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CEFTRIAXONE INCREASES THE THICKNESS OF *ESCHERICHIA COLI* BIOFILM

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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 ornonliving 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 corrosionandbiofouling 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, Minimuminhibitory 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 carbapenemasesare mainly found in nosocomial isolates of *Klebsiellapneumoniae*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, sewagewere collected in sterilized containers frompatientsreferring Teaching Laboratories/Al-Yarmookhospital 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 vitek 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 from10^8 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 *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 37Cº for 24hours. 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 37Cº 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]

Table 1: Interpretation of Biofilm production.

Average OD value	Biofilm production
≤ OD / ODc< ~ ≤ 2 x ODc	Non / weak
≤ OD / ODc< ~ ≤ 4 x ODc	Moderate
> 4 x ODc	Strong

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

Effect of ceftriaxone stress on Biofilm formation by *E. coli*

The procedure of Almeida*et al.* (2013)[20] was followed. In brief; the bacterial cells were grown in Tryptic soya brothovernight 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 37ºC in individual wells of sterile,

polystyrene, 96-well, flat-bottomedtissue culture plate in stationary condition. Thereafter, media were decanted and wells were washed thrice with D.W.Subsequently, an aliquot(200µl) of Tryptic soya broth containing double serial dilutions of ceftriaxone (2 – 1024 µg/ml) were added. Each plate was covered withthe lid supplied by the manufacturerand incubated at 37°C for24 h. Negative control wells contained sterile Trypticsoya broth.

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

Biofilms were stained by adding 200 µl of0.1% crystal violet to each well for 15minutes. After the staining reaction has been completed, excessstain was removed by repeated washing (2-3 washes) with D.W.as described above. Afterwards, 200µl of 95% ethanol wasadded to each well for 10 minutes. All assays were done in triplicates.

The amount of crystal violet extracted by the ethanol in each well wasdirectly quantifiedspectrophotometrically 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 2: Screening of the Isolate for Biofilm Formation by Tissue culture plate.

	Biofilm formation	TCM n(%)
No. of isolate (8)	High	1(12.5%)
	Moderate	6(75%)
	Weak / None	1(12.5%)

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[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[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 susceptibleAs shown in table1.

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

Isolate code	MIC (mg/ml)	interpretation
A1	>1024	Resistant
A2	2	Susceptible
A3	2	Susceptible
A4	4	Susceptible
A5	2	Susceptible
A6	8	Susceptible
A7	4	Susceptible
A8	>1024	Resistant

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 (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)[24] who also show ceftriaxone alone is not effective in the biofilm eradication which is probably due to as Donlon(2000)[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.[26]

Table 3: Effect of ceftriaxone concentration on *E. coli* biofilm.

Isolates no.	Source of isolates	MIC value	Absorbance in different MIC concentration										Absorbance for bacteria without antibiotic		
			1024	512	258	128	64	32	16	8	4	2			
1	Sewage	> 1024	0.091	0.155	0.114	0.127	0.089	0.087	0.081	0.086	0.093	0.085	0.081	0.071	0.071
2	Sewage	2	0.128	0.149	0.138	0.133	0.107	0.080	0.090	0.088	0.095	0.085	0.114	0.114	0.114
3	Sewage	2	0.127	0.123	0.110	0.115	0.119	0.098	0.102	0.148	0.156	0.111	0.123	0.152	0.152
4	Urine	4	0.135	0.140	0.123	0.122	0.135	0.120	0.131	0.109	0.144	0.148	0.124	0.129	0.129
5	Urine	2	0.120	0.140	0.123	0.173	0.203	0.136	0.142	0.140	0.141	0.165	0.118	0.137	0.137
6	Urine	8	0.123	0.111	0.127	0.132	0.117	0.111	0.167	0.128	0.109	0.103	0.153	0.135	0.135
7	Urine	4	0.157	0.214	0.179	0.176	0.153	0.141	0.144	0.141	0.152	0.154	0.127	0.121	0.121
8	Urine	> 1024	0.129	0.174	0.155	0.108	0.118	0.143	0.117	0.153	0.162	0.072	0.190	0.225	0.225

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 β-lactam antibiotics. Of these mechanisms, production of β-lactamase enzyme, which enables them to break the β-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, itcan 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.

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