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An Integrating Approach of Bacteriophage and Iron Antagonizing Molecule for the Eradication of Biofilm of *E. coli* Isolated from Clinical Samples in Sylhet City, Bangladesh

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Authors' contributions

This work was carried out in collaboration between all authors. Author SMAS designed the study and managed the analyses of the study. Author KD wrote the first draft of the manuscript and performed the statistical analysis. Author FTZ performed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Bacterial biofilms are associated with a large number of persistent and chronic infections. Biofilm-dwelling bacteria are particularly resistant to antibiotics and immune defenses, which makes it hard to eradicate biofilm-associated infections. This study aimed to analyze the effect of zinc sulphate ($ZnSO_4$) and bacteriophage treatment on *E. coli* biofilm. This study was performed in microbial biotechnology laboratory, SUST, Sylhet, Bangladesh during a period of 9 months from December 2016 to August 2017. Bacteria were isolated from clinical samples of renowned hospital and diagnostic center in Sylhet city, Bangladesh and identified using various biochemical tests. Biofilm forming isolates were screened by the crystal violet assay method in microtiter plate. Bacteriophage was isolated from drainage samples and recognized by spot test. Biofilm enhancement was studied using different concentrations of $FeCl_3$. Also, Biofilm inhibition was investigated using zinc sulphate

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($ZnSO_4$) and bacteriophage separately and in combination by the enumeration of viable cells of biofilm after treatment in six (6) days experiment. Kirby-Bauer disk diffusion method was used for antibiotic susceptibility test. The result showed that biofilm formation was enhanced by the supplementation of 10 µM $FeCl_3$ in medium. A significant decrease ($p < 0.01$) in viable cells of biofilm was occurred with highest reduction of 89±1% and 85.38±1.30% by zinc sulphate and phage treatment, respectively. Also, the combined use of zinc sulphate and phage caused significant removal ($p < 0.01$) in viable cells of biofilm with highest reduction of 97.22±1.37% in comparison to control. However, there was significant difference ($p < 0.01$) between combined treatment and single treatment. The antibiotic susceptibility test showed that selected biofilm forming isolate was resistant to antibiotics tested. Finally, it might be said that a combined approach of bacteriophage and iron antagonizing molecule is more significant than distinct approach for the eradication of *E. coli* biofilm.

Keywords: Biofilm; bacteriophage; zinc sulphate; eradication.

1. INTRODUCTION

A structured community of microorganisms enclosed in a self-produced extracellular polymer matrix by adhering to biological or nonbiological surfaces, forms biofilms [1]. The biofilms matrix composed of polysaccharides, proteins extracellular DNA, and responsible for adhesion to surfaces and for cohesion in the biofilms [2]. Sessile form (biofilm) of bacteria is predominant in nature rather than a free-swimming form (planktonic) [3]. The biofilm of *Escherichia coli* is responsible for extraintestinal infections that make it predominant in various biofilm forming bacteria [4]. About 80% of all UTI, both asymptomatic bacteriuria and symptomatic UTI caused by *E. coli* [5]. Biofilms affect human in many ways because they can form in natural and medical settings. Hancock [6] reported that treatment of chronic infections becomes more difficult because biofilm-associated bacteria can withstand host immune defenses, antibiotics and biocides. Therefore, prevention and treatment of diseases associated with biofilms require novel approaches.

Bacteriophage therapy is vital for the treatment of bacterial infections that show resistance to multi drugs [7]. Due to more host-specificity of bacteriophage than that of antibiotics, it is less possible cause of collateral damage relating to killing of healthy gut flora along with the pathogen [8]. Bacteriophage that inhibits biofilm, is known to produce or to induce enzymes that destroy the extracellular matrix [9]. It is also able to infect persistent cells and to initiate complete lysis of them [10]. However, our immune system may recognize bacteriophage and clear out of our body [11]. So, phage or antibiotics when used alone may not stand a better chance for the treatment of biofilm-associated bacterial infections. Therefore, the combination of chemically distinct antimicrobials and phages might be an effective strategy for treating biofilms.

The importance of iron for bacterial growth has been known for many years. Singh [12] reported that iron serves as a signal for development of biofilms even when there is sufficient iron for planktonic growth. Besides being a signal for biofilm development, iron stabilizes the extracellular polymeric matrix [13]. Thus, iron availability reduction has been proposed as a mean for the impairment of development of biofilm formed by *K. pneumoniae, Pseudomonas aeruginosa* etc. [14,15]. The iron antagonizing divalent metal ion Zn (II) can provide a tool to fight against biofilm [16].

Therefore, the aim of the present study was to explore the possibility of zinc sulphate ($ZnSO_4$) as an iron antagonizing molecule and bacteriophage separately and in combination to eradicate biofilm formation by *E. coli* isolated from clinical samples.

2. MATERIALS AND METHODS

2.1 Bacterial Isolates and Media

A total of 30 samples from different clinical sources such as used catheters, urine, blood and urethral swab were obtained from Medinova Diagnostic Centre and M.A.G Osmani Medical College and Hospital, Sylhet, Bangladesh from December 2016 to January 2017 and transferred to microbial biotechnology laboratory, SUST, Sylhet, Bangladesh. The organisms (*E. coli*) were identified on the basis of colonial morphology, gram staining, IMViC (Indole, Methyl red, VogesProskeaur, Citrate), oxidase, urease and catalase test. Previously studied [17] strong biofilm producer *E. coli* PHL628 and weak

biofilm producer *E. coli* PHL565 were provided by microbial biotechnology laboratory, SUST and used as positive and negative control for screening of biofilm forming isolates, respectively. For routine bacterial culture, nutrient broth was used. Biofilm generation in microtiter plate was conducted by Tryptone Yeast Extract Broth (TY) containing casein enzymic hydrolysate (5 g/l) and yeast extract (3 g/l), and as indicator iron ($FeCl_3$) was added. The iron antagonizing molecule was a divalent metal ion contained in salt, $ZnSO_4$. The concentration of $ZnSO_4$ was set at a value of 500 µM, so that it could not inhibit the growth of planktonic bacteria [6].

