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Research article

Effectiveness of some β- lactamase encoding genes on biofilm formation and slime layer production by uropathogenic *Escherichia coli*

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ABSTRACT

In present study 74 specimens of urine were collected from patients suffering from urinary tract infections. Fifty (67.56%) isolates were identified as *Escherichia coli*. 78% of isolates were identified as extended spectrum beta lactamases (ESBL) producer. Antibiotic susceptibility test was done and ceftazidime was selected to complete this study by implying stress at sub-MIC on isolate harbor high number of resistance genes (N11) and compared with sensitive isolate (S). Only four β-lactamase coding genes were detected; bla_{TEM}, bla_{PER}, bla_{VIM} and $bla_{CTX\text{-}M\text{-}2}$ and N11 had bla_{TEM}, bla_{PER}, and bla_{VIM}. It was found that the resistant isolate did not form biofilm when compared with the sensitive one, which formed moderate biofilm. In addition, ceftazidime stress reduced the ability to produce slime layer and affected the viable bacterial count in combination with pH and temperature stresses.

Keywords: Uropathogenic *E. coli*, β-lactamase, Biofilm, Slime layer.

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INTRODUCTION

Escherichia coli is one of the most important pathogenic bacteria that share the events of microbial contamination and cause about 90% of the urinary tract infection (UTI) and recurrent UTI, particularly in women. However, the importance of this pathogen comes from its ability to elaborate a wide spectrum of virulence factors. *E. coli* comprises a wide population of phenotypically and genetically highly variable organisms [1]. The discovery of antibiotics had a significant impact on lowering the incidence of UTI. On contrary, extended spectrum beta lactamases (ESBL) produced by enterobacteriaceae complicated the treatment of such infections [2]. ESBL

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covered a growing group of plasmid-mediated ß-lactamases, which confer resistance to broad spectrum beta-lactam antibiotics. The species of Enterobacteriaceae producing this class of enzymes are increasing worldwide and this triggers an irritating alarm. Furthermore, high mortality rates are associated with infections caused by ESBL producing *E. coli*. Consequently, the emergence of ESBLs establishes a complicated yet real challenge for both clinical microbiology laboratories and clinicians [3].

Biofilm is defined as a community of microorganisms attached to surface by polysaccharides, proteins and nucleic acids [4]. *E. coli* biofilm development is a complex process that leads to beautiful structures that are important for disease and for engineering applications [5]. It is clear that biofilm formation is part of the normal growth cycle of most bacteria and that, 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 [6]. Biofilm formation is therefore a major problem in many fields, ranging from industrial corrosion and biofouling to chronic and nosocomial infections [7].

On the other hand, some bacteria produce an extracellular matrix, called slime layer that is made of carbohydrate and protein molecules. A major part of the layer is called the polysaccharide intercellular adhesion. Studies show that the eradication of slime-producing bacteria is more difficult than for bacteria that do not produce slime [8].

The aim of this work is to investigate the problematic ESBL producing *E. coli* in Iraq by identifying some of ESBLs coding genes and to investigate their effect on Biofilm formation and slime layer production by UPEC.

MATERIALS AND METHODS

Specimens' collection

Seventy four midstream urine specimens (5 ml of each specimen) were collected randomly from patients suffering from urinary tract infections who attending Al-Yarmouk, Al-Numan, and Saint Raphael hospitals in Baghdad. The specimens were collected from March 2014 to April 2014. The study was conducted following approval from the scientific committee, College of Science, University of University and teaching laboratories of Al-Yarmook hospital.

Isolation and Identification of *E. coli*

All specimens were streaked onto Blood agar (HiMedia, India) and incubated at 37°C for 24 h. Thereafter, suspected colonies were streaked onto MacConkey agar (Oxoid, England) and re-incubated at 37°C for another 24 h. Pink colonies were selected and examined for Gram stainability, cultural, morphological characteristics, and conventional biochemical tests.

