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# DETERMINATION OF THE EFFICACY OF AZITHROMYCIN ON BIOFILM-FORMING UROPATHOGENIC *ESCHERICHIA COLI* ISOLATED FROM URINARY TRACT INFECTION SAMPLES

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*Key words:* Azithromycin, *E. coli*, Biofilm, Uropathogen

**Abstract**

The ability of *E. coli* to form complex surface-associated communities, called biofilms, contribute to its resistance and persistence in Urinary Tract Infection (UTI). In the present study, the ability of uropathogenic *E. coli* (n=30) isolated from UTI patients to form biofilm and the concentration of Azithromycin to inhibit or kill planktonic and biofilm phase bacteria were investigated. Effects of sub-Minimum Inhibitory Concentration (MIC) of Azithromycin on biofilm formation was investigated. An attempt was also taken to identify the quorum sensing molecule released by *E. coli* in biofilm. Among the 30 isolates, 6 (20%) were very strong, 8 (26.67%) were strong, 13 (43.33%) were moderate and 3 (10%) were weak biofilm producers. MIC and MBC (Minimum bactericidal concentration) of planktonic phase bacteria were determined and compared with MRC (Minimum regrowth concentration) and MBEC (Minimum biofilm eradication concentration) of biofilm population. MIC values ranged between 0.5 µg/ml to greater than 512 µg/ml. MBC values were measured for ten isolates and the range found was between 8-64 µg/ml. It was found that MRC values were 2-256 times greater than MIC values and MBEC values were 16-256 times greater than corresponding MBC values. After subjecting sub MIC Azithromycin levels to 10 selected isolates, 6 lost the ability to form curli fimbriae and 2 lost their capability of motility in planktonic stage, but none of this ability was lost in biofilm stage. Viable cell count of planktonic cells incubated in sub MIC Azithromycin concentration showed significant decrease of cell number whereas the number of planktonic cells without antibiotic increased significantly. In contrast, for biofilm forming cells, no change in cell number was seen with progressing time for either the presence or absence of antibiotic indicating that biofilm formation could not be inhibited by sub MIC concentrations of Azithromycin. It can be implied that Azithromycin is not the proper drug of choice for eradication of biofilm formed by *E. coli*. The present study failed to identify the quorum sensing molecule released by the test isolates by the method employed.

## Introduction

Bacterial biofilms are the predominant mode of bacterial growth, reflected in the observation that approximately 80% of all bacterial infections are related to biofilms[1]. Biofilms are defined as microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a

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matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription[2-5]. Biofilms are resistant to physical forces such as the shear forces produced by blood flow and the washing action of saliva. Organisms within biofilms can withstand nutrient deprivation, pH changes, oxygen radicals, disinfectants, and antibiotics better than planktonic organisms. Biofilms are also resistant to phagocytosis, and the phagocytes that attempt an assault on the biofilm may actually do more harm to surrounding tissues than to the biofilm itself. The chronic nature of certain infections is inarguably due to the development of a resilient biofilm.

*E. coli* is a rod-shaped Gram-negative bacterium causing a large number of nosocomial and community infections such as urinary tract infections (UTIs) and prostatitis. It has the ability to secret toxins, polysaccharide and can form biofilm both *in vivo* and *in vitro*[6]. Different environmental conditions affect the capability of *E. coli* to form biofilm[7]. Thickness of *E. coli* biofilm may be of hundreds of microns and posing a difficulty in treatment with antibiotics due to presence of exopolymers[7]. Urinary tract infections (UTIs) are among the most common bacterial infections in humans, uropathogenic *Escherichia coli* (UPEC) being the most frequent etiological agent (80%–90%)[8, 9]. Forty percent of women and 12% of men have a UTI episode at least once in their life. Besides being the major causative agent for recurrent urinary tract infections, *E. coli* biofilm is also responsible for indwelling medical device-related infectivity such as urethral and intravascular catheters, prosthetic joints and shunts and prosthetic grafts[10]. The formation of biofilm by *E. coli* on catheters makes catheter-associated urinary tract infections (CAUTI) one of the most frequent nosocomial infections[10].