2.2 Screening of *E. coli* Isolates for Biofilm Formation

According to the study of Cucarella [18] with some modifications, we screened 15 clinical isolates of *E. coli* for their capability of biofilm formation by the crystal violet assay method in microtiter plate. Isolates from overnight cultured agar plates were inoculated into 5 ml of TY broth medium followed by overnight incubation at 37°C and dilution in 1:20 with TY broth. 200 µl of diluted cultures was seeded into sterile 96-wells and incubated at 30°C for 48 h. After three times washing by 200µl of phosphate buffered saline solution, wells were dried for 20 min at 60°C. Adherent organisms forming biofilms were fixed with methanol in wells and stained with 1% crystal violet for one minute. After staining, three times washing of plate was conducted using sterile distilled water to remove unbound crystal violet. The quantitative analysis of biofilm formation was carried out by adding 200 µl 95% ethanol for the detachment of crystal violet from the bacterial biofilm. After pipetting up and down for several times, the contents of each well were then transferred to a cuvette and the optical density was measured at 570 nm using spectrophotometer (JENWAY spectro-photometer). For the compensation of background absorbance, the OD values of the controls without inoculum were averaged and subtracted from the mean OD value obtained for each isolate. For each isolate, the experiment was repeated three times and the average value was calculated.

2.3 Isolation of Bacteriophage

The isolation of phage specific to *E. coli* isolate was carried out by method of Cerveny [19] with some modifications. In that purpose, samples were collected from the drainage of M.A.G Osmani Medical College and Hospital, Sylhet, Bangladesh. This site was selected to isolate phages, as sewage was known to harbor many different bacteria and hence the possibility of prevalence of phages against different organisms. For the isolation of bacteriophage, sewage samples were centrifuged at 15000 rpm for 15 min and supernatant was sterilized with a 0.45 µm pore size filter. 1 ml of filtered supernatant and 10 ml sterile TY broth were mixed with 0.5ml overnight culture of *E. coli* (10^8 CFU/ml) followed by incubation at 37°C for 24 h. The bacteria were removed by centrifugation and supernatant was filter sterilized (0.22 µm pore size syringe filter) for the presence of phage.

2.4 Spot Test and Plaque Assay

For the detection of phage in supernatant, spot test was carried out as described by Chang [20]. In that case, the phage suspension was serially diluted (10^{-1}-10^{-3}). 0.1 ml of diluted phage suspension and 0.3 ml overnight culture of *E. coli* were mixed with 3 ml molten soft agar (0.75% agar, w/v) followed by quick pouring on solidified nutrient agar plate [21]. The numbers of plaque were counted after incubating the plate at 37°C for overnight.

2.5 Phage Purification

When plaques were identified, a pure suspension was prepared by carefully piercing agar surrounding the plaques with a sterile wire loop and picking out the agar “block” containing the plaques into microfuge tube containing 1 ml of PBS. Phage was stored in the refrigerator at 4°C for further use.

2.6 Growth of Biofilm in TY Broth Media Supplemented with Iron

100 µl of *E. coli* inoculum (10^9 CFU/ml) and 100 µl of TY broth media that was supplemented with 10 µM, 100 µM and 1000 µM $FeCl_3$ separately, were used to inoculate wells of 96-well microtiter plate. After overnight incubation at 37°C, media of all wells were removed and unadhered bacteria were rinsed off from one of wells containing TY broth media supplemented with different concentrations of $FeCl_3$ for each day. Then adherent biofilm was removed by scraping using mini cell scrapers (ABI Scientific Inc) from one well of 96 wells plate, suspended in 0.85% NaCl and vortexed for 3 min. Microbial load of biofilm was enumerated by viable cells counting

on overnight incubated nutrient agar plate. Percent increase in cells of biofilm was noted in comparison to control that was not supplemented. For remaining wells, spent media were replaced with sterile media and microtiter plate was reincubated at 37°C for overnight. This method was repeated until 6th day of experiment.

2.7 Effect of Zinc Sulphate ($ZnSO_4$) on Biofilm Growth

The effect of iron antagonizing molecule ($ZnSO_4$) was conducted according to Chhibber [22] with slight modifications. In brief, 100 µl of *E. coli* inoculum (10^9 CFU/ml) was inoculated into 96 wells of microtiter plate containing 100 µl of TY broth media supplemented with 10 µM $FeCl_3$ alone and 500 µM $ZnSO_4$ along with 10 µM $FeCl_3$ for the determination of biofilm growth depletion with $ZnSO_4$ treatment. After overnight incubation at 37°C media of all wells were removed. Then adhered bacteria were detached by scraping using mini cell scrapers (ABI Scientific Inc) from one of control wells containing 10 µM $FeCl_3$ supplemented media and wells containing media supplemented with 10 µM $FeCl_3$+$ZnSO_4$ (for each day) followed by viable cells count on overnight incubated nutrient agar plate, and viable cells reduction was noted in comparison to untreated control. For remaining wells, washed-out media were replaced with fresh media and microtiter plate was reincubated at 37°C for overnight. This method was repeated until 6th day of test.

