 1 and 7 (Fig. 3.14B). Isolated *S. typhi* phages STP 1 and 4 are more resilient to pH in the range of 3-11 with >70% plaque-forming units whereas, STP5 phage is less pH flexible as compared to the other two (Fig. 3.15A). STP 1 and 4 are significantly more heat-stable than STP5 (Fig. 3.15B).

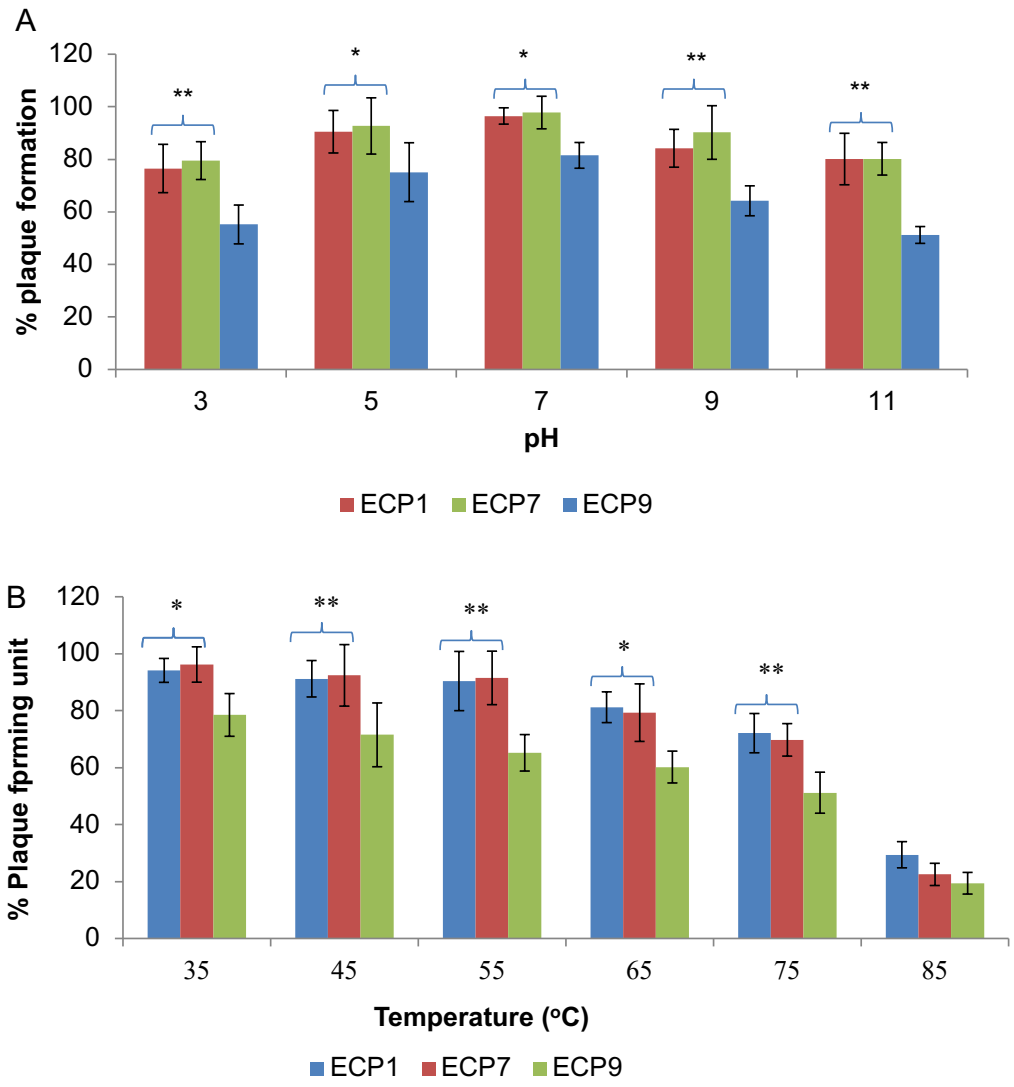

Fig. 3.14 Stability of isolated *Escherichia coli* bacteriophages ECP 1, 7 and 9. (A) pH stability (B) Thermal stability. Statistical significance of data was evaluated by using One-way ANOVA test and represented as *$p < 0.05$ and **$p < 0.01$.

