

An integrating approach of bacteriophage and iron antagonizing molecule for eradication of biofilm of *E. coli* isolated from clinical samples of Sylhet city, Bangladesh

ABSTRACT

Bacterial biofilms are associated with a large number of persistent and chronic infections. Biofilm-dwelling bacteria are particularly resistant to antibiotics and immune defences, which makes it hard to eradicate biofilm-associated infections. This study aimed to analyze the effects 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 identified by crystal violet assay method in microtiter plate. Bacteriophage was isolated from drainage samples and recognized by spot test. Biofilm enhancement was studied by using a different concentration of FeCl_3 . Also, Biofilm inhibition was investigated using zinc sulphate (ZnSO_4) and bacteriophage separately and in combination in six (6) days experiment by enumeration of viable cells of biofilm after treatment. Kirby-Bauer disk diffusion method was used for antibiotic susceptibility test. The result showed that biofilm was enhanced by $10\mu\text{M}$ FeCl_3 . A significant decrease ($p < 0.01$) in viable cells of biofilm was occurred with the highest reduction of $89 \pm 1\%$ and $85.38 \pm 1.30\%$ 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 $97.22 \pm 1.37\%$ in comparison to control. However, there was significant difference ($p < 0.01$) between the combined treatment and the single treatment. The antibiotic susceptibility test showed that selected biofilm forming isolate was resistant to antibiotics tested. Finally, it might be said that combined approach by bacteriophage and iron antagonizing molecule is more significant for eradication of *E. coli* biofilm than distinct approach.

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 and 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]. 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 humans in many ways as they can form in natural and medical settings. Hancock [6] reported that treatment of chronic infections becomes more difficult as 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 in the treatment of bacterial infections that show resistance to multi drugs [7]. Due to more host-specificity of bacteriophage than antibiotics, it is less possible cause of collateral damage relating to the 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]. They are also able to infect persistent cells and to initiate complete lysis of them [10]. However, our immune system may recognize bacteriophages and clear out of our body [11]. So, phages or antibiotics when used alone may not stand a better chance for treatment of biofilm-associated bacterial infections. Therefore, treating biofilms with combination of chemically distinct antimicrobials and phages might be an effective strategy.

The importance of iron for bacterial growth has been known for many years. Singh [12] reported that iron serves as a signal in development of biofilm 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 impairment of development of biofilm formed by *K. pneumoniae*, *Pseudomonas aeruginosa* etc. [14, 15]. The iron antagonizing divalent metal ion Zn(II) could provide a tool to fight 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 as well as in combination to eradicate biofilm formation by *E.coli* isolates 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, VogesProskauer, 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 were used as positive and negative control respectively for screening of biofilm forming isolates. 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 (5g/l) and yeast extract (3g/l), and as indicator iron (FeCl_3) was added. The iron antagonizing molecule was a divalent metal ion containing salt, ZnSO_4 . The concentration of ZnSO_4 was set at a value of $500\mu\text{M}$, so that it does 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 crystal violet assay method in microtiter plate. Isolates from overnight cultured agar plates were inoculated in 5 ml of TY broth medium followed by overnight incubation at 37°C and dilution in 1:20 with TY broth. $200\mu\text{l}$ of diluted cultures were seeded in sterile 96-wells and incubated at 30°C for 48 h. After three times washing by $200\mu\text{l}$ 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 plates was conducted by using sterile distilled water to remove unbound crystal violet. The quantitative analysis of biofilm formation was carried out by adding $200\mu\text{l}$ 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 spectrophotometer). For 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* isolates was carried out by method of Cervený [19] with some modifications. In this purpose, samples were collected from drainage of M.A.G Osmani Medical College and Hospital, Sylhet, Bangladesh. This site was selected to isolate phages, as sewage is known to harbor many different bacteria and hence the possibility of prevalence of phages against different organisms. For isolation of bacteriophage, sewage samples were centrifuged at 15000 rpm for 15 min and supernatant was sterilized with a $0.45\mu\text{m}$ pore size filter. 1 ml of filtered supernatants and 10 ml sterile TY broth were mixed with 0.5ml overnight culture of *E. coli* (10^8 CFU/ml) and incubated at 37°C for 24h. The bacteria were removed by centrifugation and supernatant was filter sterilized ($0.22\mu\text{m}$ 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 this 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) and poured quickly on top of the solidified nutrient agar plate [21]. The numbers of plaques were counted after incubating the plates overnight at 37°C.

