ABSTRACT

The term ‘biofilm’ was described in 1978 by Costerton.[1] It is clear that biofilm formation is part of the normal growth cycle of most bacteria. A biofilm can be defined as a sessile community, surface-associated microorganism characterized by cells that are irreversibly attached to a living or nonliving substratum to form a multilayered cell clusters that embedded in a matrix of extracellular polysaccharide (slime), that they have produced, which facilitates the adherence of these microorganisms to the surfaces and protect them from host immune system and antimicrobial therapy.[2-4] Biofilm formation is therefore a major problem in many fields, ranging from industrial corrosion and biofouling to chronic and nosocomial infections.[5] Hence this work aimed to investigate the resistance of E. coli biofilm cells to one of the third generation cephalosporin; ceftriaxone in comparison to planktonic cells. Although that, certain antimicrobial agents could significantly reduce the biofilm layer.[6] These effects appear to depend on the particular strain and antimicrobial agent under investigation. For instance, certain levels of antibiotic were shown to increased biofilm formation. The impact of Ceftriaxone on biofilm was investigated and it was found that the biofilm of E. coli has increased with the increase of Ceftriaxone minimum inhibition concentration.

KEYWORDS: Biofilm, Ceftriaxone, Minimum inhibitory concentration, Uropathogenic E. coli, Cephalosporin, Sewage.
INTRODUCTION
Several studies showed occurrence of high rates of antimicrobial resistance among E. coli.[7] In E. coli, β-lactamase production is the most important mediator of resistance to broad spectrum of β-lactams. ESBLs confer resistance to several antibiotics including third- and fourth-generation cephalosporin and monobactams.[8] The vast majority of ESBLs belong to the TEM-, SHV- and CTX-M-type enzymes. TEM- and SHV-type ESBLs arise via substitutions in strategically positioned amino acids from narrow-spectrum enzymes, whereas all known CTX-M enzymes have expanded-spectrum activity.[9] Carbapenem resistance in Enterobacteriaceae is a new emerging problem caused primarily by plasmid-encoded carbapenemases are mainly found in nosocomial isolates of Klebsiella pneumoniae and E. coli.[10]

Biofilm is defined as a community of microorganisms attached to a surface by polysaccharides, proteins and nucleic acids.[11] Moreover, in the biofilm phase, bacteria exhibit greater resistance to a variety of stresses; these stresses include high salt, oxidizing agents, and low pH, as well as antibiotics used in treating common infections; which are usually ineffective at eradicating them.[12]

Hence this work aimed to investigate the resistance of E. coli biofilm cells to one of the third generation cephalosporin; ceftriaxone in comparison to planktonic cells.

MATERIALS AND METHODS
Specimens collection
Through the period extending from first November 2015 till April January 2016, Fifty mid-stream urine specimens, sewage were collected in sterilized containers from patients referring Teaching Laboratories/Al-Yarmook hospital in Baghdad.

Identification of E. coli isolates
Identification was carried by standard microbiological procedures (Gram staining, colonial morphology, catalyses test, cytochrome oxidase reaction, motility, biochemical tests)[13] which carried out depending on Berge's manual of systematic Bacteriology,[14] also by analytical profile index (API) 20 E system and vitex 2 system.[15]
Determination of minimal inhibitory concentration (MIC)

Using Mueller Hinton broth, double serial dilutions (2-1024µg/ml) of ceftriaxone were prepared form a stock solution previously prepared in addition to positive and negative controls. 20µl from $10^8$ CFU/ml bacterial suspension was added to all wells except negative control wells and incubated at 37°C for 24hr. The lowest concentration that inhibit bacterial growth was considered as the MIC.\[16\]

Biofilm formation assays by using tissue culture plate (TCP) method

This quantitative test described by Hassan \textit{et al} (2011)\[17\], considered the gold standard method for biofilm detection. Organisms isolated from fresh agar plates were inoculated in 10 ml of trypticase soy broth with 1% glucose w/v. Broths were incubated at 37°C for 24 hours. The culture were then diluted 1:100 with fresh medium and inoculated individual wells of sterile 96 well- flat bottom polystyrene tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37°C for 24hrs. After incubation content of each well were removed by gentle tapping. The wells were washed with sterile distilled water once. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were stained by (0.1%) w/v crystal violet. Excess stain was removed by using distilled water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA auto reader (model 680, Biorad, UK) at wavelength 630 nm, and the interpretation of the results was conducted as shown in table 1. The experiment was performed in triplicate and repeated three time.\[18,19\]

\[\text{Table 1: Interpretation of Biofilm production.}\]

<table>
<thead>
<tr>
<th>Average OD value</th>
<th>Biofilm production</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\leq \text{OD} / \text{OD}_{c}$</td>
<td>Non / weak</td>
</tr>
<tr>
<td>$\leq \text{OD} / \text{OD}<em>{c}&lt; \sim \leq 2 \times \text{OD}</em>{c}$</td>
<td>Moderate</td>
</tr>
<tr>
<td>$&gt; 4 \times \text{OD}_{c}$</td>
<td>Strong</td>
</tr>
</tbody>
</table>