Antibiotic susceptibility test

Kirby-Bauer method was used as described by Morello *et al*. [9] to carry out the antibiotics susceptibility test for 7 different β lactam antibiotics including: ampicillin, ampicillin/ sulbactam, amoxicillin/ clavulanic acid, cefalothin, ceftazidime (all purchased form Bioanalyse, Turkey), imipenem, and meropenem (both of them were provided by Mast, England). Each isolate was interpreted as susceptible, intermediate, or resistant to a particular antibiotic by comparison with standards inhibition zones [10].

Determination of minimal inhibitory concentration (MIC)

Double serial dilutions (16-2048 µg/ml) were prepared form a stock solution in addition to positive and negative controls. 100 µl from10^8 CFU/ml bacterial suspension was added to all tubes except negative control tube and incubated at 37°C for 24 h. The lowest concentration that inhibits bacterial growth was considered as the MIC [10].

Detection of extended spectrum β-lactamase production (ESBL)

Disk replacement method was used according to that described by Al-Jasser [3] with some modifications. Two amoxicillin/ clavulanate disks were applied onto Mueller-Hinton plate and inoculated with the test organism (*E. coli*). After one hour of inoculation at room temperature, the antibiotic disks were removed and replaced on the same spot by disks containing ceftazidime and aztreonam. Control disks of these two antibiotics were simultaneously placed at least 30 mm from these locations. A positive test was indicated by an increase of zone of inhibition by **≥** 5 mm for the disks which were replaced the amoxicillin/clavulanate disks compared to the control disks, which placed alone directly on inoculated Muller-Hinton plates. Inhibition zones were measured and recorded by a metric ruler [3].

Molecular detection of β-lactamases

ESBL-producing *E. coli* isolates were tested for bla_{TEM}, bla_{SHV}, $bla_{OXA\text{-}like}$, $bla_{CTX\text{-}M}$, bla_{PER}, bla_{VIM}, bla_{IMP} and bla_{KPC} genes by polymerase chain reaction (PCR). DNA was extracted from *E. coli* clinical isolates using Genomic DNA Mini Kit (Geneaid, Thailand). Purity and concentration of DNA were measured by Microspectrophotometer NAS99 (ACT Gene, USA).

Primer preparation

Forward and reverse primers (BioCorp, Canada) were chosen from previously published DNA sequences of *E. coli* described by Dallenne *et al.* (2010) [11]. **Table 1** lists the sequences, names of the mentioned primer pairs as well as the molecular size of amplicons.

Table 1. Fragments of β-lactamases genes primers used in polymerase chain reaction [11].

id	Primer name	Primer sequence5'→3'	Gene targeted	Amplicon size(bp)
1	TEM_for	CATTTCCGTGTCGCCCTTATTC	*bla*$_{TEM}$	800
	TEM_rev	CGTTCATCCATAGTTGCCTGAC		
2	SHV_for	AGCCGCTTGCAAATTAAAC	*bla*$_{SHV}$	713
	SHV_rev	ATCCCGCAGATAAATCACCAC		
3	OXA_for	GGCACCAGATTCAACTTTCAAG	*bla*$_{OXA-1}$, *bla*$_{OXA-4}$, *bla*$_{OXA-30}$	564
	OXA_rev	GACCCCAAGTTTCCTGTAAGTG		
4	CTXM1_for	TTAGGAARTGTGCCGCTGYA*	*bla*$_{CTX-M}$ group-1	688
	CTXM1_rev	CGATATCGTTGGTGGTRCCAT*		
5	CTXM2_for	CGTTAACGGCACGATGAC	*bla*$_{CTX-M}$group-2	404
	CTXM2_rev	CGATATCGTTGGTGGTRCCAT*		
6	CTXM9_for	TCAAGCCTGCCGATCTGGT	*bla*$_{CTX-M}$group-9	561
	CTXM9_rev	TGATTCTCGCCGCTGAAG		
7	PER_for	GCTCCGATAATGAAAGCGT	*bla*$_{PER-1}$, *bla*$_{PER-3}$	520
	PER _rev	TTCGGCTTGACTCGGCTGA		
8	IMP_for	TTGACACTCCATTTACDG*	*bla*$_{IMP}$	139
	IMP_rev	GATYGAGAATTAAGCCACYCT*		
9	VIM_for	GATGGTGTTTGGTCGCATA	*bla*$_{IVIM}$	390
	VIM_rev	CGAATGCGCAGCACCAG		
10	KPC_for	CATTCAAGGGCTTTCTTGCTGC	*bla*$_{IKPC}$	538
	KPC_rev	ACGACGGCATAGTCATTTGC		

Primers utilized in this study were provided in lyophilized form and dissolved in sterile TE-Buffer to give a final concentration of 100 picomole/µl as recommended by the provider and stored in a deep freeze (-20°C) until use.