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 1ml 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 10µM, 100 µM and 1000µM FeCl_3 separately, were used to inoculate wells of 96-well microtiter plate. After overnight incubation at 37°C, medium of all wells were removed and unadhered bacteria were rinsed from one of wells containing TY broth media supplemented with different concentration of FeCl_3 for each day. Adherent biofilms were removed by scraping using mini cell scrapers (ABI Scientific Inc) from one well of 96 wells plate supplemented with different concentration of FeCl_3 , suspended in 0.85% NaCl and vortexed for 3 min. Microbial load of biofilm was enumerated by viable cell counting on overnight incubated nutrient agar plates. Percent increase of cells was noted in comparison to control that is not supplemented. For remaining wells, spent medium was replaced with sterile media and plate was re-incubated at 37°C for overnight. This method was repeated until 6th day of the experiment.

2.7 Effect of zinc sulphate (ZnSO_4) on biofilm growth

The effect of iron antagonizing molecule was conducted according to Chhibber [22] with slight modifications. In brief, 100 µl of *E.coli* inoculum (10^9 CFU/ml) was inoculated in 96 wells of microtiter plate containing 100 µl of TY broth media supplemented with 10µM FeCl_3 alone and 500µM ZnSO_4 along with FeCl_3 for determination of biofilm growth depletion with ZnSO_4 treatment. After overnight incubation at 37°C medium of all wells were removed and adhered bacteria were detached by scraping using mini cell scrapers (ABI Scientific Inc) from one of control wells containing 10µM FeCl_3 supplemented medium and wells containing supplementation of 10 µM FeCl_3 with 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 medium was replaced with fresh media and plate was reincubated at 37°C overnight. This method was repeated until 6th day of testing.

2.8 Effect of bacteriophage alone and in combination with ZnSO₄ on biofilm growth

The bacteriophage treatment on biofilm was conducted according to Chhibberet [22] with slight modifications. For determination of the effectiveness of bacteriophage alone and in combination with the ZnSO₄ in treating the *E. coli* biofilm, 100µl of bacterial inoculum was inoculated in microtiter plate containing 100µl of media supplemented with 10µM FeCl₃ alone and with combination of 500µM ZnSO₄. After overnight incubation at 37°C, from one of wells supplemented with 10µM FeCl₃ and wells supplemented with 10 µM FeCl₃ in combination with 500µM ZnSO₄, unadhered bacteria were removed. Subsequently, bacteriophage at multiplicity of infection [M.O.I: ratio of infectious agent (phage) to infection target (bacterial cell)] of 1 was used for expose to biofilms for 6 h followed by washing with 0.85% NaCl and viable bacterial cells counting (one well for each day). One of wells containing biofilm grown in iron supplemented TY broth media served as control. For remaing wells, washed-out medium was replaced with fresh media and plate was reincubated at 37°C overnight. This method was repeated until 6th day of testing.

2.9 Antibiotic susceptibility test

Biofilm forming *E. coli* isolate was tested for their 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 5ml Luria Bertani Broth and incubated 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 the help of 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 zones of inhibition around the antibiotic disc. 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 in case of *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 FeCl₃ and biofilm reduction by different

treatments were 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 used for biofilm assay. Biofilm formation was assayed by using crystal violet assay method in microtiter plate. The average values of O.D. at 570 nm of different *E. coli* isolates along with the *E. coli* PHL628 and *E. coli* 565 used as positive control and negative control respectively, shown in figure 1. From the result, it was found that isolate no. 6 was strong biofilm producer as 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 no. 6 showed significantly higher ($p < 0.01$) optical density than that of other isolates except isolate 3, isolate 7 and *E. coli* PHL628.