2.8 Effect of Bacteriophage alone and in Combination with $ZnSO_4$ on Biofilm Growth

The treatment of biofilm with bacteriophage was conducted according to Chhibberet [22] with slight modifications. For the determination of effectiveness of bacteriophage alone and in combination with $ZnSO_4$ in treating the biofilm of *E. coli*, 100 µl of bacterial inoculum was inoculated into microtiter plate containing 100 µl of media supplemented with 10 µM $FeCl_3$ alone and combination of 500 µM $ZnSO_4$ with 10 µM $FeCl_3$. Unadhered bacteria were removed from one of wells only supplemented with 10 µM $FeCl_3$ and wells supplemented with 500 µM $ZnSO_4$+10 µM $FeCl_3$ after overnight incubation at 37°C. Subsequently, bacteriophage at multiplicity of infection [M.O.I: ratio of infectious agent (phage) to infection target (bacterial cell)] of 1 was exposed to biofilm for 6 h followed by washing with 0.85% NaCl and viable bacterial cells counting (one well for each day). The depletion in cells of biofilm was recorded in comparison to control. One of wells containing biofilm grown in iron supplemented TY broth media served as control. For remaing wells, washed-out media were replaced with fresh media and microtiter plate was reincubated at 37°C for overnight. This method was repeated until 6th day of test.

2.9 Antibiotic Susceptibility Test

Biofilm forming *E. coli* isolate was tested for its susceptibility to various antibiotics such as ampicillin (25 µg), ceftriaxone (30 µg), gentamicin (10 µg), tetracycline (30 µg), azithromycin (05 µg) by the Kirby–Bauer disc diffusion method with modification. For the experiment, biofilm forming *E. coli* isolate was inoculated into the sterile 5 ml Luria Bertani broth followed by incubation at 37°C for 24 h. After incubation, a lawn was made by inoculating 0.1 ml of *E. coli* inoculum (1.5×10^8 CFU/ml) on Mueller Hinton agar plate with a sterile cotton swab. Then antibiotic discs were placed on the plate with gentle press. The plate was incubated at 37°C for 24 h followed by examination of the plate for the presence of zone inhibition around the antibiotic discs. The diameter of the inhibition of zone was measured and compared with antimicrobial susceptibility testing standards (Table 1) provided by the Clinical and Laboratories Standards Institute for *E. coli* [23]. The susceptibility of weak biofilm former (*E. coli* PHL565) was tested to compare with strong biofilm former *E. coli* (isolate 6).

Table 1. CLSI* provided zone inhibition values in millimeter (mm) for antibiotics

Antibiotic	Resistant	Intermediate	Susceptible
Ampicillin (10 µg)	≤13	14-16	≥17
Ceftriaxone (30 µg)	≤13	14-20	≥21
Gentamicin (10 µg)	≤12	13-14	≥15
Tetracycline (30 µg)	≤11	12-14	≥15
Azithromycin	-	-	-

CLSI: Clinical and Laboratory Standards Institute [24]*

2.10 Statistical Analysis

The results of optical density, biofilm enhancement and biofilm reduction were expressed as means±standard deviations (SD) of three independent experiments. Viable cells enhancement and reduction in biofilm presented as percentage of control. The optical density of different selected isolates, biofilm enhancement by different concentrations of $FeCl_3$ and biofilm reduction by different treatments was evaluated by the Student's t-test and $p < 0.05$ was considered significant. Data were explored using microsoft excel software.

3. RESULTS

3.1 Prevalence and Biofilm Assay of *E. coli* Isolates

Out of 30 clinical samples, *E. coli* was reported in 15 cases through standard cultural and biochemical tests. Resulting *E. coli* isolates were considered for biofilm assay. Biofilm formation was assayed by the crystal violet assay method in microtiter plate. The average values of O.D. at 570 nm of different *E. coli* isolates along with *E. coli* PHL628 and *E. coli* 565 used as positive control and negative control respectively, shown in Fig. 1. From the result, it was found that isolate 6 was strong biofilm producer because its optical density (0.983±0.01) was most near ($p > 0.05$) to positive control (strong biofilm producer) and it was used for further study. Also, isolate 6 showed significantly higher ($p < 0.01$) optical density than other isolates except isolate 3, isolate 7 and *E. coli* PHL628.

3.2 Bacteriophage Isolation

Bacteriophage produced small clear plaques on bacterial culture contained on nutrient agar plate in plaque assay as shown in Fig. 2. The phage titer count showed 1×10^8 PFU/ml for biofilm former *E. coli* (isolate 6) on overnight incubated nutrient agar plate.