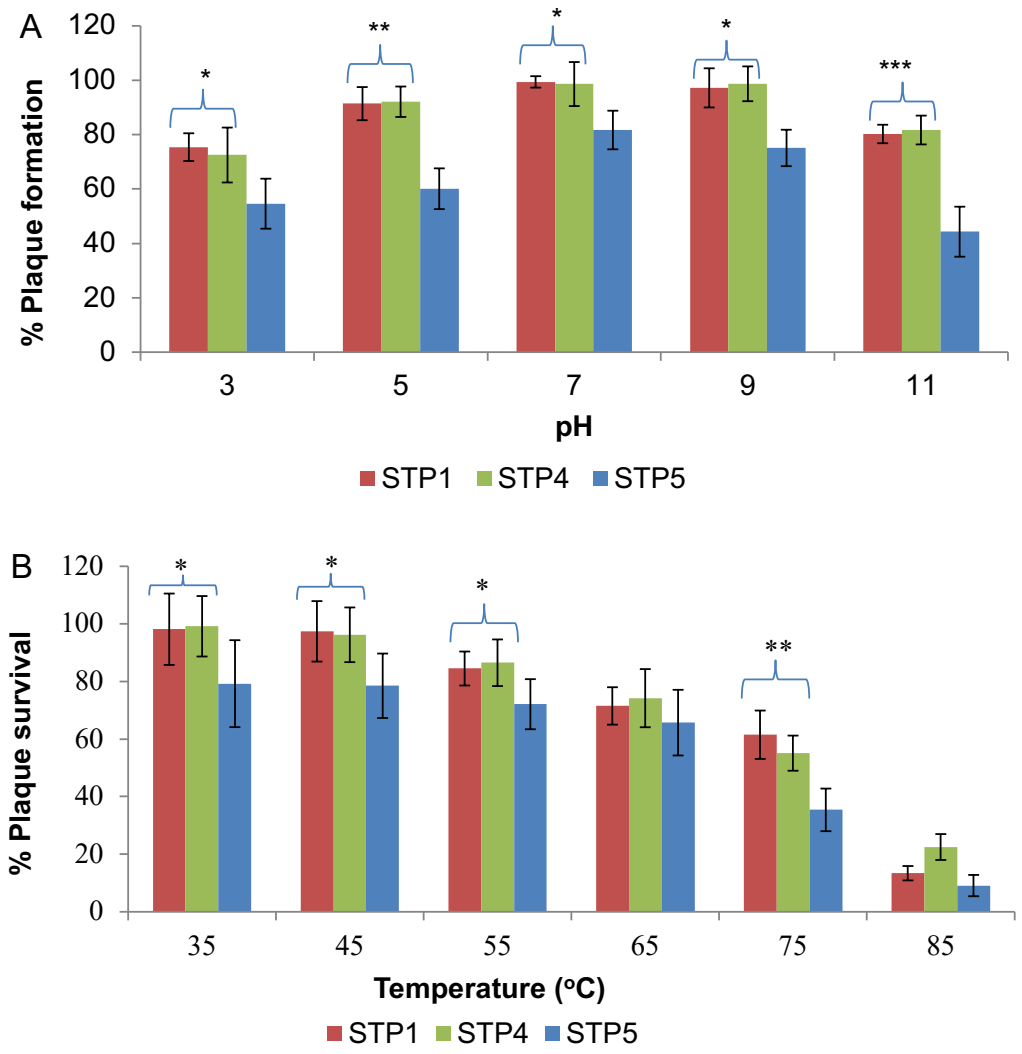

Fig. 3.15: Stability of isolated *Salmonella typhi* bacteriophages STP 1, 4 and 5. (A) pH stability (B) Thermal stability. Statistical significance of data was evaluated by using One-way ANOVA test and represented as **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

3.6 Biofilm Eradication Potential

Biofilm formation by bacteria is one of the crucial problems to chocking of the membranes used to filter water in water treatment plants. Biofilm formation by different microbes is responsible for impaired function of RO membrane, such as blocking and clogging, thus requires high energy consumption for water pumping system with decreased permeate quality (Flemming 2002; Bereschenko *et al*., 2010). Biofilm eradication potential of the isolated phages were also determined using crystal violet assay and microscopic analysis. ECP1 has less potential to eradicate preformed biofilms of *E. coli*, whereas, rest of the phages, ECP7, STP 1 and 4, have significantly higher biofilm eliminating potential than that of ECP1 (Fig 3.16 A and B).