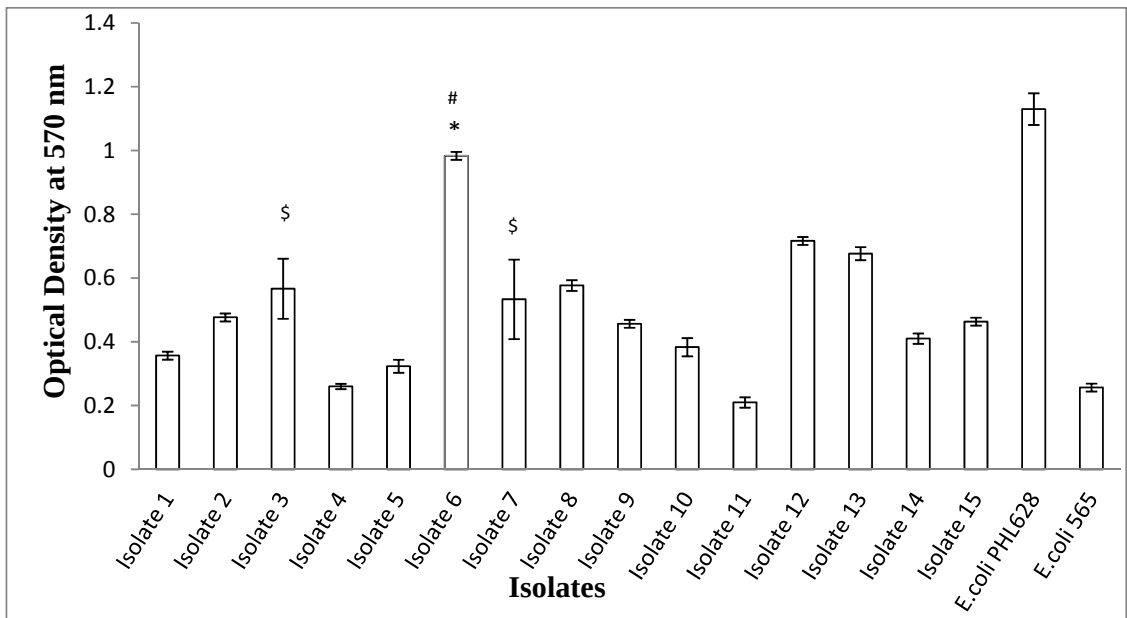


Fig. 1. The optical density (O.D) at 570nm of different *E. coli* isolates was represented by subtraction of the mean O.D value obtained for each isolate from mean O.D value of controls without inoculum. The highest optical density of isolates at 570nm indicates strong biofilm producer. Isolate 6 shows highest value of optical density except *E. coli* PHL628. *E. coli* PHL628 and *E. coli* 565 serve 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).

3.2 Bacteriophage isolation

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

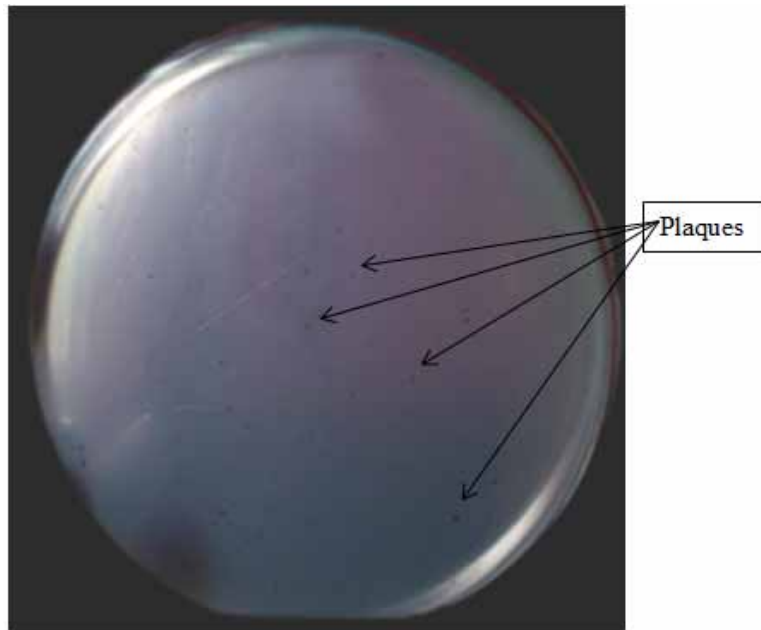


Fig. 2. Clear zone on bacterial culture containing nutrient agar plate indicates the presence of bacteriophage that is specific to the selected biofilm forming *E. coli* isolate.