3.3 Biofilm Formation in Iron Supplemented Media

The effect of supplementation of $FeCl_3$ in TY media at different concentrations was studied on

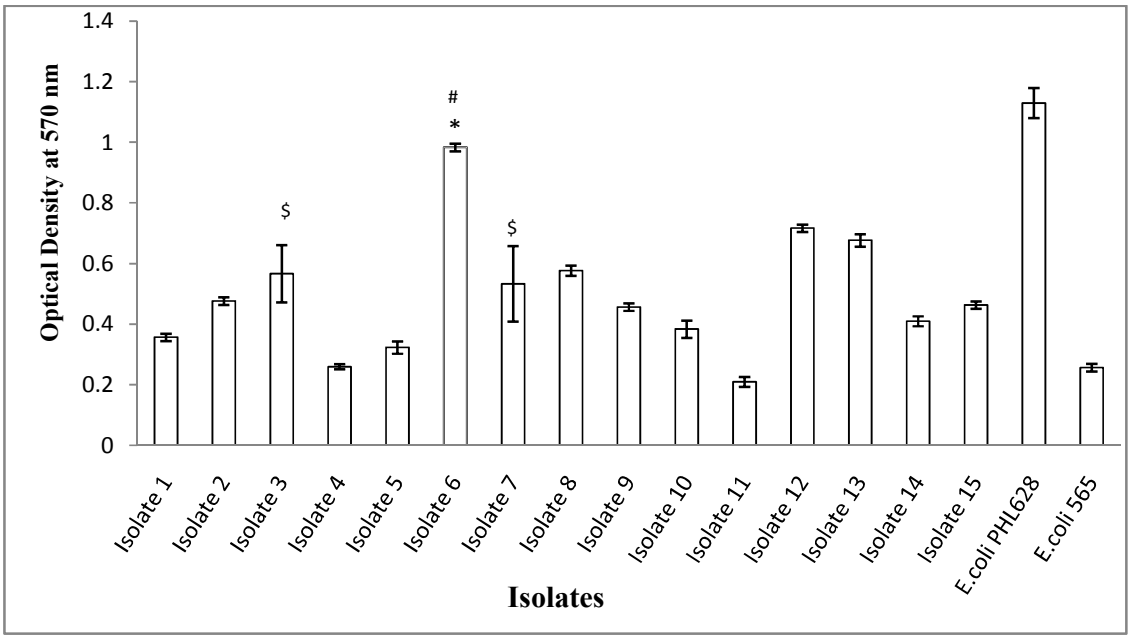

Fig. 1. The optical density (O.D) at 570nm of different *E. coli* isolates was represented by subtraction of the mean O.D value of control without inoculum from mean O.D value obtained for each isolate. Highest optical density of isolates at 570nm indicated strong biofilm producer. Isolate 6 showed highest value of optical density except *E. coli* PHL628. *E. coli* PHL628 and *E. coli* 565 served as positive and negative control for biofilm development. *p <0.01 (Isolate 6 vs Isolates except Isolate 3, Isolate 7), #p >0.05 (Isolate 6 vs *E. coli* PHL628), $p<0.05 (Isolate 3/ Isolate 7 vs *E. coli* PHL628)

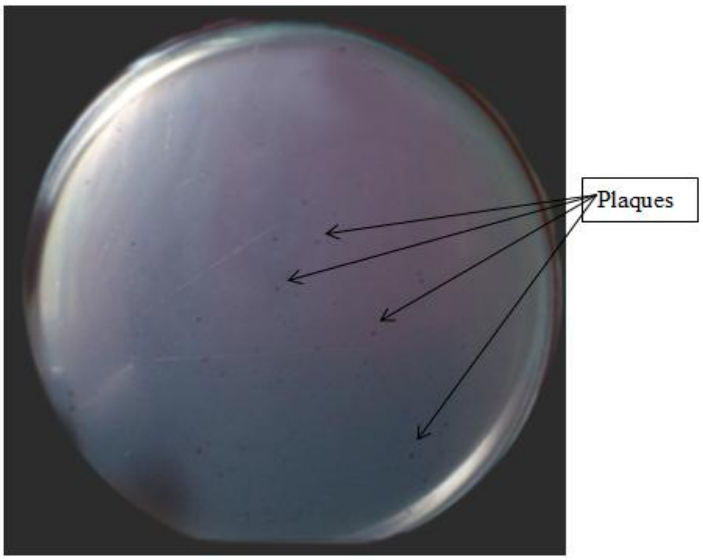

Fig. 2. Clear zone on bacterial culture contained on nutrient agar plate indicated the presence of bacteriophage that was specific to the selected biofilm forming *E. coli* isolate

the biofilm growth. The significant increase ($p < 0.05$) in bacterial count was take placed by 10 µM $FeCl_3$ supplementation in media in comparison to non-iron supplemented control in all (6) days with highest increase (13.13±0.56%) of cells count on 3rd day (Fig. 3). The supplementation of media with 100 µM and 1000 µM $FeCl_3$ showed highest increase of 8.83±0.62% and 8.13±0.50% in bacterial count of biofilm on 4th day and 3rd day, respectively. However, 10 µM $FeCl_3$ supplementation significantly enhanced ($p < 0.05$) the bacterial count in comparison to other supplementations and it was used for further study.