PCR

Reactants concentrations and conditions for multiplex PCR (ABI, USA) were summarized in **Tables 2, 3 and 4**; while the monoplex PCR were listed in **Tables 5, 6 and 7**.

Table 2. Reactants volumes and concentrationsused for the PCR amplification of *bla*$_{TEM}$ and *bla*$_{PER}$

Reactant	Volume (µl)	Final concentration
Free nuclease water	14	-
Kapa Multiplex	25	-
DNA template	3	25 - 50 ng
TEM for	2	10 pmol
TEM rev	2	10 pmol
PER for	2	10 pmol
PER rev	2	10 pmol
Final concentration	50	-

Table 3. PCR program followed to amplify *bla*$_{TEM}$ and *bla*$_{PER}$

Step	Number of Cycle	Time	Temperature (°C)
Initial denaturation	1	3 Min	95
Denaturation	35	18 Sec	95
Primer nnealing		38 Sec	59
Polymerization		38 Sec	72
Final extension	1	10 Sec	72

Reagents concentrations of *bla*$_{KPC}$ and *bla*$_{IMP}$ were similar to those described in **Table 2**, except for the conditions of KPC primers were 0.2 pmole and 0.5 pmole for IMP primers. Amplification conditions are listed in **Table 4**.

Table 4. PCR program followed to amplify *bla*$_{KPC}$ and *bla*$_{IMP}$ [11].

Step	Number of Cycle	Time	Temperature (°C)
Initial denaturation	1	10 min	94
Denaturation	30	40 sec	94
Primer annealing		40 sec	55
Polymerization		1 min	72
Final extension	1	7 min	72

Reagents for *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-9}$ were similar to those listed in **Table 2**, except primers conditions for *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-9}$ were 0.4, 0.2, 0.4, and 0.4 pmol, respectively. Amplification conditions were similar to **Table 4**, in exception to the annealing was at 60°C and the final extension for 10 min.

Table 5. Reactants volume and concentration employed for *bla*$_{VIM}$ amplification

Reactant	Volume (µl)	Final concentration
Free nuclease water	5.5	-
Kapa Multiplex	12.5	-
DNA template	3	25 - 50 ng
VIM for	2	10 pmol
VIM rev	2	10 pmol
final volume	25	-

Reactants volumes and concentrations employed for *bla*$_{CTX-M-2}$ amplification were the same as those described in **table 5**. PCR amplification conditions for *bla*$_{CTX-M-2}$were similar to those described in **table 6**, except the annealing was at 52°C for 38 sec and initial extension for 38 sec.

Reactants volumes and concentrations employed for *bla*$_{SHV}$ amplification were similar to those described in **table 6**, except the primer concentrations were 0.4 pmol for both

reverse and forward primers. Amplification conditions used for *bla*$_{SHV}$ were as listed in **table 7**.

Table 6. PCR amplification conditions for *bla*$_{VIM}$

Step	Number of Cycle	Time	Temperature (°C)
Initial denaturation	1	3 Min	95
Denaturation	35	18 Sec	95
Primer annealing		32 Sec	60
Polymerization		32 Sec	72
Final extension	1	10 Sec	72

Table 7. PCR amplification conditions used for *bla*$_{SHV}$

Step	Number of Cycle	Time	Temperature (°C)
Initial denaturation	1	10 min	94
Denaturation	30	40 sec	94
Primer annealing		40 sec	60
Polymerization		1 min	72
Final extension	1	10 min	72

Reactants volumes and concentrations employed for *bla*$_{OXA}$ amplification were same conditions as those described for *bla*$_{SHV}$ amplification. PCR amplification conditions of *bla*$_{OXA}$ were the same as those described in **table 7**. Amplicons were visualized after running at 100 V for 1 h on a 1.5% agarose gel containing ethidium bromide. A 100 and 800 bp DNA ladder were used as a size marker [11].