Azithromycin has been successful in eradicating the biofilm of Gram negative *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Porphyromonas gingivalis*. In Bangladesh, Azithromycin is a commonly used readily available over-the-counter drug which is also within the affordable limit of the general mass. Hence, the use of Azithromycin to treat UTI is also a common phenomenon. In this study, effect of Azithromycin was investigated on biofilm formation of *E. coli.* Minimum Biofilm Eradication Concentration (MBEC) assays were developed for rapid and reproducible antimicrobial susceptibility testing for bacterial biofilms in the anticipation that the MBEC would be more reliable for selection of clinically effective antibiotics[11]. MRC and MBEC methods make it possible to study the influence of different concentrations of antibiotics on biofilm phase bacteria. Comparing them with MIC and MBC gives a clear idea of antibiotic susceptibility or resistance against the respective antibiotic.

During biofilm formation many species of bacteria are able to communicate with one another through a mechanism called quorum sensing[6]. It is a system of communication with other cells to co-ordinate gene expression and response related to the density of their local population. During quorum sensing, signaling molecules attach to receptors of

new bacteria and help in transcription of genes within a single species of bacteria as well as between different bacterial species[12]. Many clinically associated bacteria use QS for the regulation of the collective production of virulence factors. Detection of the molecule involved in quorum sensing is necessary because biofilm formation can be curbed by inhibiting/inactivating the quorum sensing molecule. In view of these circumstances, the present study was designed to determine the effect of Azithromycin on biofilm forming *E. coli*. The findings from this study will help the treatment regime, establish the ability of biofilm formation by *E. coli* and to evaluate the effect of Azithromycin against planktonic and biofilm phase bacteria. The study also aims to identify quorum sensing molecules released by biofilm forming *E. coli* in the presence of Azithromycin, so that future studies could be designed to reduce biofilm forming ability of these bacteria by inactivating/ inhibiting the molecules involved.

## Materials and Methods

*Isolation of clinical bacteria:* A total of forty-three clinical samples were collected from a renowned hospital of Dhaka city. Samples were maintained in glycerol broth as a stock. Samples from glycerol broth were revived in Trypticase Soy Broth (TSB). After culturing on Trypticase Soy Agar (TSA), an isolated colony was streaked on to MacConkey and EMB agar for presumptive identification of the isolate. Both the plates were incubated at 37°C for 24 h. Pink colonies and colonies with green metallic sheen in MacConkey and EMB agar respectively were indication of *E. coli*. Specific biochemical reactions such KIA test, MIU, MR and citrate test were used to identify *E. coli*.

*Determination of MIC and MBC:* Microtitre plate was used and each well of a 96-well microtiter plate was aliquoted with 50 µl of Mueller-Hinton broth (MHB). As sterility control, 100 µl of MHB was used. Growth control media contained MHB with 10% Dimethylsulfoxide (DMSO). Azithromycin was initially dissolved in 95% ethanol and diluted in MHB. A serial 2-fold dilution was performed starting from 128µg/ml. Broth inoculum was standardized according to the protocol by Wiegand *et al*.[13] McFarland (0.5 standard) broth inoculum was diluted to the ratio of 1 : 100 and added into 1st-11$^{th}$ well to achieve the final inoculum size at $5 \times 10^5$ CFU/ml. Then the microtiter plate was kept overnight at room temperature. At the end of the incubation period, 15 µl of 0.02% resazurin were added into all wells and plates were re-incubated for additional one hour. Color change of the dye from blue to pink indicated cell viability. MIC was considered as the lowest concentration of antibiotic where pink color was absent. For MBC determination subculturing was done in nutrient agar plate taking concentration from and above MIC. Antibiotic concentration where there was no growth in the plate was considered as MBC.

Biofilm formation was optimized using different conditions. TSB medium was selected for biofilm production after optimization. *Escherichia coli* ATCC 25922 was used

as positive control in this study. Growth medium without bacteria was used as negative control. Bacteria from TSA plates were inoculated into 2 ml of TSB in a 5 ml vial and incubated at 37ºC for 24 h. Overnight cultures of bacteria in TSB were diluted (1 : 20) with fresh TSB media by adding 10 µl of bacterial cultures into 190 µl TSB media in microtitre plate. For each sample this dilution was run in triplicate. Microtitre plate was incubated in a rotary shaker at 37ºC at 150 rpm for 48h. After incubation microtitre plate containing bacterial cultures were removed and washed with a continuous spray of 1X PBS. Next 200 µl of 1% crystal violet stock solution was added to each vial and incubated for 30 min at room temperature. Then all of the liquid was aspirated and the vials were washed thoroughly with 1X PBS for 3 times (It was ensured to empty the wells completely after the last wash). Biofilm was immersed in 33% acetic acid to extract the crystal violet. Two hundred µl of 33% acetic acid stock solution was added to each vial and incubated for 15 min with gentle shaking at room temperature. Dye retained by the bacterial cells was measured at 600 nm by an ELISA plate reader (Epoch, USA). Triplicate OD values were obtained for each sample.