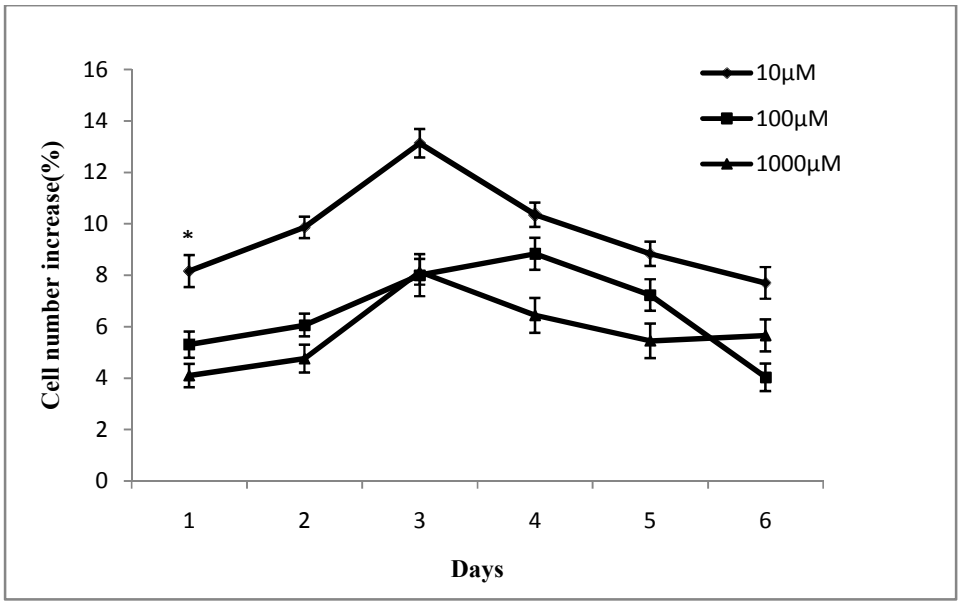

Fig. 3. Percent increase of cells number in *E. coli* biofilm grown in iron supplemented TY media and control had no cells number increase. *$p<0.05$ (10 µM $FeCl_3$ vs control/ 100 µM $FeCl_3$/ 1000 µM $FeCl_3$) in each day

3.4 Effect of $ZnSO_4$ on Biofilm Growth

E. coli biofilm grown in TY media supplemented with 500 µM $ZnSO_4$ and 10 µM $FeCl_3$ was affected by iron antagonizing molecule $ZnSO_4$, and it was observed that although biofilm growth was improved by 10 µM $FeCl_3$ supplementation but addition of 500 µM $ZnSO_4$ alone showed inhibitory effect on biofilm growth in comparison to control well containing iron. The result presented in Fig. 4 showed that there was significant loss ($p < 0.01$) of 89±1% to 80.53±1.50% in viable cells of biofilm in comparison to control in first 3 days. In rest days, significant loss ($p < 0.01$) of 80.38±1.45% to 77±1% was found in viable cells.

3.5 Effect of Bacteriophage alone and in Combination with $ZnSO_4$ on Biofilm Growth

For the determination of efficiency of bacteriophage in reducing the biofilm of *E. coli,* it was added at MOI of 1 alone and in combination with 500 µM $ZnSO_4$ to wells containing TY media supplemented with 10 µM $FeCl_3$.The result of the study showed that addition of phage to the wells containing 10 µM $FeCl_3$ caused significant loss ($p < 0.01$) of 85.38±1.30% to 72.28±2.48% in 1-3 day old biofilm in comparison to control wells supplemented with 10 µM $FeCl_3$ alone (Fig. 4). In remaining days, the reduction in viable cells of biofilm was noted 72.5±1.34% to 65.86±1.52 ($p < 0.01$). The reduction in cells of biofilm by phage treatment was significantly less ($p < 0.05$) than that of $ZnSO_4$ treatment in all days except 2nd day. Addition of 500 µM $ZnSO_4$ and phage to 10 µM $FeCl_3$ supplemented wells resulted in significant eradication ($p < 0.01$) of 97.22±1.37% to 94.31±1.69% in viable cells of biofilm in comparision to control in 1-3 day (Fig. 4). But in the old biofilm (4-6 day old), the reduction was 85.08±1.95% to 79.83±1.31% ($p < 0.01$). The combined treatment caused significantly higher reduction ($p < 0.01$) in cells of biofilm than $ZnSO_4$ treatment in first 3 days. In remaining days, there was not significant reduction ($p > 0.05$) of biofilm. Also, significantly higher reduction ($p < 0.01$) of biofilm was occurred by combined treatment than phage treatment.