Effect of ceftazidime stress on biofilm formation by UPEC

This method carried out according to Almeida *et al*. (2013) [12] and applied on isolates that possess highest and lowest numbers of β-lactamase genes (**table 8**).

Table 8. Distribution of *E. coli* isolates on media type for the biofilm detection.

Set of wells	Medium	*E. coli* isolate
A	Nutrient Broth	Sensitive isolate(S)
B	Nutrient Broth supplemented with sub MIC of Ceftazidime	Resistant isolate (N 11)
C	Nutrient Broth	Resistant isolate (N 11)

Effect of ceftazidime stress on slime layer production

This test carried out according to Handke *et al.* (2004). This test was used to identify ability of bacterial isolates to produce slime layer [13]. A set of Congo red agar (CRA) plates supplemented with sub MIC of ceftazidime was inoculated with an overnight *E. coli* N 11suspensions (1.5 × 10^8 CFU/ml). Furthermore, another two sets of antibiotic free CRA plates were streaked with an overnight *E. coli* N 11and *E. coli* S suspensions (1.5 × 10^8 CFU/ml), respectively, and incubated at 37ºC for 24 h. Colonies developed over CRA medium were categorized as red, weak black (Bordeaux; red center surrounded by black rim), black, very black. The black colonies were considered as slime-producing isolates; whereas, red colonies were classified as non-slime-producing strains.

Effect of ceftazidime stress on slime layer production on CRB

This protocol was done according to Handke *et al*. (2004) but CRB was used rather than CRA [13].

Effect of pH and eftazidime stress on *E. coli* growth

This experiment was achieved according to Yuxin [14] with applying some modifications. Three sets of tubes (**table 9**) containing Nutrient broth were prepared. First set was adjusted at different pH values (ca. 6, 7, and 8) and supplemented with sub MIC of Ceftazidime. This set was inoculated with resistant *E. coli* isolate (N 11) compared to McFaraland standard No. 0.5. Second set was adjusted for these pH values but was free from Ceftazidime. This set was cultured by N 11 compared to McFaraland standard No. 0.5. The third set of tubes contained free antibiotic sterile Nutrient broth which was inoculated with sensitive *E. coli* strain S compared to McFaraland standard No. 0.5. Eventually, all tubes were incubated at 37°C for 24 h. Bacterial growth was measured by viable plate count.

Table 9. Distribution of *E. coli* isolates on media type for both pH and sub-MIC antibiotic effect detection.

Tube set	Type of media	*E. coli* isolate
1	Nutrient Broth supplemented with sub MIC of ceftazidime and 6, 7, 8 pH values were adjusted.	Resistant isolate (N 11)
2	Nutrient Broth free from antibiotic and pH was adjusted at 6, 7, 8.	Resistant isolate (N 11)
3	Nutrient Broth free from antibiotic and pH was adjusted at 6, 7, 8.	Sensitive isolate (S)

Effect of temperature on *E. coli* growth

Similar protocol mentioned in pH was followed [14]. Nevertheless, pH was adjusted at 7; while, the incubation temperatures were 20, 37, and 45 ^{o}C for 24 h.

RESULTS

Results showed that 50 specimens were identified to harbor *E. coli,* which is tested by conventional morphological methods and biochemical analysis. A high percentage of resistance was identified against ampicillin where it reached 96%. However, 82% and 84% of isolates were resistant to β lactam/ β lactamase inhibitor antibiotic; ampicillin/sulbactam, amoxicillin/ clavulanic acid (**fig. 1**). These findings indictate the capacity of these isolates to produce β-lactamases. About 52% of the isolates were considered as ESBL due to resisting ceftazidime. Nevertheless, 82% of total isolates have the ability to produce β lactamase. While, 92% of these isolates were sensitive to carbapenems (**fig 1**).