For MRC determination, biofilm was produced in 96 well microtiter plate containing 142.5 µl fresh Adherence Test Medium (ATM) inoculated with 7.5 µl (1 : 20 dilution) bacterial culture from TSB. Stock solution of azithromycin at a concentration of 2 mg/ml was prepared according to described guideline[14]. After biofilm production, wells containing bacterial cultures were removed and washed with 1X PBS three times under aseptic condition. The microtiter plate was kept in an inverted position for 5 min to dry the plate. Volumes of 200 µl of appropriate dilutions of the Azithromycin TSB broth following the antibiotic concentration of 2048, 1024 and 512 µg/ml were prepared from stock solution and transferred into the wells with established biofilms. For each sample antibiotic dilution in each concentration was run in duplicate. A positive control (untreated group) using TSB broth without antibiotic and negative control (control of Microtiter plate sterility; only diluted antibiotics) were included in all experiments. The plates were incubated for 24 h at 37ºC. Resazurin, which changes colour according to cell viability, was added to detect MRC.

For MBEC determination, each well containing antibiotic treated biofilm was washed three times with 1X PBS under aseptic conditions and filled with 10 µl 0.1% Buffered Peptone Water (BPW). For MBEC determination a protocol described earlier was followed[15]. Sterile cotton swabs were moistened with 0.1% BPW. Biofilm layers were collected by scraping the biofilm area with sterile cotton swabs and vigorously mixed in the wells to release biofilm forming cells. The wells were then filled with 200 µl of fresh TSB. Samples were cultivated for one day at 37ºC and the presence of viable bacteria was determined by subculturing in nutrient agar plate.

*Effects of sub-MIC concentration of Azithromycin on biofilm:* Effects of sub-MICs of antimicrobial agents on curli expression of planktonic cells was assessed[16]. Briefly, each

strain was grown overnight with the sub-MICs of antibiotics and then inoculated onto a plate containing nutrient agar supplemented with Congo red and the same sub-MICs. Curli-producing *E. coli* bound Congo red dye and formed red colonies on Congo red, whereas curli-negative bacteria formed white colonies. Control cultures contained no antibiotics.

The effect of sub-MIC of antimicrobial agents on curli expression of biofilm cells was observed. In order to do this, biofilm was grown in TSB medium in the presence of 0.5 × MIC of the antibiotic for 48 h and 37°C. Each bacterial culture was washed three times with PBS to remove non-adherent bacteria. Bacterial biofilm was treated with trypsin EDTA to isolate the biofilm cells. Biofilm cells were plated on Congo red agar. The plates were incubated for 24 h at 37°C. Control cultures contained no antibiotics.

To determine effect of sub-MICs of antimicrobial agents on motility of planktonic cells, assays were performed on each strain grown overnight with the sub-MICs of antibiotics. Using a sterile needle, bacteria cells were stab-inoculated into Motility Agar containing the same sub-MICs and incubated at 37°C for 24 h. Non-motile bacteria grew only where they were inoculated. Motile bacteria grew along the stab and also swam from the stabbed area. Control cultures contained no antibiotics.

The effect of sub-MICs of antimicrobial agents on motility of biofilm cells was observed as follows. Following 48 h incubation at 37°C in the presence of 0.5 x MIC of antibiotic, each bacterial culture was washed three times with PBS to remove non-adherent cells. Bacterial biofilm was treated with trypsin EDTA to isolate the biofilm cells. Bacterial culture was inoculated into the tube with Motility Agar.

*Detection of quorum sensing molecule:* Quorum sensing molecules were detected by Liquid-liquid extraction (LLE) method followed by FT-IR. The bacterial isolates were grown overnight in 5 ml of sterile Muller-Hinton broth (Merck, Germany) at 37ºC; then, 1.5 ml of the overnight cultures were aseptically transferred into sterile centrifuge tubes (Eppendorf, Germany) and centrifuged in a bench-top centrifuge (Eppendorf, Germany) at 10,000 rpm for 15 min. The cell pellets were discarded and the procedure was repeated three times. The supernatants were filtered through 0.2 μm membrane filters (Sartorius, Germany) to remove the cell debris. Then, filtrate was mixed with of ethyl acetate and shaken for 10 min. The mixture was allowed to stand for 5 min in a separating funnel to form upper (organic) and lower (aqueous) immiscible. The upper part was collected in sterile tubes and the lower portion was extracted two more times as described above. The upper portion from each sample were pooled and dried in an oven at 40ºC and stored at −20°C for further analyses. Fourier Transform Infrared Spectroscopy (FTIR) facility from Center for Advanced Research in Sciences (CARS) was utilized to identify the chemical bonds present in the extracted quorum sensing molecules.