3.3 Biofilm formation in iron supplemented media

The effect of supplementation with different concentrations of FeCl_3 in TY media was studied on the biofilm growth. The significant increase ($p < 0.05$) in bacterial count was taken place by $10 \mu\text{M}$ FeCl_3 supplementation in medium in comparison to non-iron supplemented control in all (6) days with highest increase ($13.13 \pm 0.56\%$) of cells count on 3rd day (figure 3). The supplementation of medium with $100 \mu\text{M}$ and $1000 \mu\text{M}$ FeCl_3 caused highest increase of $8.83 \pm 0.62\%$ and $8.13 \pm 0.50\%$ in bacterial count of biofilm on 4th day and 3rd day, respectively. $10 \mu\text{M}$ FeCl_3 supplementation significantly enhanced ($p < 0.05$) the bacterial count in comparison to other supplementations and it had been used in further study.

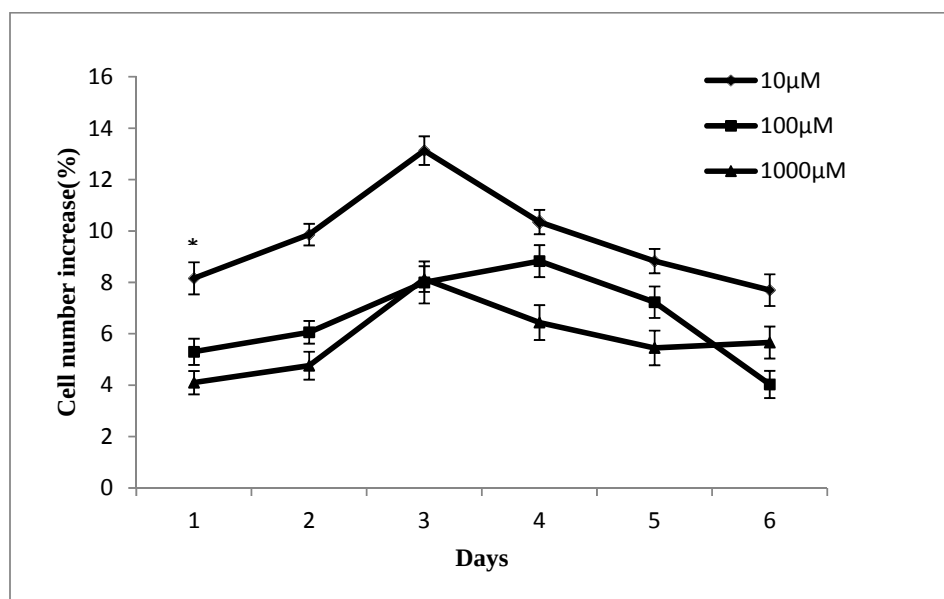


Fig. 3. Percent increase in cell number in *E. coli* biofilm grown in iron supplemented TY media and control has no cell number increase. * $p < 0.05$ (10µM FeCl_3 vs control/ 100µM FeCl_3 / 1000µM FeCl_3) in each day.