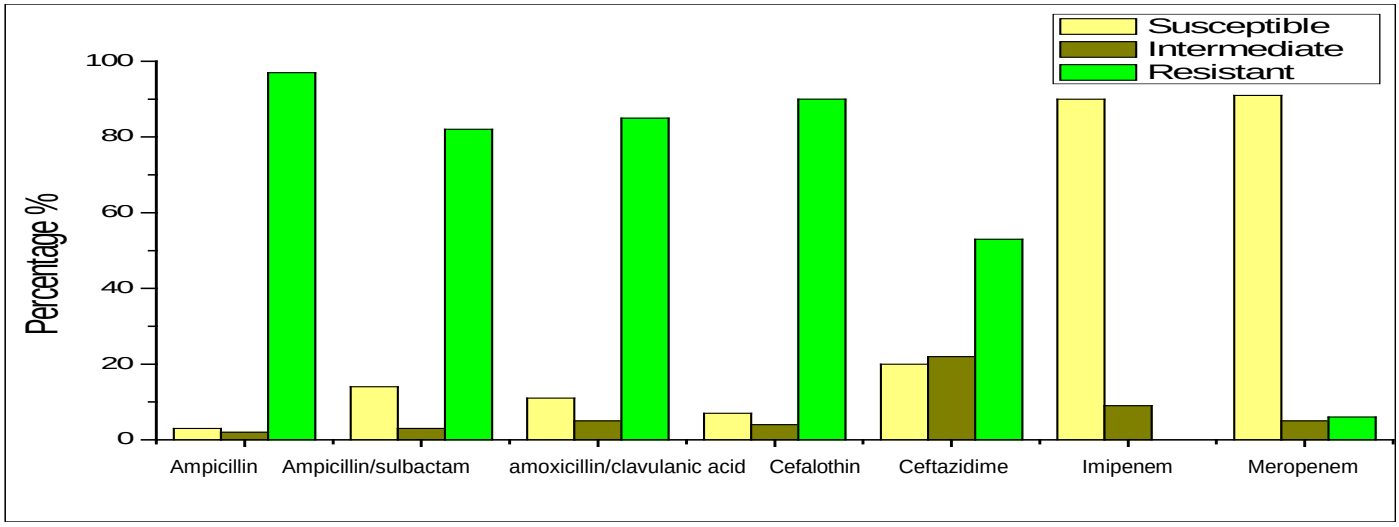

Fig 1. Antibiotic resistance of 50 *E. coli* clinical isolates isolated from 60 patients.

Results are presented in **fig. 2, 3, 4** demonstrated only four β-lactamase genes out of ten β-lactamase genes under investigation were detected; *bla*$_{PER}$ (520 bp), *bla*$_{TEM}$ (800 bp), *bla*$_{VIM}$ (390 bp) and *bla*$_{CTX-M-2}$ (404 bp), which comprised 4, 10, 12, and 18% of isolates, respectively. Nevertheless, *bla*$_{SHV}$, *bla*$_{OXA-1-like}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-9}$, *bla*$_{IMP}$ and *bla*$_{KPC}$ were not detected.

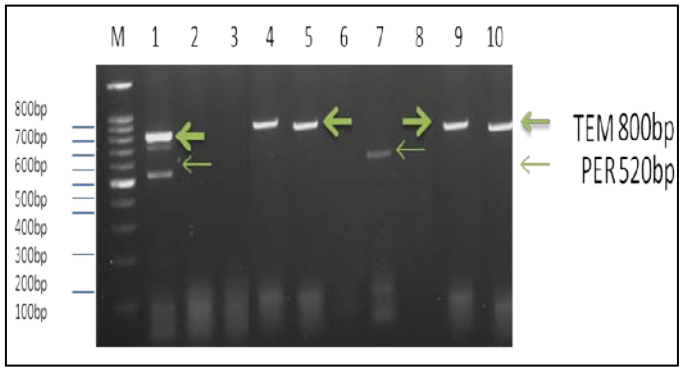

Fig 2. Analysis of the presence of *bla*$_{TEM}$ and *bla*$_{PER}$among *E. coli* clinical isolates by PCR and run on agarose gel (1.5%) at 5 V/cm for 1 hour, stained with ethidium bromide and visualized on a UV transilluminator documentation system. Lane M: ladder, lanes 1-10 represent *E. coli* isolates 11, 12, 21, 22, 24, 25, 27, 28, 31, and 32, respectively.