**Results and Discussion**

The main focus of this study was to assess the effectiveness of Azithromycin against *E. coli* biofilm production and detection of quorum sensing molecule involved in biofilm formation. For this purpose, all the isolates were collected from UTI patients. Out of 30 isolates tested, all were capable of biofilm formation. Comparison of biofilm formation in different media and under static and shaking conditions showed that the best condition for biofilm formation by the test *E. coli* was obtained by using TSB media incubated at 37°C for 48 h (Figs 1 and 2).

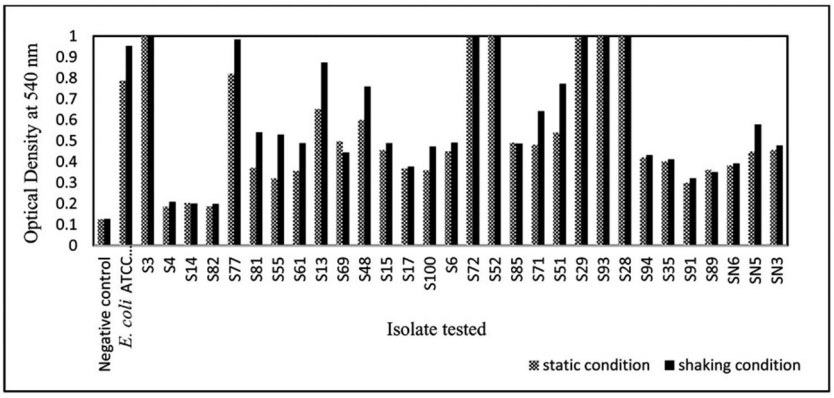


Fig. 1. Optimization of medium for biofilm formation. NB=Nutrient Broth, LB=Luria Bertani Broth, TSB=Trypticase Soy Broth and MHB=Mueller Hinton Broth.

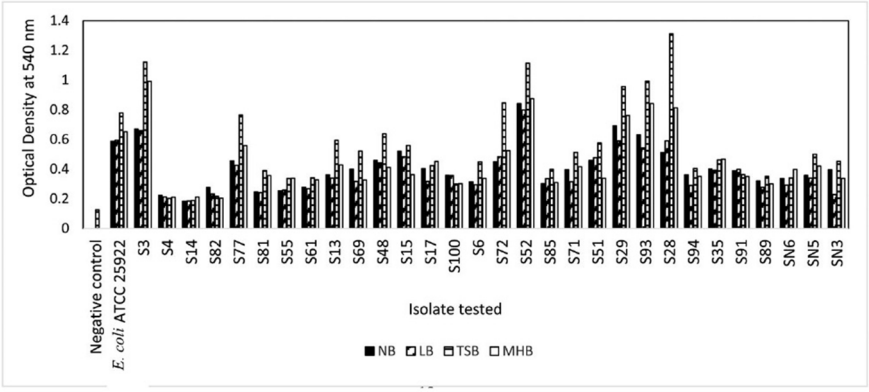


Fig. 2. Comparison of biofilm formation under incubation in static and shaker incubator. NB=Nutrient Broth, LB=Luria Bertani Broth, TSB=Trypticase Soy Broth and MHB=Mueller Hinton Broth.

All isolates of *E. coli* were tested in triplicate and were found to be capable of biofilm formation to different extents. The degree of crystal violet retention is an indication of the number of bacteria in the biofilm which bind to crystal violet. Hence, the absorbance at 600 nm correlates positively with the number of biofilm producer. The cut-off OD was

taken as two standard deviation above mean of the negative control. Figure 3 depicts the mean OD values of the crystal violet retained by *E. coli* isolates. As all of them can produce biofilm to different extent, they were able to settle more strongly in urinary system than planktonic cell. So, biofilm production provided them an extra advantage in UTI. The present study has demonstrated a clear difference in antibiotic susceptibility between planktonic populations and biofilm populations of *E. coli*.