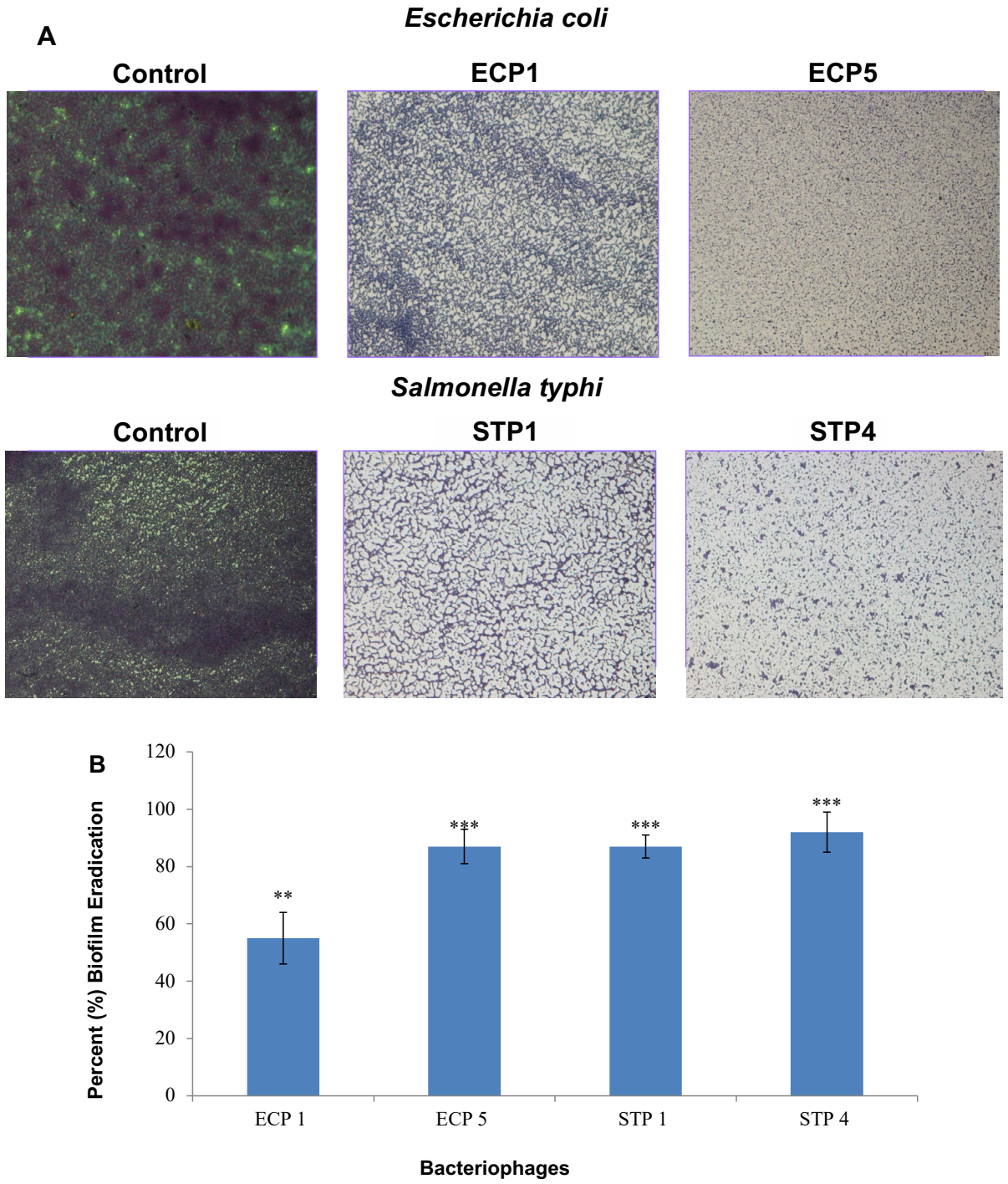

Fig. 3.16: Biofilm Eradication Potential of isolated *E. coli* and *S. typhi* bacteriophages. (A) Microscopic images of biofilm eradication (B) Bar graph of percent biofilm eradication. Statistical significance of data was evaluated by using One-way ANOVA test and represented as **$p < 0.01$ and ***$p < 0.001$.