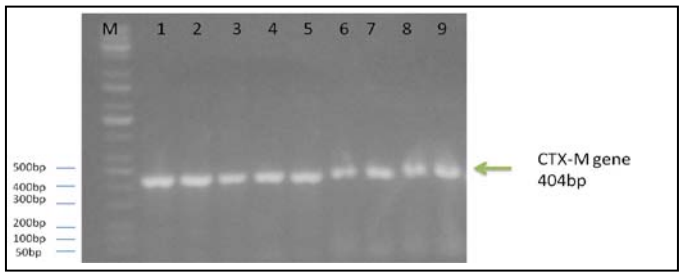

Fig 3. Analysis of the presence of *bla*$_{CTX-M-2}$among *E. coli* clinical isolates by PCR and run on agarose gel (1.5%) at 5 V/cm for 1 hour, stained with ethidium bromide and visualized on a UV transilluminator documentation system. Lane M: ladder, lanes 1-9 represent *E. coli* isolates 3, 26, 30, 31, 32, 35, 41, 48, and 49, respectively.

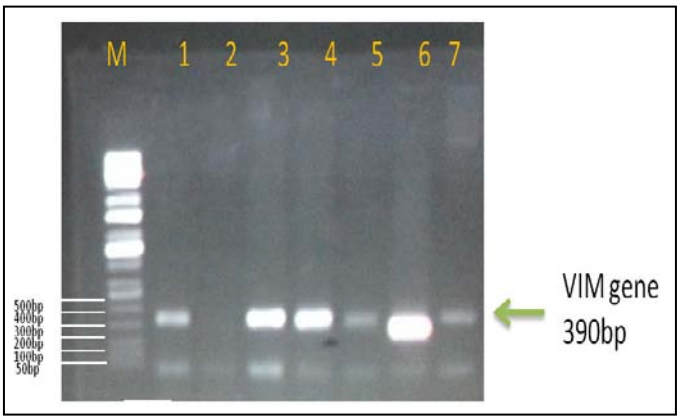

Fig 4. Analysis of the presence of *bla*$_{VIM}$among *E. coli* clinical isolates by PCR and run on agarose gel (1.5%) at 5 V/cm for 1 hour, stained with ethidium bromide and visualized on a UV transilluminator documentation system. Lane M: ladder, lanes 1-7 represent *E. coli* isolates 9, 10, 11, 23, 27, 38, and 46, respectively.

In a local study done by Aziz (2013), the result revealed that the most common ESBL in *E. coli* were *bla*$_{CTX-M}$ (CTX-M-14 and CTX-M-15) and *bla*$_{TEM}$, which represented 18 and 11% of total isolates tested, respectively [15]. The current results can be strongly similar to the results revealed by Garrec *et al.* (2011) who found that TEM was found at about 7.4% of *E. coli* isolates, and no isolate showed band of the presence of *bla*$_{SHV}$ which, in turn, support our results since that local isolates appeared to lack this gene [16]. Regarding *bla*$_{CTX-M}$, they found that 18 isolate of *E. coli* out of 107 (19.26%) enterobacteriaceae isolates revealed the existence of *bla*$_{CTX-M.}$ In conclusion, ESBL *E. coli* local isolates are found to exist relatively at a high level among clinical isolates derived from UTI patients.

Effect of ceftazidime stress on biofilm formation by UPEC

The results of effect of ceftazidime stress on biofilm formation by UPEC were shown in **table 10.**

Table 10. Results of biofilm formation in microtiter plate. SD standard deviation.

