

*Original Research Article*

# **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 defenses, which makes it hard to eradicate biofilm-associated infections. For isolation of biofilm forming bacteria, clinical samples were collected from renowned hospital and diagnostic center in Sylhet city, Bangladesh. Development of biofilm is solely dependent on iron. So, the present study was performed with the addition of zinc sulphate to inhibit iron action on the development of biofilm production. Further, we employed bacteriophage approach along with zinc sulphate for the effective reduction of biofilm. The result showed a decrease of 89% and 83.60%-77% of viable cells in biofilm in first day and last five days respectively with only Zinc sulphate (ZnSO<sub>4</sub>) treatment. Bacteriophage treatment caused 85.38%-80.05% and 72.28%-65.86% loss of viable cells in first two days and last four days respectively. But 97.22%-94.31% and 85.08%-79.83% loss of viable cells occurred in biofilm treated with integrated approach of bacteriophage and zinc sulphate in first three days and last three days respectively. 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 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 ( $\text{ZnSO}_4$ ) 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. MATERIAL 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. 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. *E. coli* PHL628 and *E. coli* PHL565 were used as positive and negative control for biofilm development. For routine bacterial culture, nutrient broth was used. Biofilm experiment in 96 well microtiter plates was conducted in Tryptone Yeast Extract Broth (TY) containing casein enzymic hydrolysate (5g/l) and yeast extract (3g/l), and as indicator iron( $\text{FeCl}_3$ ) was added. The iron antagonizing molecule was a divalent metal ion containing salt,  $\text{ZnSO}_4$ . The concentration of  $\text{ZnSO}_4$  was set at a value of 500 $\mu\text{M}$ , so that it does not inhibit the growth of planktonic bacteria [6].

## 74 **2.2 Screening of *E. coli* isolates for biofilm formation**

75 According to the study of Cucarella [17] with some modifications, we screened 15 clinical isolates of  
 76 *E.coli* for their capability of biofilm formation by microtiter plate method. Isolates from overnight  
 77 cultured agar plates were inoculated in 5 ml of TY broth medium followed by overnight incubation at  
 78 37°C and dilution in 1:20 with TY broth. 200 µl of diluted cultures was seeded in sterile 96-wells and  
 79 incubated at 300C for 48 h. After three times washing by 200µl of phosphate buffered saline solution,  
 80 wells were dried for 20 min at 60°C. Adherent organisms forming biofilms were fixed with methanol in  
 81 wells and stained with 1% crystal violet for one minute. After rinsing three times with distilled water, all  
 82 wells were filled by 200µl 95% ethanol to release the dye. The contents of each well were then  
 83 transferred to a cuvette and the optical density was measured at 570 nm using spectrophotometer  
 84 (JENWAY spectrophotometer). For compensation of background absorbance, the OD values of the  
 85 controls were averaged and subtracted from the mean OD value obtained for each strain. For each  
 86 strain, the experiment was repeated three times and the average value was calculated.

## 87 **2.3 Isolation of Bacteriophage**

88 The isolation of phage specific to *E. coli* isolates was carried out by method of Cerveny [18] with some  
 89 modifications. In this purpose, samples were collected from drainage of M.A.G Osmani Medical  
 90 College and Hospital, Sylhet, Bangladesh. This site was selected to isolate phages, as sewage is  
 91 known to harbor many different bacteria and hence the possibility of prevalence of phages against  
 92 different organisms. For isolation of bacteriophage, sewage samples were centrifuged at 15000 rpm  
 93 for 15 min and supernatant was filter sterilized with Millipore filter (0.45 µm pore size). 1 ml of filtered  
 94 supernatants and 10 ml sterile TY broth were mixed with 0.5ml overnight culture of *E. coli* ( $10^8$   
 95 CFU/ml) and incubated at 37°C for 24h. The bacteria were removed by centrifugation and supernatant  
 96 was filter sterilized (0.22 µm pore size syringe filter) for the presence of phage.