3.7 Bacteriophage Cocktail Preparation for Dual Species Treatment

The third phase of the project was to formulate a bacteriophage cocktail from the isolated phages, which can be effective against mono-species and multiple species model in the presence of autoclaved sewage. This was done to evaluate the phage

efficacy in an actual environment. Cocktail provides a solution to target different species of one genus with varying types of virulence or it can be used to target different genuses of bacteria in one go. Secondly, we don't need to evaluate the safety profile of each bacteriophage independently so that it will save time (Forti *et al*., 2018).

Different studies suggest the importance of using bacteriophage cocktail instead of using single phage treatment (Chen *et al*., 2018; Forti *et al*., 2018; Manohar *et al*., 2019). In this project, four different phage cocktails (ECP1 – STP1; ECP1 – STP4; ECP7 – STP1; ECP7 – STP4) were prepared and evaluated for the bacterial reduction in the presence of sewage. All the prepared cocktails significantly reduced the log colony-forming units of bacteria individually. Still, cocktail ECP1 - STP1 was found to be more active in reducing bacterial count as compared to the other cocktail preparations (Fig. 3.17, 3.18).

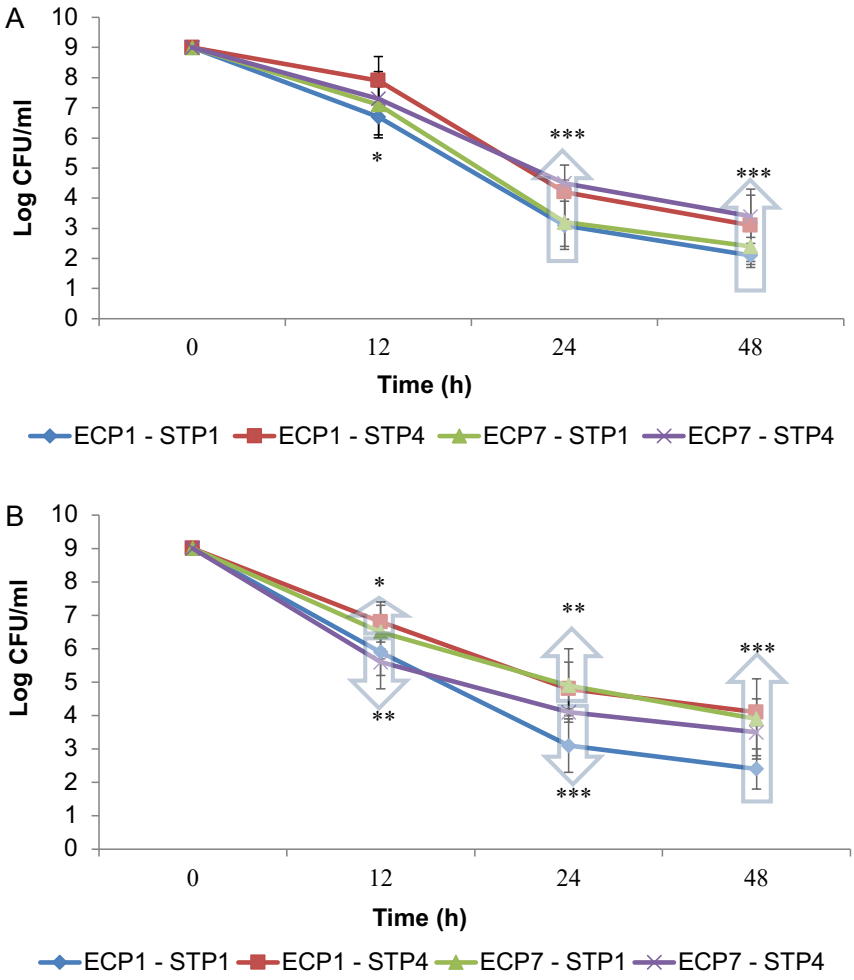

Fig. 3.17: Reduction of bacterial viability against individual cultures of *E. coli* after bacteriophage cocktail treatment in the presence of autoclaved sewage (A) EC59 (B) EC99. Statistical significance of data was evaluated by using One-way ANOVA test and represented as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