Optical density cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control\[10\]

Effect of ceftriaxone stress on Biofilm formation by \textit{E. coli}

The procedure of Almeida \textit{et al}. (2013)\[20\] was followed. In brief; the bacterial cells were grown in Tryptic soya broth overnight at 37°C under aerobic conditions. A suspension of bacterial isolate that equivalent to the McFarland No.0.5 turbidity standard were inoculated in Tryptic soya broth and incubated for 24 hours at 37°C in individual wells of sterile,
polystyrene, 96-well, flat-bottomed tissue culture plate in stationary condition. Thereafter, media were decanted and wells were washed thrice with D.W. Subsequently, an aliquot (200 μl) of Tryptic soya broth containing double serial dilutions of ceftriaxone (2 – 1024 μg/ml) were added. Each plate was covered with the lid supplied by the manufacturer and incubated at 37°C for 24 h. Negative control wells contained sterile Tryptic soya broth.

After incubation, assay plates were uncovered and liquid culture was removed from each well, and non-adherent bacteria were removed by washing each well 2–3 times with D.W.

Biofilms were stained by adding 200 μl of 0.1% crystal violet to each well for 15 minutes. After the staining reaction has been completed, excess stain was removed by repeated washing (2–3 washes) with D.W. as described above. Afterwards, 200 μl of 95% ethanol was added to each well for 10 minutes. All assays were done in triplicates.

The amount of crystal violet extracted by the ethanol in each well was directly quantified spectrophotometrically by measuring the OD$_{630}$ using a micro plate reader. Cut off value was estimated as the control OD$_{630}$ + 3SD.$^{[21]}$

RESULTS AND DISCUSSION

Among 50 urine samples were collected only 8 isolates were E. coli. Among 8 isolates of TCP; 1 was produced strong biofilm, 6 were moderate and 1 was weak or non–biofilm. The number of isolates produced biofilm formation were 7(87.5%) and none or weak biofilm producer was 1 (12.5%). As shown in table 2:

<table>
<thead>
<tr>
<th>Number of isolate (8)</th>
<th>Biofilm formation</th>
<th>TCM n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>1(12.5%)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>6(75%)</td>
<td></td>
</tr>
<tr>
<td>Weak / None</td>
<td>1(12.5%)</td>
<td></td>
</tr>
</tbody>
</table>

Hassan et al (2011)$^{[17]}$, also showed that out of 110 isolates tested, the number of biofilm producers were 70(64.7%) and non or weak biofilm producers were 40(36.3%). The difference in biofilm thickness result from different reasons such as differences in isolates capacity to form biofilm, Perhaps the primary number of cells that succeeded in adherence and the differences of quality and quantity of auto inducers (Quorum sensing signaling molecules) that produced from each isolate and play an essential as well as important role in
biofilm formation\cite{22}, *E. coli* isolates developed high MIC value exceeded 1024 µg/ml. Whereas other isolates fluctuated between 2 and 8µg/ml (Table 1 and Figure 1). However, the breakpoint of ceftriaxone for enterobacteriaceae according to CLSI\cite{23} is ≤ 8 µg/ml for susceptible, 16-32 µg/ml for intermediate, and ≥ 64 µg/ml for resistant isolates. Consequently, only two isolates were resistant while all other isolates were susceptible as shown in table 1.

**Table 1: Ceftriaxone MIC of planktonic *E. coli* isolates.**

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>MIC (mg/ml)</th>
<th>interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>&gt;1024</td>
<td>Resistant</td>
</tr>
<tr>
<td>A2</td>
<td>2</td>
<td>Susceptible</td>
</tr>
<tr>
<td>A3</td>
<td>2</td>
<td>Susceptible</td>
</tr>
<tr>
<td>A4</td>
<td>4</td>
<td>Susceptible</td>
</tr>
<tr>
<td>A5</td>
<td>2</td>
<td>Susceptible</td>
</tr>
<tr>
<td>A6</td>
<td>8</td>
<td>Susceptible</td>
</tr>
<tr>
<td>A7</td>
<td>4</td>
<td>Susceptible</td>
</tr>
<tr>
<td>A8</td>
<td>&gt;1024</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

Nevertheless, isolate no. 8 was an exception; given that it was affected by the presence of ceftriaxone since its biofilm thickness has declined with the increase of ceftriaxone concentration. Obviously, findings depicted in Figure 2 revealed that biofilm thickness (\(\text{OD}_630\) measurement) increased with the increase of ceftriaxone concentration. Although theirs MICs were within susceptible limits (except for isolates A1 and A8 which were reported as resistant).

The increasing of biofilm thickness after the exposure of increasing concentration of ceftriaxone in this experiment is also reported by Manu and anurag(2012)\cite{24} who also show ceftriaxone alone is not effective in the biofilm eradication which is probably due to as Donlon(2000)\cite{25} reported that EPS contributes to the antimicrobial resistance properties of biofilms by impeding the mass transport of antibiotics through the biofilm, Or due to the differentiation of classes of extracellular proteins have been described as part of an adaptive response to a change in the environment.\cite{26}
Table 3: Effect of ceftriaxone concentration on *E. coli* biofilm.