## 98 **2.4 Spot test and plaque assay**

99 For the detection of phage in supernatant, spot test was carried out as described by Chang [19]. In  
 100 this case, the phage suspension was serially diluted ( $10^{-1}$ - $10^{-3}$ ). 0.1 ml of diluted phage suspension  
 101 and 0.3 ml overnight culture of *E. coli* were mixed with 3 ml molten soft agar (0.75 % agar, w/v) and  
 102 poured quickly on top of the solidified nutrient agar plate [20]. The numbers of plaques were counted  
 103 after incubating the plates overnight at 37°C.

## 105 **2.5 Phage purification**

106 When plaques were identified, a pure suspension was prepared by carefully piercing agar  
 107 surrounding the plaques with a sterile wire loop and picking out the agar “block” containing the  
 108 plaques into microfuge tube containing 1ml of PBS. Phage was stored in the refrigerator at 4°C for  
 109 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 separately, were used to inoculate wells of 96-well microtiter plate. After overnight incubation at 37°C, substances of all wells were removed and unadhered bacteria were rinsed from one of wells containing TY broth media supplemented with different concentration of FeCl<sub>3</sub> for each day. Adherent biofilms were removed from one well of 96 wells plate supplemented with different concentration of FeCl<sub>3</sub>, 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 experiment.

## 2.7 Effect of zinc sulphate (ZnSO<sub>4</sub>) on biofilm growth

The effect of iron antagonizing molecule was conducted according to Chhibber [21] 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<sub>3</sub> alone and 500µM ZnSO<sub>4</sub> along with FeCl<sub>3</sub> for determination of biofilm growth depletion with ZnSO<sub>4</sub> treatment. After overnight incubation at 37°C substances of all wells were removed and adhered bacteria were detached from one of control wells and wells containing supplementation of 10 µM FeCl<sub>3</sub> with ZnSO<sub>4</sub> (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 ZnSO<sub>4</sub> on biofilm growth

The bacteriophage treatment on biofilm was conducted according to Chhibberet [21] with slight modifications. For determination of the effectiveness of bacteriophage alone and in combination with the ZnSO<sub>4</sub> in treating the *E. coli* biofilm, 100µl of bacterial inoculum was inoculated in microtiter plate containing 100µl of media supplemented with 10µM FeCl<sub>3</sub> alone and with combination of 500µM ZnSO<sub>4</sub>. After overnight incubation at 37°C, from one of wells supplemented with 10µM FeCl<sub>3</sub> and wells supplemented with 10 µM FeCl<sub>3</sub> in combination with 500µM ZnSO<sub>4</sub>, 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 hours 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 remaining wells, previously mentioned procedure was followed.

## 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 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 ( $1.5 \times 10^8$  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. The susceptibility of a non-biofilm former (*E. coli* 565) was tested to compare with biofilm former.

## 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 microtitre plate method. 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 was most near to positive control and it was used for further study.