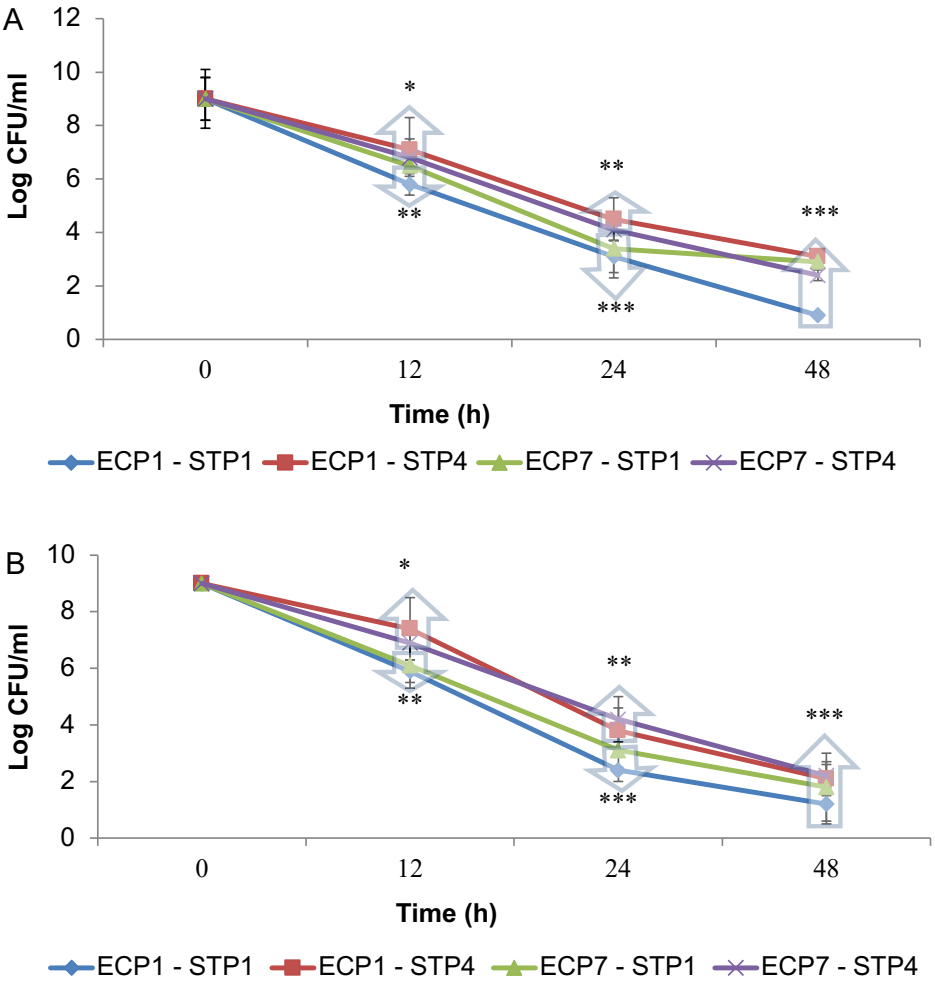

Fig. 3.18: Reduction of bacterial viability against individual cultures of *Salmonella typhi* after bacteriophage cocktail treatment in the presence of autoclaved sewage (A) ST60 (B) ST68. Statistical significance of data was evaluated by using One-way ANOVA test and represented as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

The prepared bacteriophages also reduced the *E. coli* and *S. typhi* as dual-species model significantly (Fig. 3.19). Again, the cocktail containing ECP1 and STP1 reduced the bacterial load more efficiently as compared to the other cocktails (Fig. 3.19). Costa *et al*. (2019) also showed the cocktail preparation, which reduced dual-species model of *E coli* and *S. typhi*. Various other groups have also reported the efficacy of different phage cocktails to treat waterborne and other infectious pathogens (Moye *et al*., 2018; Kalatzis *et al*., 2018; Remirez *et al*., 2018). The prepared bacteriophage cocktail was further screened for toxicological studies in mice. No mortality was observed. At the organ level, no histopathological changes were found in the liver and spleen, which also showed that this could be used to treat human diarrheal infections related to the studied pathogens.