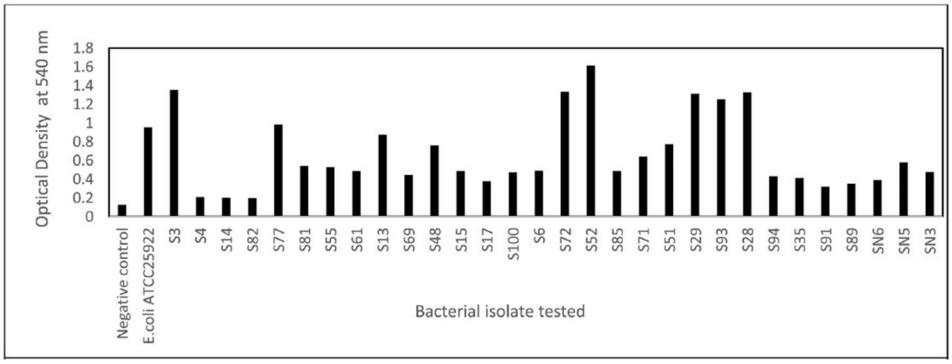


Fig. 3. Measurement of biofilm formation under optimized conditions.

Based on the binding of crystal violet by the biofilm forming bacteria the isolates were categorized as very strong biofilm producer, strong biofilm producer and moderate biofilm producer using a modified convention[18]. With some modifications, the isolates were classified as follows: non-producing, weak, moderate, strong and very strong-producing, based on the following optical density average values: OD (isolate) ≤ OD (control) = non-biofilm-producer; OD (control) ≤ OD (isolate) ≤ 2OD (control) = weak biofilm producer; 2OD (control) ≤ OD (isolate) ≤ 4OD (control) = moderate biofilm producer; 4OD (control) ≤ OD (isolate) ≤ 8OD (control) = strong biofilm producer; 8OD (control) ≤ OD (isolate) = very strong biofilm producer (Table 1). Among the 30 isolates of identified *E. coli*, 6 (20%) were very strong, 8 (26.67%) were strong, 13 (43.33%) were moderate and 3 (10%) were weak biofilm producers.

From the isolates ten specimens were taken and their MBC and MBEC were determined. The MIC and MBC values obtained for planktonic *E. coli* were compared to the MRC and MBEC values obtained for biofilm bacteria. It was found that the MRC values were between 2 to 256 times greater than the MIC values. In a similar pattern, the MBEC values for biofilm *E. coli* were between 16 to 256 times greater than the corresponding MBC values. These findings indicate that *in vitro* MIC and MBC values determined for Azithromycin to be effective against planktonic *E. coli* may not be sufficient to treat the same bacteria in biofilm form (Table 2).

**Table 1. Categorization of Biofilm producers based on biofilm strength.**

| Isolate | Biofilm category | OD control/OD sample |
|---|---|---|
| *E. coli* ATCC 25922 | Strong | 4OD (control) ≤ OD (isolate) ≤ 8OD (control) |
| S3, 72, 52, 29, 93, 28 | Very strong | 8OD (control) ≤ OD (isolate) |
| S77, 81, 55, 13, 48, 71, 51, N5 | Strong | 4OD (control) ≤ OD (isolate) ≤ 8OD (control) |
| S61, 69, 15, 17, 100, 6, 85, 94,91,89, N6, N3, 35 | Moderate | 2OD (control) ≤ OD (isolate) ≤ 4OD (control) |
| S4, 14, 82 | Weak | OD (control) ≤ OD (isolate) ≤ 2OD (control) |