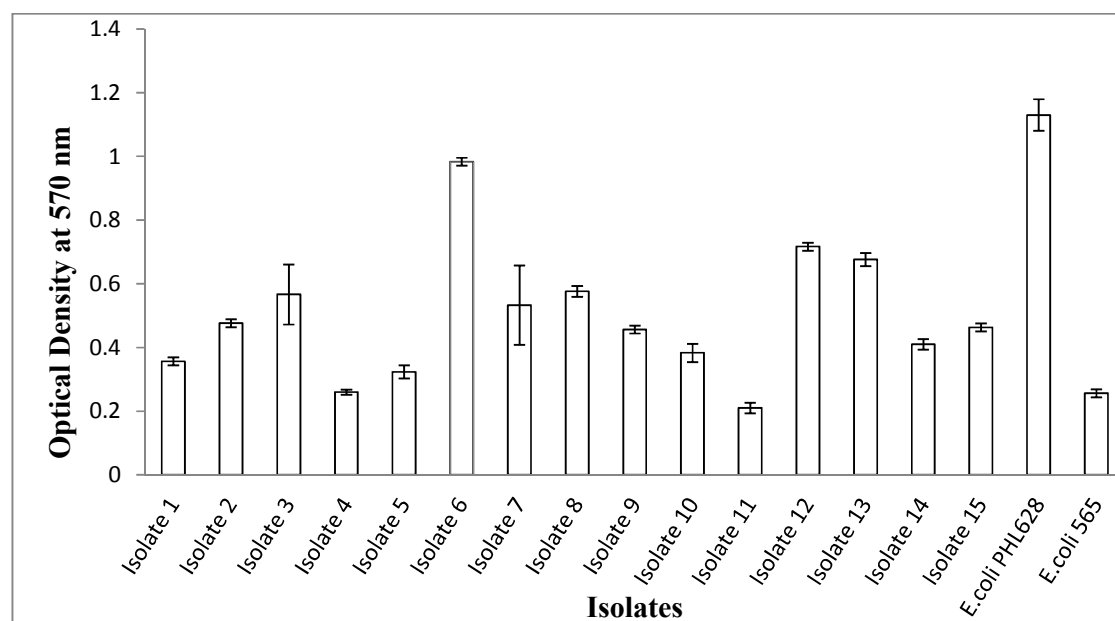


**Fig. 1. The optical density (O.D) at 570nm of different *E. coli* isolates was represented by subtraction of the mean O.D value obtained for each isolate from mean O.D value of controls. Highest optical density of isolates at 570nm indicates strong biofilm producer. Isolate 6 shows highest value of optical density except *E. coli* PHL628. *E. coli* PHL628 and *E. coli* 565**

serve as positive and negative control for biofilm development. Bar represent mean±standard error (SE) for three replicates.

### 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  $1 \times 10^8$  PFU/ml for biofilm former *E. coli* on overnight incubated nutrient agar plate.

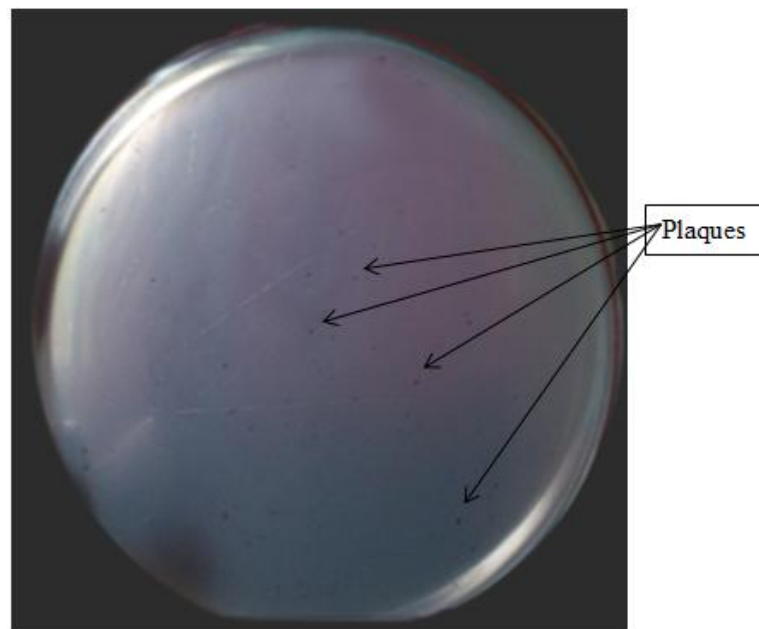


**Fig. 2. Clear zone on bacterial culture containing nutrient agar plate indicates the presence of bacteriophage that is specific to the selected biofilm forming *E. coli* isolate.**