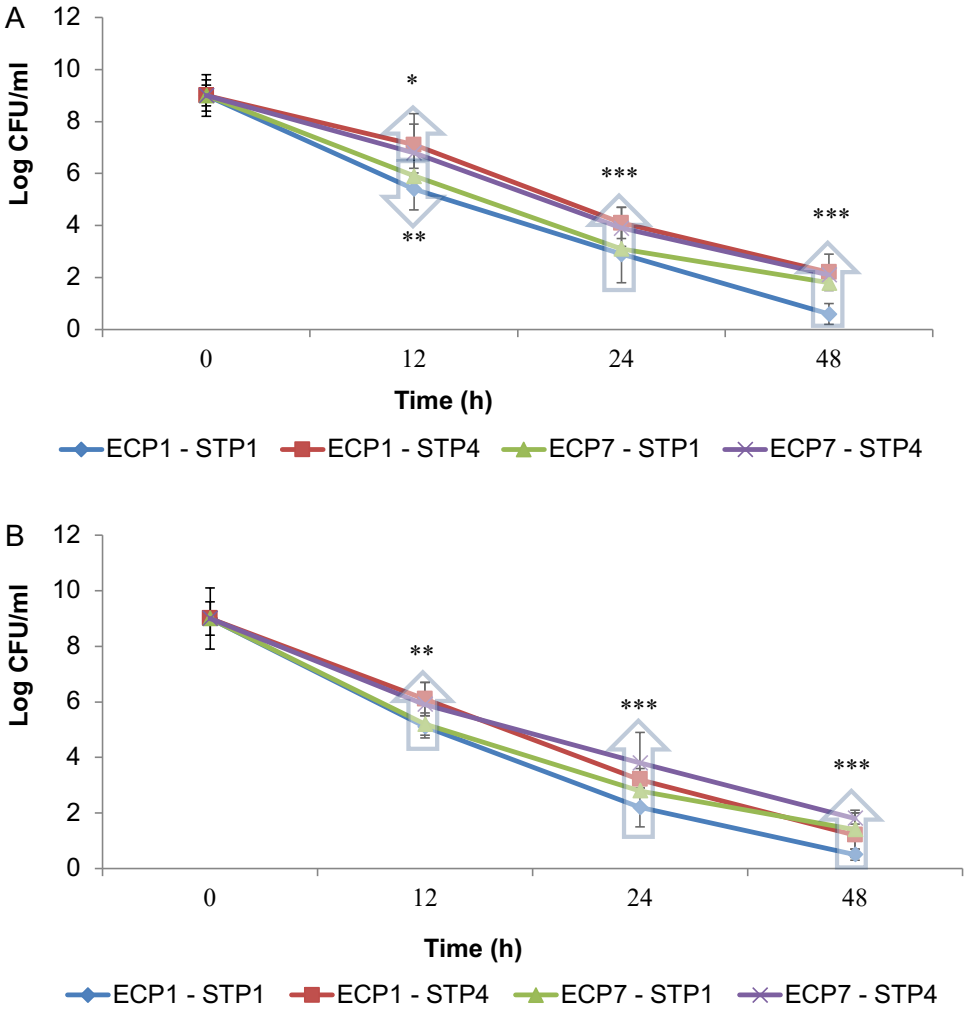

Fig. 3.19: Reduction of bacterial viability against mixed cultures of *E. coli* (EC 59 & 99) and *S. typhi* (ST 60 & 68) after bacteriophage cocktail treatment (A) ST60 (B) ST68. Statistical significance of data was evaluated by using One-way ANOVA test and represented as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

3.8 Research Output

3.8.1 MS/PhD completed with student name and title of thesis

One M. Phil study regarding the isolation of bacteriophage to conquer human pathogen is in the final steps, thesis writing is in progress after achieving the research data.