<table>
<thead>
<tr>
<th>Isolates no.</th>
<th>Source of isolates</th>
<th>MIC value</th>
<th>Absorbance in different MIC concentration</th>
<th>Absorbance for bacteria without antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1024</td>
<td>512</td>
<td>258</td>
</tr>
<tr>
<td>1</td>
<td>Sewage</td>
<td>&gt; 1024</td>
<td>0.091</td>
<td>0.155</td>
</tr>
<tr>
<td>2</td>
<td>Sewage</td>
<td>2</td>
<td>0.128</td>
<td>0.149</td>
</tr>
<tr>
<td>3</td>
<td>Sewage</td>
<td>2</td>
<td>0.127</td>
<td>0.123</td>
</tr>
<tr>
<td>4</td>
<td>Urine</td>
<td>4</td>
<td>0.135</td>
<td>0.140</td>
</tr>
<tr>
<td>5</td>
<td>Urine</td>
<td>2</td>
<td>0.120</td>
<td>0.140</td>
</tr>
<tr>
<td>6</td>
<td>Urine</td>
<td>8</td>
<td>0.123</td>
<td>0.111</td>
</tr>
<tr>
<td>7</td>
<td>Urine</td>
<td>4</td>
<td>0.157</td>
<td>0.214</td>
</tr>
<tr>
<td>8</td>
<td>Urine</td>
<td>&gt; 1024</td>
<td>0.129</td>
<td>0.174</td>
</tr>
</tbody>
</table>
The origin of such resistant bacterial strains appears to be the hospital environment and the selective pressure responsible for expanding such bacterial populations in hospitals must have been using drugs in humans and not from their use in the veterinary and agriculture field.

**The increase of antibiotic resistant isolated *E. coli* in the hospitals effluents to.**

1. Selection of antibiotic resistant strains originated from the effluent in presence of the antibiotics.
2. Genetic mutation that makes them resistant to the antibiotics.
3. Horizontal transfer of antibiotic resistance genes from other bacteria existing in the effluents.\(^{[27]}\)

*E. coli* has different mechanisms of resistance for \(\beta\)-lactam antibiotics. Of these mechanisms, production of \(\beta\)-lactamase enzyme, which enables them to break the \(\beta\)-lactam ring and effectively abolishes the antibiotic’s effectiveness\(^{[28]}\)

Krumpermann (1993)\(^{[29]}\) suggested that the observed resistance to some drugs is a probable indication of earlier exposure of the isolates to these drugs, which may have enhanced resistant development. Furthermore, the uncontrolled sale of antibiotics in Iraq and resultant self-treatment with antibiotics could result in this resistance.

Regarding isolate no. 8, it can be said that resistance genes played important role to fit the surrounding environment by switching off biofilm formation responsible genes in order to render these cells persist and survive because of what is known as a fitness cost.

**CONCLUSION**

Upon the findings of the present work, it can be concluded that the susceptibility to ceftriaxone is 80% of all study isolates. All isolates are capable to form biofilm. Biofilm thickness increased with the increase of ceftriaxone concentration.

**REFERENCES**


23. NLSI,(Clinical Laboratory Standard Institute), Performance standard for antimicrobial susceptibility testing; Twenty- first informational supplement., 2011; 31(1): M100-S21.
Escherichia coli is one of the most frequent causes of many common bacterial infections, including cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI), and traveler's diarrhea, and other clinical infections such as neonatal meningitis and pneumonia. The genus Escherichia is named after Theodor Escherich, who isolated the ty... Medication Summary. E coli meningitis requires antibiotics, such as third-generation cephalosporins (eg, ceftriaxone). E coli pneumonia requires respiratory support, adequate oxygenation, and antibiotics, such as third-generation cephalosporins or fluoroquinolones. E coli cholecystitis/cholangitis requires antibiotics such as third-generation cephalosporins that cover E coli and Klebsiella organisms. Ceftriaxone increases the thickness of escherichia colibiofilm. HK Tawfeeq, AM Husein, HJ Fahad. 2016. Effect of combination of d-glycin and antibiotics on biofilm. MHM Al-Khafaji, HK Tawfeeq, BA Mahdii. 2016. The effect of D and L-amino Acids on Biofilm Formation in Different Microorganisms. Ceftriaxone-netilmicin combination in single-daily-dose treatment of experimental Escherichia coli endocarditis. Article (PDF Available) in Antimicrobial Agents and Chemotherapy 33(5):767-70 · June 1989 with 28 Reads. DOI: 10.1128/AAC.33.5.767 · Source: PubMed. In vitro, a greater rate of killing and an increased trough serum bactericidal titer (P less than 0.01) were achieved with the combination. In vivo, the combination had a greater bactericidal effect (P less than 0.01) and resulted in a greater number of sterile vegetations (P less than 0.05) than single-drug therapy.