### 3.3 Biofilm formation in iron supplemented media

The effect of supplementation with different concentrations of  $\text{FeCl}_3$  in TY media was studied on the biofilm growth. The increase in bacterial count of young 2nd day biofilm was 9.86% followed by a peak on day 3 (13.13%) and 7.7-10.35% from 4th to 6th day when media was supplemented with  $10\mu\text{M}$   $\text{FeCl}_3$ . But in the case of  $100\mu\text{M}$   $\text{FeCl}_3$  supplementation, highest increase of bacterial count in the biofilm was 8.83% on 4th day and remaining days showed increase with a range of 4.03-8%. Also,  $1000\mu\text{M}$   $\text{FeCl}_3$  supplementation caused cell number increase by 8.13% on 3rd day and remaining days showed increase with a range of 4.1-6.44% (figure 3). Therefore, addition of  $10\mu\text{M}$   $\text{FeCl}_3$  enhanced the growth in the bacterial count in comparison to non-iron supplemented control and other supplemented wells.

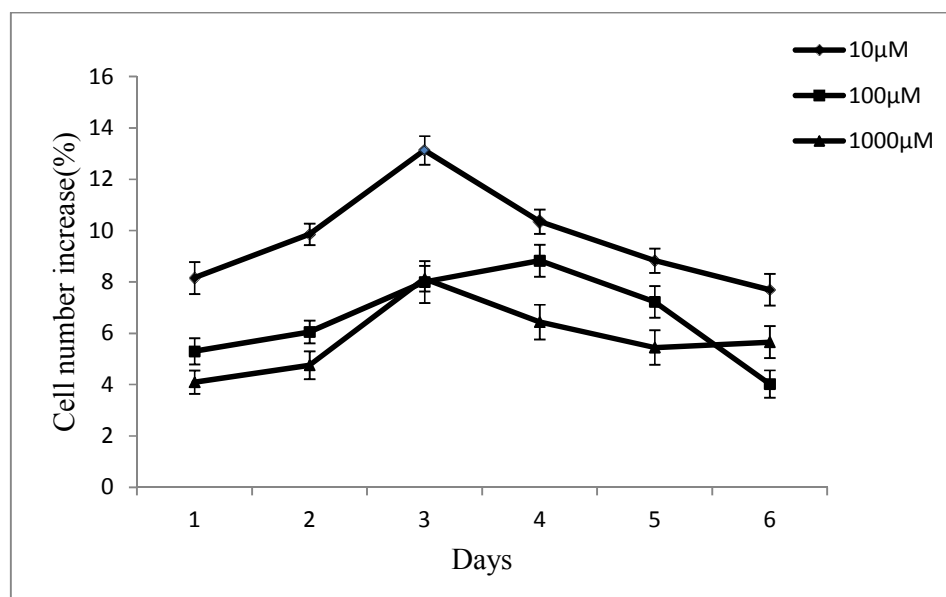


**Fig. 3. Percent increase of cell number in *E. coli* biofilm grown in iron supplemented TY media. Bar represent mean±standard error (SE) for three replicates.**

### 3.4 Effect of ZnSO<sub>4</sub> on biofilm growth

*E. coli* biofilm grown in TY media supplemented with 500µM ZnSO<sub>4</sub> and 10µM FeCl<sub>3</sub> was affected by iron antagonizing molecule e.g ZnSO<sub>4</sub> and it was observed that although biofilm growth was improved by 10µM FeCl<sub>3</sub> supplementation but addition of 500µM ZnSO<sub>4</sub> alone showed inhibitory effect on biofilm growth in comparison to control wells containing iron. The results presented in figure 4 showed that there were 89%, 83.60% and 80.53% loss of viable cells on 1st, 2nd and 3rd day respectively when ZnSO<sub>4</sub> was used. On 4th, 5th and 6<sup>th</sup> day loss of viable cells was observed 80.38%, 77.37% and 77% respectively.