3.8.2 Project-based papers/ posters presented in conferences/ workshops

Project based poster regarding “Molecular epidemiology of water borne bacterial pathogens and their bacteriophage remedies” has been accepted for poster presentation in European Congress of Clinical Microbiology and Infectious Diseases 2020, Paris.

3.8.3 Papers published in journals

One manuscript entitled “Molecular epidemiology of *Salmonella* strains and *Escherichia coli* isolated from water” have been prepared and will be submitted in Molecular Pathogenesis Journal. One more paper will be prepared and submitted accordingly.

3.8.4 Project impact

The prepared phage cocktail and singular phage preparation will be further tested for toxicity evaluation in human cell lines, and in rat and mice. After such testing this preparation will be presented to authorities to be tested in bulk water treatment palnts.

4. CONCLUSION AND RECOMMENDATIONS

4.1 Conclusion

In conclusion, bacteriophage has the potential to treat water pathogens and can be prepared in the formulation to target multiple species as one therapy. This study, for the first time, reported the efficacy of isolated indigenous phages to treat indigenous microbial species of *E. coli* and *S. typhi* isolated from drinking and tap water. The isolation rate of *E. coli* and *S. typhi* from the collected water samples suggested the presence of fecal contamination. Majority of the isolated pathogens are multidrug-resistant, which can cause diarrhea to infants for whom immunity is not well established. These water pathogens, due to improper drinking water treatment, cause enhanced mortality and morbidity in infants. So, in this study, the bacteriophages (bacterial killers) were isolated to decipher its potential to infect isolated microbial strains. Out of 10, three bacteriophages significantly reduced the bacterial load and are heat and pH stable. These bacteriophages in a cocktail preparation significantly reduced the dual-species model in the presence of autoclaved sewage. Thus, these cocktails can be used as a possible measure to be used in water treatment processes. However, there are some limitations of this study; i.e., phage resistance was not evaluated for a more extended period of time as well as the resilience of the phage cocktail is not determined. It was not tested on human pathogens to make it more beneficial to treat both water-related problems and also cure diarrhea among individuals. Its toxicity profile needs to be evaluated in growing mice pubs for whose immune system is not well established. Further research is required to keep monitoring the prevalent pathogens of water and to formulate more cocktail options to treat such issues.

4.2 Recommendations

Based on the results achieved in this study, following recommendations are formulated:

Technical:

1. The bacteriophage isolated can be used as a disinfectant tool after proper preclinical studies to avoid water contaminant.
2. More characterized phage should be isolated and can be used to treat water in treatment plant to kill disease causing pathogens.
3. The contaminated water showed the presence of human pathogens such as *Escherichia coli* and *Salmonella* strains clearly suggesting waste water contamination, which should be stopped.

General:

1. The sewage water pipeline or fresh water supply pipe line must be repaired properly to stop leakage and contamination.
2. The pressurized, safe, controlled and continuous water supply system may be maintained to all parts of the Karachi to avoid spread of disease.
3. Drinking water quality may be monitored regularly.

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Appendix-1 Reagents used

S. No	Buffers and stock solutions	Composition
1.	SM buffer	200 mM Tris HCl, 10 mM $MgSO_4$, 10 mM $CaCl_2$, and 100 mM NaCl in distilled water. Adjust pH to 7.5 with HCl. Sterilize by autoclaving at 121 °C for 15 min. The buffer can be kept for up to one month at room temperature
2.	Phage buffer	100m M NaCl, 10 mM $MgCl_2$, 50m M Tris-HCl, and 0.01% gelatin (pH 7.5). **OR** 100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-Cl (pH 7.5), and 0.01% gelatin
3.	PBS buffer	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4 in distilled water. Adjust pH to 7.4 with HCl. Sterilize by autoclaving at 121 °C for 15 min. The buffer can be kept for up to one month at room temperature
4.	$CaCl_2$ solution	Dissolve $CaCl_2$ into distilled water at a final concentration of 1 M. Sterilize by autoclaving at 121 °C for 15 min. The solution can be kept for up to one month at room temperature.
5.	$MgSO_4$ solution	Dissolve $MgSO_4$ into distilled water at a final concentration of 1 M. Sterilize by autoclaving at 121 °C for 15 min. The solution can be kept for up to one month at room temperature
6.	PEG 8000 solution	Dissolve PEG 8000 into distilled water to a final concentration of 30% (*w*/*v*). Sterilize by autoclaving at 121 °C for 15 min. The solution can be kept for up to one month at room temperature.
7.	NaCl solution	Dissolve NaCl into distilled water to a final concentration of 5 M. Sterilize by autoclaving at 121 °C for 15 min. The solution can be kept for up to one month at room temperature.