**Table 2. Determination of MIC and MRC of different isolates.**

| Sample no. | MIC (µg/ml) | MRC (µg/ml) | MRC/MIC | MBC | MBEC | MBEC/MBC |
|---|---|---|---|---|---|---|
| S3 | 128 | 1024 | 8 | - | - | - |
| S4 | 4 | 128 | 32 | - | - | - |
| S14 | 4 | 256 | 64 | - | - | - |
| S82 | 8 | 256 | 32 | - | - | - |
| S77 | > 512 | >1024 | - | - | - | - |
| S81 | 16 | 256 | 16 | - | - | - |
| S55 | 128 | 256 | 2 | - | - | - |
| S61 | 16 | 512 | 32 | 64 | 1024 | 16 |
| S13 | 4 | 512 | 128 | 32 | 1024 | 32 |
| S69 | 4 | 256 | 64 | 64 | 1024 | 16 |
| S48 | 64 | 512 | 8 | - | - | - |
| S15 | 0.5 | 64 | 128 | 16 | 512 | 32 |
| S17 | 2 | 36 | 18 | 8 | 512 | 64 |
| S100 | 2 | 128 | 64 | 8 | 2048 | 256 |
| S6 | 8 | 128 | 16 | - | - | - |
| S72 | > 512 | > 1024 | | - | - | - |
| S52 | > 512 | > 1024 | | - | - | - |
| S85 | 16 | 256 | 16 | - | - | - |
| S71 | 64 | 512 | 8 | - | - | - |
| S51 | 64 | 512 | 8 | - | - | - |
| S29 | 2 | 512 | 256 | 8 | 1024 | 128 |
| S93 | 256 | 512 | 2 | - | - | - |
| S28 | 16 | 512 | 32 | - | - | - |
| S94 | 4 | 128 | 32 | 16 | 1024 | 64 |
| S35 | 8 | 512 | 64 | 32 | 2048 | 64 |
| S91 | 2 | 256 | 128 | 8 | 1024 | 128 |
| S89 | 4 | 512 | 128 | 16 | 1024 | 64 |
| SN6 | 4 | 256 | 64 | - | - | - |
| SN5 | 4 | 256 | 64 | - | - | - |
| SN3 | 2 | 128 | 64 | - | - | - |

Ten *E. coli* isolates were subjected to sub MIC treatment to compare the post incubation changes between the planktonic and biofilm forming cells. Among the 10 isolates, 6 lost the ability to express curli fimbriae in planktonic form in presence of sub MIC Azithromycin concentration whereas every strain expressed curli fimbriae in absence of antibiotic. But all of them retained their ability to express curli fimbriae in biofilm stage. In case of motility, 2 isolates lost their motility in presence of sub MIC in planktonic cell but none lost this character in biofilm stage. Curli fimbriae and motility are two notable virulent factors for *E. coli* to cause disease. Results were obtained by MRC (Minimum regrowth concentration) and MBEC (Minimum biofilm eradication concentration) assays.

The present study showed that the antibiotic, Azithromycin, was able to kill planktonic phase bacteria at a lower concentration that was not effective in killing biofilm bacteria. In our test, the range of MRC of *E. coli* isolates was 2 to 256 times greater than MIC, and MBEC values were 16 to 256 times greater than MBC. Range of MBEC was between 512 and 2048, and MBEC values were 16 to 128 times greater than MBC. These findings indicate that *in vitro* MIC and MBC values determined for Azithromycin to be effective against planktonic *E. coli* may not be sufficient to treat the same bacteria in biofilm form. From the results obtained it can be asserted that sub MIC Azithromycin treatment can be possible for planktonic stage but not in biofilm stage. In the present study, we investigated the effect of sub MIC concentrations of Azithromycin on biofilm formation by *E. coli*. Sub MIC treatment was also done to observe the difference in viable cell number at definite time intervals for biofilm and planktonic cells. For planktonic cell without antibiotic, there was an increase in cell number in the absence and a decrease in number in the presence of the antibiotic. In contrast, for biofilm forming cells, both sub MIC antibiotic and absence of antibiotic, had the same effect on the cells. It means biofilm formation was not at all affected by Azithromycin. We came to conclusion that Azithromycin is suitable for treating *E. coli* in planktonic form but not suitable for the same cell in biofilm stage. So we averted our attention to find the molecule involved in quorum sensing. But we could not identify the molecule involved by the same method used by another group for Uropathogenic *E. coli* (UPEC).

Due to the resistance by *E. coli* isolates against Azithromycin we tried to determine the molecule involved in quorum sensing. For this purpose, Fourier Transform Infrared Spectroscopy (FT-IR) was used. After FT-IR we got several peaks resembling different functional groups. We performed FT-IR of ten samples but in every case the sample resembled to negative control (Data not shown). So, it might indicate that the experimented UPEC specimen did not use AHL as their quorum sensing signal molecule, or if we would adopt different measures other than liquid-liquid extraction then it might be possible to identify the molecule involved.

Future directions for research of the present investigation may consider inclusion of more clinical isolates to increase the statistical validity of the results and testing the behavior of the test bacteria *in vivo* e.g. in tissue culture cells or animal models to compare the correlation of laboratory based experiments with *in vivo* results. Also, a different strategy could be employed to identify the quorum sensing molecule released by UPEC.

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