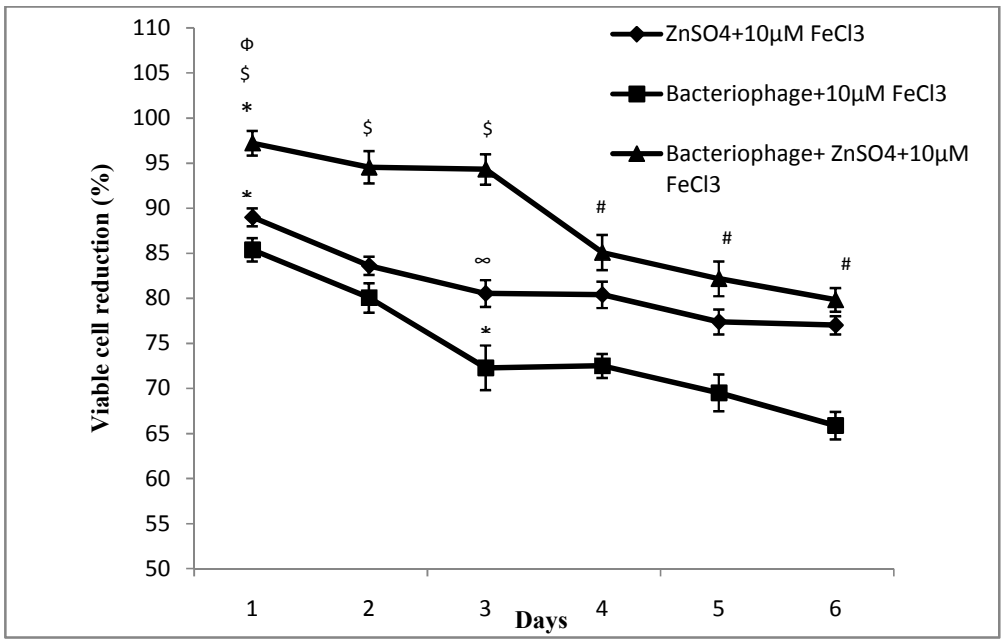

Fig. 4. Percent reduction of viable cells in *E. coli* biofilm treated with 500 µM zinc salt ($ZnSO_4$) and bacteriophage separately as well as in combination. TY media supplemented with 10 µM $FeCl_3$ served as control and it had no cells number reduction. *p <0.01 (Bacteriophage+$ZnSO_4$+10 µM $FeCl_3$/ $ZnSO_4$+10 µM $FeCl_3$/ Bacteriophage+10 µM $FeCl_3$ vs 10 µM $FeCl_3$) in each day, $^{\$}$p <0.01 (Bacteriophage+$ZnSO_4$+10 µM $FeCl_3$ vs $ZnSO_4$+10 µM $FeCl_3$), $^{\#}$p >0.05 (Bacteriophage+$ZnSO_4$+10 µM $FeCl_3$ vs $ZnSO_4$+10 µM $FeCl_3$), $^{\Phi}$p <0.01 (Bacteriophage+$ZnSO_4$+10 µM $FeCl_3$ vs Bacteriophage+10 µM $FeCl_3$) in each day, ∞p <0.05 ($ZnSO_4$+10 µM $FeCl_3$ vs Bacteriophage+10 µM $FeCl_3$) in all days except 2nd day

3.6 Antibiotic Susceptibility Test

Antibiotic susceptibility of strong biofilm producer *E. coli* (isolate 6) and weak biofilm producer (*E.coli* PHL565) was observed using different antibiotics e.g. ampicillin, ceftriaxone, gentamicin, tetracycline and azithromycin. From the result, it was found that *E. coli (*isolate 6) was resistant to ampicillin, ceftriaxone, gentamicin and tetracycline (Table 2). In addition, *E. coli* PHL565 was resistant to antibiotics except tetracycline and showed comparatively large zone of inhibition. The susceptibility of biofilm forming *E. coli* to azithromycin could not be determined since diameter of zone inhibition by azithromycin in case of *E. coli* had not been indicated by CLSI antimicrobial susceptibility testing standards.

4. DISCUSSION

Biofilms cause life threatening diseases through recurrent and chronic infections because of higher level of drug resistance caused by biofilm structures. Previous studies showed that the sessile bacterial cells exhibited higher resistance to antibiotics than planktonic cells [25,26]. Therefore, it is essential to exploit an alternative antibacterial therapy for combating nuisance bacteria. Various strategies have been reported to destroy biofilms. Biofilm-associated *E. coli* cells are particularly desperate for iron [6]. For the growth of both planktonic and biofilm mode of bacteria, iron is essential [15]. Therefore, iron antagonizing agents have been reported to inhibit biofilm growth. For iron up taking in biofilm development, master controller protein i.e. 'Fur' is essential and it has higher affinity (10,000-fold) for Zn (II) and Co (II) than iron [6]. Thus they are important for the reduction of biofilm formation by infectious isolates e.g *Klebsiella and* UTI *E. coli* [6]. In the present study, reduced biofilm formation of our *E. coli* isolate was observed in presence of $ZnSO_4$. From the study, we found that $ZnSO_4$ treatment showed significant loss ($p < 0.01$) in viable cells of biofilm in all days causing highest loss of 89±1%. Besides $ZnSO_4$, another study reported $ZnCl_2$ to reduce biofilm formation by *Actinobacillus pleuropneumoniae, Haemophilus parasuis and Salmonella typhimurium,* and $ZnCl_2$ at concentration of 500 μ mol l^{-1} significantly reduced ($p < 0{\cdot}001$) *E. coli* biofilm formation by 80% [16]. The reduction of biofilm might be caused by the impairment of Fur-controlled iron uptake systems like enterobactin, ferric dicitrate, aerobactin involved in biofilm formation through metal ion Zn (II) [27]. Even metal ions such as Zn (II) or Co (II) impaired biofilm could not be restored using iron supplement [6].

In addition to iron antagonizing molecule, bacteriophage therapy was reported as increasingly attractive way to control human infections since many antibiotics had shown declined efficacy [28]. Doolittle [29] observed that the extracellular matrix of *E. coli* biofilm did not protect the bacterial cells from infection with phage T_4 and that phage-infected cells were associated with the biofilm surface. When we used bacteriophage in the treatment of biofilm for 6 h, significant decrease ($p < 0.01$) of viable cells was occurred in *E. coli* biofilm with highest loss of 85.38±1.30%. On the other hand, previous study showed a significant reduction (90%, $P<0.0001$) of *P. mirabilis* and *E. coli* biofilm on bacteriophage-treated catheter, and another significant removal ($p < 0.05$) of 99.9–99.99% in cells of established biofilm [30]. Also, the reduction of biofilm in our study was higher than reduction of biomass of *Klebsiella pneumoniae* biofilm in which 40.98% and 32.39% loss of biomass occurred by Siphoviridae bacteriophage treatment for 4 h in 24 h and 72 h old biofilm,

Table 2. Antibiotic susceptibility test by the disk diffusion method for *E. coli* (isolate 6) and *E. coli* PHL565

Antibiotics	*E. coli* (isolate 6)		*E. coli* PHL565	
	Diameter of zone inhibition (mm)	Susceptibility	Diameter of zone inhibition (mm)	Susceptibility
Ampicillin	-	Resistant	7	Resistant
Ceftriaxone	-	Resistant	9	Resistant
Gentamicin	5	Resistant	12	Resistant
Tetracycline	6	Resistant	13	Intermediate
Azithromycin	-	~	12	~

(-)-no zone of inhibition, (~)- not determined

respectively [31]. Phage treatment using a titer of 1×10^6 PFU/well caused 96% reduction of *Mycobacterium smegmatis* biofilm [32].