3.4 Effect of ZnSO_4 on biofilm growth

E. coli biofilm grown in TY media supplemented with 500µM ZnSO_4 and 10µM FeCl_3 was affected by iron antagonizing molecule e.g ZnSO_4 and it was observed that although biofilm growth was improved by 10µM FeCl_3 supplementation but addition of 500µM ZnSO_4 alone showed inhibitory effect on biofilm growth in comparison to control wells containing iron. The results presented in figure 4 showed that there was significant loss ($p < 0.01$) of $89 \pm 1\%$ to $80.53 \pm 1.50\%$ in viable cells in first 3 days in comparison to control, when ZnSO_4 was used. In rest days, significant loss ($p < 0.01$) of $80.38 \pm 1.45\%$ to $77 \pm 1\%$ was occurred 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 500µM of ZnSO_4 in wells of TY media supplemented with 10µM FeCl_3 . The results from the study showed that addition of phages to the wells containing 10µM FeCl_3 caused significant loss ($p < 0.01$) of $85.38 \pm 1.30\%$ to $72.28 \pm 2.48\%$ in 1-3 day old biofilm in comparison to control wells supplemented with 10µM FeCl_3 alone (figure 4). In the remaining days, viable cells reduced to $72.5 \pm 1.34\%$ to 65.86 ± 1.52 ($p < 0.01$). The reduction of cells in biofilm by phage treatment is significantly less ($p < 0.05$) than ZnSO_4 treatment in days except 2nd day. Addition of 500µM ZnSO_4 as well as phages in 10µM FeCl_3 supplemented wells resulted in significant eradication ($p < 0.01$) of $97.22 \pm 1.37\%$ to $94.31 \pm 1.69\%$ in viable cells of biofilm in comparison to control in 1-3 day (figure 4). But in the old biofilm (4-6 day old) the reduction was $85.08 \pm 1.95\%$ to $79.83 \pm 1.31\%$ ($p < 0.01$). The combined treatment caused significantly higher reduction ($p < 0.01$) of cells in 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 occurred by combined approach than that of phage treatment.

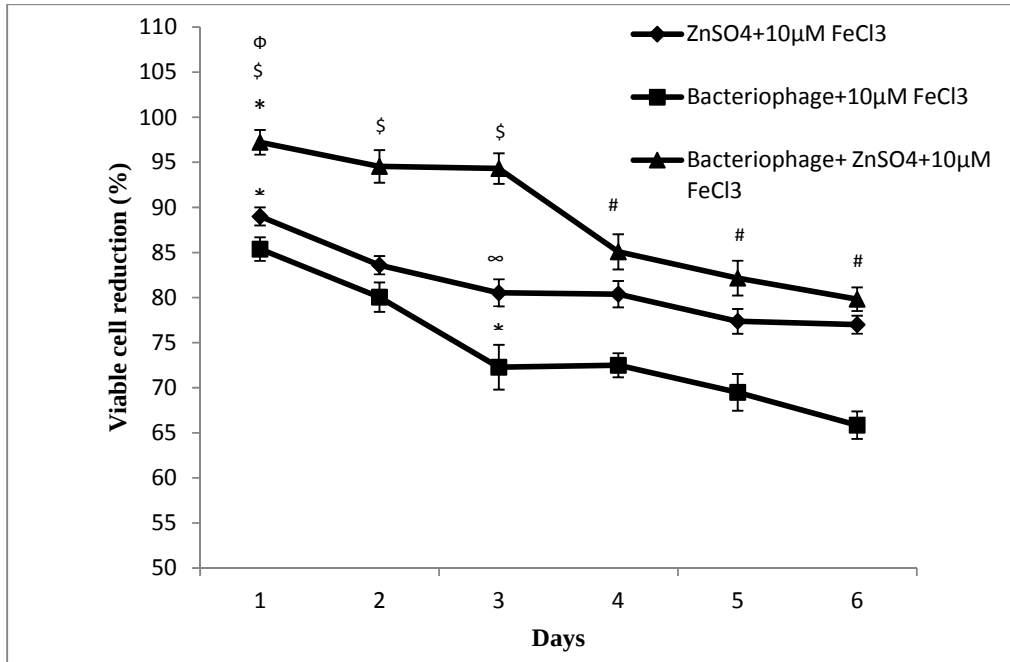


Fig. 4. Percent reduction of viable cells in *E. coli* biofilm treated with 500µM zinc salt (ZnSO₄) and bacteriophage alone as well as in combination. TY media supplemented with 10µM FeCl₃ served as control and it has no cell number reduction. * $p < 0.01$ (Bacteriophage+ZnSO₄+10µM FeCl₃/ ZnSO₄+10µM FeCl₃/ Bacteriophage+10µM FeCl₃ vs 10µM FeCl₃) in each day, $^{\$}p < 0.01$ (Bacteriophage+ZnSO₄+10µM FeCl₃ vs ZnSO₄+10µM FeCl₃), $^{\#}p > 0.05$ (Bacteriophage+ZnSO₄+10µM FeCl₃ vs ZnSO₄+10µM FeCl₃), $^{\Phi}p < 0.01$ (Bacteriophage+ZnSO₄+10µM FeCl₃ vs Bacteriophage+10µM FeCl₃) in each day, $^{\infty}p < 0.05$ (ZnSO₄+10µM FeCl₃ vs Bacteriophage+10µM FeCl₃) in days except 2nd day..