### 3.5 Effect of bacteriophage alone and in combination with ZnSO<sub>4</sub> 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<sub>4</sub> in wells of TY media supplemented with 10µM FeCl<sub>3</sub>. The results from the study showed that addition of phages to the wells containing 10µM FeCl<sub>3</sub> caused 85.38%, 80.05% and 72.28% loss of viable cells on 1st, 2nd and 3rd day respectively in comparison to control wells supplemented with 10µM FeCl<sub>3</sub> alone. In the rest days, reduction of cells was within a range of 65.86-72.5% that was less than the reduction of cells in first three days (figure 4). Addition of 500µM ZnSO<sub>4</sub> as well as phages in 10µM FeCl<sub>3</sub> supplemented wells resulted in eradication of 97.22%, 94.55% and 94.31% of viable cells in biofilms on 1st, 2nd and 3rd day respectively. But in the old biofilms (4-6 day old) the reduction was 79.83%-85.08% (figure 4).

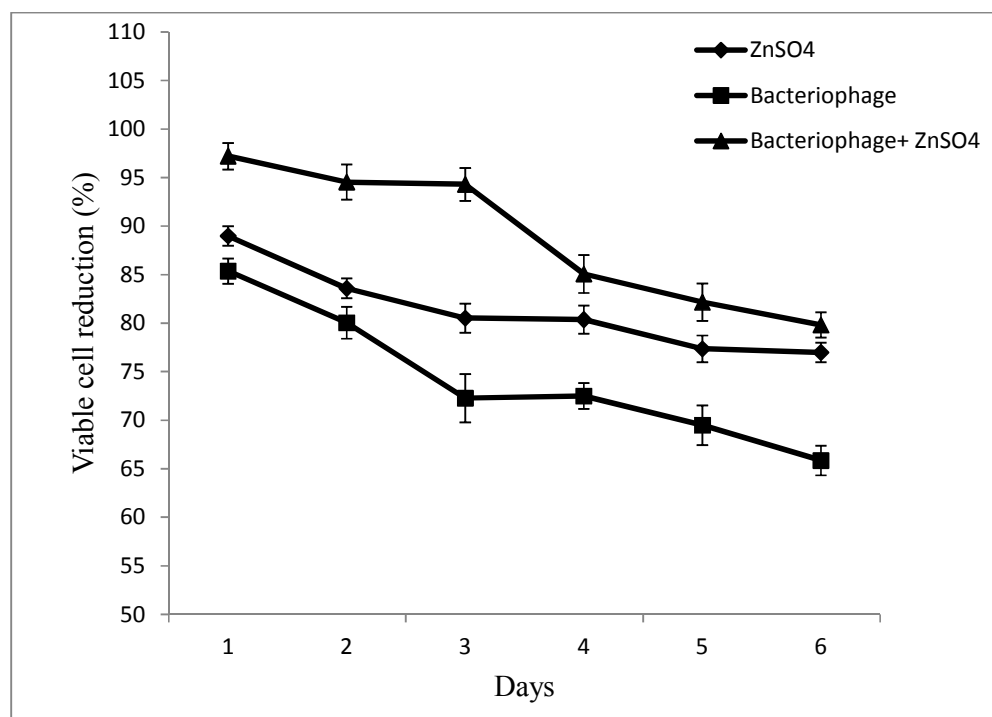


**Fig. 4. Percent reduction of viable cells in *E. coli* biofilm treated with 500μM zinc salt (ZnSO<sub>4</sub>) and bacteriophage alone as well as in combination. TY media supplemented with 10μM FeCl<sub>3</sub> served as control. Bar represent mean±standard error (SE) for three replicates.**

### 3.6 Antibiotic susceptibility assay

Antibiotic susceptibility of strong *E. coli* biofilm producer (isolate 6) obtained from our study and non-biofilm producer (*E. coli* 565) was observed using different antibiotics e.g. Azithromycin, Ceftriaxone, Gentamicin, Tetracycline, Ampicillin. From the test, it was found that antibiotics (Ampicillin, Ceftriaxone, Azithromycin) showed no zone of inhibition in lawn of biofilm producer when tested (Table 1). In addition, comparatively large zone of inhibition was found in antibiotic susceptibility test of non-biofilm producer.