The combination of phage and $ZnSO_4$ was used in *E. coli* biofilm treatment. We found that a significant loss ($p < 0.01$) in cells of treated biofilm occurred with highest removal of 97.22±1.37%. However, the combined use of phage and zinc sulphate was more effective ($p < 0.01$) in decreasing biofilm load compared to the single approach of treatment. The report by Zhang [33] showed that combined treatment of phage (3×10^7 PFU/mL) and chlorine (210 mg/L) reduced *Pseudomonas aeruginosa* biofilm development by 94 ± 2%, and there was significant difference ($p < 0.02$) between combined treatment and single treatment. In addition, the combination of cobalt sulfate ($CoSO_4$) and bacteriophage was studied by Chhibber [22] for the inhibition of *Klebsiella pneumoniae* B5055 biofilm, and significant removal ($p < 0.005$) of biofilm was reported. Therefore, combined treatment using bacteriophage and antimicrobial chemical is promising.

Resistance is the growth ability of a microorganism in a condition remaining higher level of antimicrobial [34]. Multidrug resistant tendency has been seen in approximately 80% of the biofilm forming strains [35] and biofilm formed by these strains can be up to 1000-fold more resistant to antibiotics than planktonic cells [36]. Therefore, the present study needed to investigate antibiotic susceptibility of selected bacteria. Biofilm forming *E. coli* (isolate 6) showed resistance to antibiotics tested, but *E. coli* PHL565 showed resistance to antibiotics except tetracycline. May [37] reported that *E. coli* in biofilm showed high level resistance to ampicillin and tetracycline. This resistance might be caused by the matrix of biofilm through forming physical and chemical barriers to antibiotics [38]. Another study by Kirby [39] showed that the density and physiological state of the culture might be responsible for antibiotic resistance instead of residing of bacteria within biofilm.

5. CONCLUSION

At last, from our study we might say that iron antagonizing molecule and bacteriophage alone or in combination are able to eradicate *E. coli* biofilm. But the results suggest that a combination of $ZnSO_4$ and phage is more effective in inhibiting biofilm. The finding on antibiofilm activity of $ZnSO_4$ and bacteriophage is significant and further study needs to be carried out to determine the potential use of bacteriophage therapy and $ZnSO_4$ treatment for the eradication of biofilm.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: A common cause of persistent infections. Science. 1999;284: 1318–1322.
2. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol. 2010;8:623–633.
3. Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. Annu Rev Microbiol. 2002;56:187–209.
4. Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. Nat Rev Microbiol. 2004;2:123-140.
5. Ronald A. The etiology of urinary tract infection: Traditional and emerging pathogens. Dis Mon. 2003;49:71–82.
6. Hancock V, Dahl M, Klemm P. Abolition of biofilm formation in urinary tract *Escherichia coli* and *Klebsiella* isolates by metal interference through competition for Fur. Appl Environ Microbiol. 2010;72: 3836–3841.
7. Górski A, Miedzybrodzki R, Borysowski J, Weber-Dabrowska B, Lobocka M, Fortuna W, Letkiewicz S, Zimecki M, Filby G. Bacteriophage therapy for the treatment of infections. Curr Opin Investig Drugs. 2009;10:766-74.
8. Clark JR, March JB. Bacteriophages and biotechnology: Vaccines, gene therapy and antibacterials. Trends in Biotechnology. 2006;24:212-218.
9. Harper D, Parracho H, Walker J, Sharp R, Hughes G, Werthén M, Lehman S,

Morales S. Bacteriophages and biofilms. Antibiotics. 2014;3:270-284.
DOI: 10.3390/antibiotics3030270