8.	Tris HCl 100 mM pH 7.4	Dissolve Tris HCl into distilled water to a final concentration of 100 mM. Adjust pH to 7.4 with HCl. Sterilize by autoclaving at 121 °C for 15 min. The solution can be kept for up to one month at room temperature.
9.	Calcium chloride ($CaCl_2$) for LB	Prepare a 1 M stock solution and add a final concentration of 0.001 M to desired volume of the LB broth that will be used for the liquid lysate. Autoclave, 0.02 µm filter-sterilize before use, and store at room temperature.
10.	Magnesium chloride ($MgCl_2$)	Prepare a 1 M stock solution and add a final concentration of 0.001 M to desired volume of the LB broth that will be used for the liquid lysate. Autoclave, 0.02 µm filter-sterilize before use, and store at room temperature.
11.	2,3,5-triphenyl-2H-tetrazolium chloride	TTC powder dissolve in sterile distilled water at a concentration of 5 mg/mL at room temperature then filter through 0.22 µm Whatman filter paper and store at −20 °C until used.
12.	STE	For 100 ml, add 1 ml 1 M Tris (pH 8), 0.2 ml 0.5 M EDTA (pH 8), 2 ml 5 M NaCl. Autoclave.
13.	PEG	For 100 ml, add 20 gm PEG-8000 and 14.6 gm NaCl, filter sterilize.
14.	BS (1x) or Tris Buffer Saline	50 mM Tris-HCl pH 7.5, 150 mM NaCl, sterile, made 1x from commercial 10x stock solution (prevent possible protease contaminations).
15.	PEG/NaCl (5x) stock solution	PEG-8000 20%, NaCl 2.5 M. Dissolve 100 g PEG-8000 (20% w/v) and 75 g NaCl (2.5 M) in 400 ml ddH_20 and bring to a final volume of 500 ml by stirring at RT. The solution can be autoclaved (optional) but mixing during the cooling period is required to prevent a phase separation. Store at RT.

About the Authors

Dr. Ayaz Ahmed is currently working as an Assistant Professor at Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi. He did his PhD from Dalian Medical University, Dalian, P. R. China and Post Doctorate from Hunan Drug Safety Evaluation Center. He has more than 10 year teaching and research experience. He has published more than 30 papers in peer reviewed journal of international repute and also presented his work in several national and international conferences. Under his supervision two students have completed their PhD and one student completed her M. Phil degree. He is also selected as a Visiting Professor at Affiliated T.C.M. Hospital of Southwest Medical University, Luzhou, P.R. China. Beside this project, he also won national and international research grant as an early career scientist.

Dr. Adnan Khan is currently working as an Associate Professor at Department of Microbiology, University of Karachi. He completed his PhD from the same department in 2010 after winning indigenous scholarship for PhD from Higher Education Commission. After PhD, he joined Professor Dr. David Kelvin research group as Postdoctoral fellow at the International Institute of Infection and Immunity in 2015, followed by his Post-Doctoral studies, in 2016 at the University of Toronto. During his postdoctoral studies he worked on influenza transmission and its vaccine efficacy testing. He holds various trainings in the field of Biosafety and Security, a core component in microbiological research.

Main thrust of Applied Research component of the Water Center is to stimulate an environment that promotes multi-disciplinary research within the broader context of water-development nexus to support evidence-based policy making in the water sector. This is pursued using the framework provided by the six targets of the Sustainable Development Goal on Water i.e. SDG-6.

Contact:

U.S.-Pakistan Centers for Advanced Studies in Water
Mehran University of Engineering and Technology, Jamshoro-76062, Sindh - Pakistan

92 22 210 9145 water.muet.edu.pk /USPCASW /USPCASW