Treatment	OD_{630}± SD
N 11 with antibiotic(at sub MIC)	0.0536 ± 0.0005
N 11 without antibiotic	0.0593 ± 0.002
S isolate	0.103 ± 0.005
Control (Nutrient broth with antibiotic)	0.054 ± 0.002
Control (Nutrient broth without antibiotic)	0.067 ± 0.004

The current results showed that the tested isolate which harbor 3 β-lactamase resistance genes (N 11) showed non adherent cells both when treated with CAZ and without antibiotic treatment when compared with control (which composed of antibiotic and nutrient broth and nutrient broth alone).While, sensitive isolate revealed formation of moderately biofilm when compared with control.

Effect of ceftazidime stress on slime layer production

The ability of *E. coli* resistant and sensitive isolates for the slime layer production was investigated both in agar and liquid media. Current results demonstrated that bacterial production of slime layer was significantly affected when the isolate was challenged with antibiotic at sub-MIC; which was demonstrated by appearance of pink colonies instead of black colonies which were developed in antibiotic free on CRA (**fig. 5a,b,c**) and (**fig. 6**) .

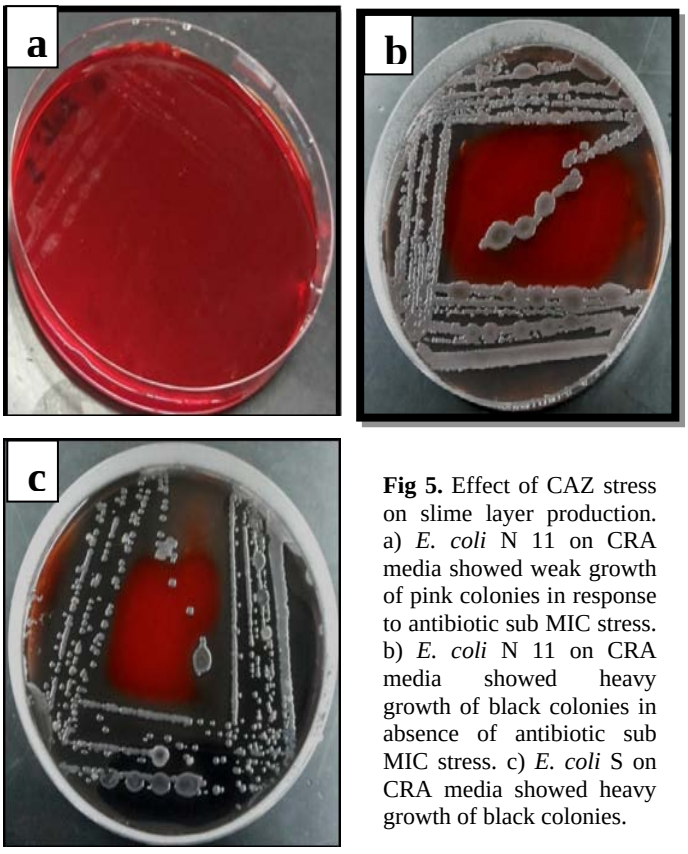

Fig 5. Effect of CAZ stress on slime layer production. a) *E. coli* N 11 on CRA media showed weak growth of pink colonies in response to antibiotic sub MIC stress. b) *E. coli* N 11 on CRA media showed heavy growth of black colonies in absence of antibiotic sub MIC stress. c) *E. coli* S on CRA media showed heavy growth of black colonies.