3.6 Antibiotic susceptibility assay

Antibiotic susceptibility of strong *E. coli* biofilm producer (isolate 6) obtained from our study and weak biofilm producer (*E. coli* PHL565) was observed using different antibiotics e.g. ampicillin, ceftriaxone, gentamicin, tetracycline and azithromycin. The obtained diameter of zone inhibition was compared with antimicrobial susceptibility testing standards (table 1) for *E. coli* and it was found that *E. coli* (isolate 6) were 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.

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

Antibiotics	<i>E. coli</i> (isolate 6)		<i>E. coli</i> 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

4. DISCUSSION

Biofilm causes life threatening diseases by recurrent and chronic infections because of higher level of drug resistance caused by biofilm structures. Previous studies showed that the sessile bacterial cells exhibit higher resistance to antibiotics than that of planktonic cells [25, 26]. Therefore, it is essential to exploit an alternative antibacterial therapy for combating nuisance bacteria. Various strategies had been used 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 and thus they are important for reduction of biofilm formation by infectious isolates e.g *Klebsiella* and UTI *E. coli* [6]. Reduced biofilm formation of our *E. coli* isolate in the presence of ZnSO₄ was observed in the present study. From study, we found that ZnSO₄ treatment showed significant loss ($p < 0.01$) in biofilm in all days causing highest loss of $89 \pm 1\%$. Besides ZnSO₄, another study reported ZnCl₂ to reduce biofilm formation of *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis* and *Salmonella typhimurium*, and ZnCl₂ at concentration of $500 \mu \text{mol l}^{-1}$ significantly reduced ($p < 0.001$) *E. coli* biofilm formation by 80% [16]. To reduce biofilm, metal ions such as Co(II), Mn(II), Zn(II), Cd(II) are possible cause and they impair Fur-controlled iron uptake systems like enterobactin, ferric dicitrate, aerobactin involved in biofilm formation [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 therapeutics is showing increasingly attractive to control human infections as many antibiotics efficacy is declined and successful treatment of infections in both humans and animals with bacteriophage therapy was reported [28]. Doolittle [29] observed that the extracellular matrix of *E. coli* biofilm did not protect the bacterial cells from infection with phage T₄ 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 occurred in *E. coli* biofilm with highest loss of $85.38 \pm 1.30\%$. Although biofilm reduction by bacteriophage in our study differs from previous study that showed 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 established biofilm [30], yet it might be quite good. Also, reduction of biofilm in our

study is higher than reduction of biomass of *Klebsiella pneumoniae* biofilm in which 40.98% and 32.39% loss of biomass occurred for 24h and 72h old biofilm respectively by Siphoviridae bacteriophage treatment for 4 h [31]. Phage treatment using a titer of 1×10^6 PFU/well caused 96% reduction of *Mycobacterium smegmatis* [32].

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

Resistance is the growth ability of a microorganism in a condition remaining higher level of antimicrobial [34]. Multidrug resistant tendency has been shown 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, present study needs to investigate antibiotic susceptibility of selected bacteria. Biofilm forming *E. coli* (isolate 6) showed resistant 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 may be caused by the matrix of biofilm forming physical and chemical barrier to antibiotics [38]. Another study by Kirby [39] showed that the density and physiological state of the culture may 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 molecules and bacteriophages alone or in combination are able to eradicate *E. coli* biofilm. But the results suggest that a combination of ZnSO_4 and phages is more effective in inhibiting biofilm. The finding of antibiofilm activities 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 biofilms.

Consent Disclaimer: NA

Ethical Approval: NA

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