**Table 1. Antibiotic susceptibility of both biofilm former and non-biofilm former of *E. coli* isolates by the disk diffusion method.**

| <i>E. coli</i> isolates                       | Name of the antibiotics and zone of inhibition (mm in diameter) |     |     |    |     |
|-----------------------------------------------|-----------------------------------------------------------------|-----|-----|----|-----|
|                                               | AMP                                                             | CTR | GEN | TE | AZM |
| Biofilm former<br>( <i>E. coli</i> isolate 6) | -                                                               | -   | 5   | 6  | -   |
| Non-biofilm former<br>( <i>E. coli</i> 565)   | 7                                                               | 9   | 12  | 13 | 12  |

AMP-Ampicillin, CTR-Ceftriaxone, GEN-Gentamicin, TE-Tetracycline, AZM-Azithromycin, (-)-no zone of inhibition.

#### 4. DISCUSSION

Biofilms cause life threatening diseases and they are resistant to antibiotics. The antibiotic resistance study of our *E. coli* isolates showed that the biofilm forming isolate was more resistant to the antibiotics in comparison with non-biofilm forming isolate (*E. coli* 565). This result shows agreement with the previous studies showing that the sessile bacterial cells exhibit higher resistance to antibiotics than the planktonic cells [22, 23]. 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 ZnSO<sub>4</sub> was observed in the present study. From study, we found that ZnSO<sub>4</sub> treatment caused highest loss (89%) of viable cells in biofilm on 1st day in comparison to control. Although on the rest days a loss of viable cells in biofilm occurred but it was comparatively less than the first day. Besides ZnSO<sub>4</sub>, another study reported ZnCl<sub>2</sub> to reduce biofilm formation of *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis* and *Salmonella typhimurium* and ZnCl<sub>2</sub> at concentration of 500 μmol l<sup>-1</sup> reduced *E. coli* biofilm formation by 80% [16](Wu et al. 2013). However, according to our study *E. coli* biofilm reduction by Zinc (II) ion is comparatively high. 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 [24]. 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 [25]. Doolittle [26] observed that the extracellular matrix of *E. coli* biofilm did not protect the bacterial cells from infection with phage T<sub>4</sub> and that phage-infected cells were associated with the biofilm surface. When we used bacteriophage at MOI-1 in the treatment of biofilm for 6 hours, highest loss (85.38%) of viable cells occurred in *E. coli* biofilm. Although biofilm reduction by bacteriophage in our study differs from previous study that showed reduction (90%) of *P. mirabilis* and *E. coli* biofilm [27], 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 [28]. Phage treatment using a titer of 1 × 10<sup>6</sup> PFU/well caused 96% reduction of *Mycobacterium smegmatis* [29].

The combination of phage and ZnSO<sub>4</sub> on *E. coli* biofilm treatment was used. We found that a significant loss (97.22%-94.31%) of cells occurred in younger biofilm. There was also a loss (79.83%-85.08%) of viable cells in older biofilm. The report by Zhang [30] showed that combined treatment of phages ( $3 \times 10^7$  PFU/mL) and chlorine (210 mg/L) reduced *Pseudomonas aeruginosa* biofilm development by  $94 \pm 2\%$ . In addition, the combination of cobalt sulfate (CoSO<sub>4</sub>) and bacteriophage was used by Chhibber [21] for inhibition of *Klebsiella pneumoniae* B5055 biofilm. Therefore, combined treatment by bacteriophage and antimicrobial chemicals is promising.

## 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<sub>4</sub> and phages is more effective in inhibiting biofilm. The finding on antibiofilm activities of ZnSO<sub>4</sub> and bacteriophage is significant and further study needs to be carried out to determine the potential use of bacteriophage therapy and ZnSO<sub>4</sub> treatment for the eradication of biofilms.

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