10. Pearl S, Gabay C, Kishony R, Oppenheim A, Balaban NQ. Nongenetic individuality in the host-phage interaction. PLoS Biol. 2008;5:e120.
DOI: 10.1371/journal.pbio.0060120
11. Kutter E, Vos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, Abedon ST. Phage therapy in clinical practice: Treatment of human infections. Curr Pharm Biotechnol. 2010;11:69-86.
12. Singh PK, Parsek MR, Greenberg EP, Welsh MJ. A component of innate immunity prevents bacterial biofilm development. Nature. 2002;417:552–555.
13. Berlutti N, Morea C, Battistoni A, Sarli S, Cipriani P, Superti F, Ammendolia MG. Iron availability influences aggregation, biofilm adhesion and invasion of *Pseudomonas aeruginosa* and *Burkholderiace nocepacia*. Int J Imunopathol Pharmacol. 2005;18:661–670.
14. Banin E, Vasil ML, Greenberg EP. Iron and *Pseudomonas aeruginosa* biofilm formation. Proc Natl Acad Sci. 2005;102:11076–11078.
15. O'May CY, Sanderson K, Roddam LF, Kirov SM, Reid DW. Iron binding compounds impair *Pseudomonas aeruginosa* biofilm formation especially under anaerobic conditions. J Med Microbiol. 2009;58:765–773.
16. Wu C, Labrie J, Tremblay YD, Haine D, Mourez M, Jacques M. Zinc as an agent for the prevention of biofilm formation by pathogenic bacteria. J Appl Microbiol. 2013;115:30-40.
17. Sayem SM, Manzo E, Ciavatta L, Tramice A, Cordone A, Zanfardino A, de Felice M, Varcamonti M. Anti-biofilm activity of an exopolysaccharide from a sponge-associated strain of *Bacillus licheniformis*. Microb. Cell Fact. 2011;10:74.
DOI: org/10.1186/1475-2859-10-74
18. Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penadés JR. Bap a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol. 2001;183:2888-2896.
19. Cerveny KE, Depaola A, Duckworth DH, Gulig PA. Phage therapy of local and systemic disease caused by *Vibrio vulnificus* in iron- dextran -treated mice. Infect Immun. 2002;70:6251-6262.
20. Chang HC, Chen CR, Lin JW, Shen GH, Chang KM, Tseng YH, Weng SF. Isolation and characterization of novel giant *Stenotrophomonas maltophilia* phage ΦSMA5. Appl Environ Microbiol. 2005;71: 1387–1393.
21. Adams M, (Ed). Bacteriophages. Interscience Publishers, London, United Kingdom; 1959.
22. Chhibber S, Nag D, Bansal S. Inhibiting biofilm formation by *Klebsiella pneumonia* B5055 using an iron antagonizing molecule and a bacteriophage. BMC Microbiology. 2013;13:174.
23. Gloria A, Cheryl B, John E. Manual for the laboratory identification and antimicrobial susceptibility testing of bacterial pathogens of public health importance in the developing world, centers for disease control and prevention and World health organization, Department of communicable disease surveillance and response, Atlanta, Ga, USA; 2003.
24. Clinical and Laboratory Standards Institute, Performance Standards for Antimicrobial Susceptibility Testing, Seventeenth Informational Supplement, Wayne, Pa, USA, M100-S17.CLSI; 2010.
25. Mirnejad R, Jeddi F, Kiani J, Khoobdel M. Etiology of spontaneous bacterial peritonitis and determination of their antibiotic susceptibility patterns. Pac J Trop in Iran. Asian Dis. 2011;1(2):116-118.
26. Sauer K, Rickard AH, Davies DG. Biofilm and biocomplexity. Microbe. 2007;7:347-353.
27. Donlan RM, Costerton JW. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002;15:167–193.
28. Sulakvelidze A, Alavidze Z, Morris JG. Bacteriophage therapy. Antimicrob Agents Ch. 2001;45:649–659.
29. Doolittle MM, Cooney JJ, Caldwell DE. Lytic infection of *Escherichia coli* biofilms by bacteriophage T4. Can J Microbiol. 1995;41:12–18.
30. Carson L, Sean P, Gorman, Gilmore BF. The use of lytic bacteriophages in the prevention and eradication of biofilms of *Proteus mirabilis* and *Escherichia coli*. FEMS Immunol Med Microbiol. 2010;59: 447–455.
31. Jamal M, Hussain T, Das CR, Andleeb S. Inhibition of clinical multi-drug resistant *Klebsiella pneumoniae* biofilm by

Siphoviridae bacteriophage. Z. Sci Lett. 2015;3(3):122-126.
32. Kiefer B, Dahl JL. Disruption of *Mycobacterium smegmatis* biofilms using bacteriophages alone or in combination with mechanical stress. Advances in Microbiology. 2015;5:699-710.
33. Zhang Y, Hu Z. Combined treatment of *Pseudomonas aeruginosa* biofilms with bacteriophages and chlorine. Biotechnol Bioeng. 2013;110(1):286–295. DOI: 10.1002/bit.24630
34. Kim L. Riddle of biofilm resistance. Antimicrob Agents Chemother. 2001;45(4): 999–1007. DOI: 10.1128/AAC.45.4.999-1007.2001
35. Pramodhini S, Niveditha S, Umadevi S, Kumar S, Stephen S. Antiobiotic resistance pattern of biofilm-forming uropathogens isolated from catheterised patients in Pondicherry, India. Australas Med J. 2012;5(7):344–348. DOI: 10.4066/AMJ.2012.1193.
36. Soto SM. Importance of biofilms in urinary tract infections: New therapeutic approaches. Adv Biol. 2014;543974. DOI:http://dx.doi.org/10.1155/2014/543974
37. May T, Ito A, Okabe S. Induction of multidrug resistance mechanism in *Escherichia coli* biofilms by interplay between tetracycline and ampicillin resistance genes. Antimicrob Agents Chemother. 2009;53(11):4628–4639. DOI: 10.1128/AAC.00454-09
38. Taraszkiewicz A, Fila G, Grinholc M, Nakonieczna J. Innovative strategies to overcome biofilm resistance. BioMed Research International. 2013;2013: 150653. DOI:http://dx.doi.org/10.1155/2013/150653
39. Kirby AE, Garner K, Levin BR. The relative contributions of physical structure and cell density to the antibiotic susceptibility of bacteria in biofilms. Antimicrob Agents Chemother. 2012;56(6):2967–2975. DOI: 10.1128/AAC.06480-11

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