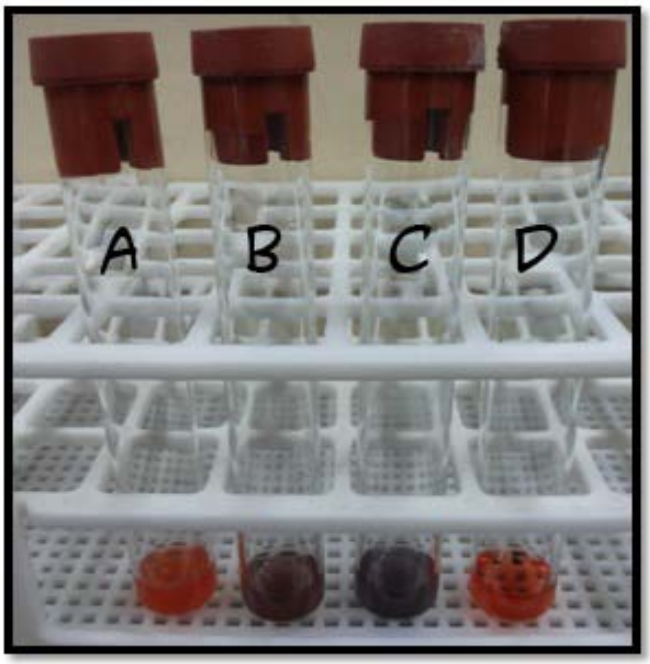

Fig 6. Slime layer production in CRB media. Tube A represents *E. coli* N 11 with antibiotic stress that showed no production of slime layer. Tube B represents *E. coli* N 11 without antibiotic stress that showed production of slime layer. Tube C represents *E. coli* S without antibiotic stress that showed production of slime layer and tube D represents CRB media with antibiotic that considered as control.

Effect of pH and ceftazidime stress on *E. coli* growth

Apparently, present study demonstrated that pH values variability affected *E. coli* growth rate. Resistant isolate (N 11) was highly influenced by both pH changes in presence of CAZ at sub MIC. **Table 11** shows results of pH value change on *E. coli* growth.

Table 11. Effect of pH value changes on *E. coli* growth depending on viable plate count. CFU, colony forming unit. TMTC= too many to be counted. N11 isolate harbours 3 genes (bla_{TEM}, bla_{PER} and bla_{VIM}). S isolate is sensitive to all antibiotic under investigation.

Id	*E. coli* Isolate	pH value	Count of bacteria with antibiotic (CFU/ml)	Count of bacteria without antibiotic (CFU/ml)
1	N 11	6	No growth	1.2×10^{14}
2		7	0.0000116×10^{14}	TMTC
3		8	No growth	0.00007×10^{14}
4	S	6	–	0.52×10^{14}
5		7	–	TMTC
6		8	–	0.0009×10^{14}

Effect of temperature and ceftazidime stress on *E. coli* growth

Current results demonstrated that temperature degree changes had high impact on bacterial growth in presence of antibiotic when compared with the same temperatures but in absence of antibiotic. As well as, temperature stress alone showed high influence on *E. coli* isolates growth when compared with their growth in

optimal temperature. **Table 12** shows results of temperature changes on *E. coli* growth.

Table 12. Effect of temperature change on *E. coli* growth depending on viable plate count. CFU= colony forming unit. TMTC= too many to be counted. N11 isolate harboured3 genes (bla_{TEM}, bla_{PER} andbla_{VIM}). S isolate is sensitive to all antibiotic under investigation.

Id	*E. coli* isolate	Temperature (°C)	Count of bacteria with antibiotic (CFU/ml)	Count of bacteria without antibiotic (CFU/ml)
1	N 11	20	4240	6.4×10^{11}
2		37	4.8×10^{11}	TMTC
3		45	No growth	0.00000088×10^{11}
4	S	20	–	0.04×10^{11}
5		37	–	TMTC
6		45	–	No growth

Obviously, at low temperature, bacterial count was higher than that obtained when bacterial isolates were grown at high temperature in both cases of the presence and absence of antibiotic resistance genes. Also, it was clear that at optimum temperature, bacterial growth was high; however the presence of sub-MIC antibiotic stress when compared with growth results at low and high temperatures in addition to antibiotic stress for the same isolate (*E. coli* N 11). As well as temperature stress, sub-MIC antibiotic appeared to inhibit bacterial growth for resistant isolate when compared with bacterial growth in free antibiotic conditions. Viable count of sensitive isolate showed mild decrease at low temperature when compared with the optimum growth temperature, whereas, high temperature revealed high influence on bacterial growth that showed full inhibition and bacterial isolates were prevented to grow.

DISCUSION

Effect of ceftazidime stress on biofilm formation by UPEC

Since the formation of biofilms on surfaces can be regarded as a universal bacterial strategy for survival and for optimum positioning to effectively use available nutrients [17], the current results can be explained by that the isolate N 11 which contained bla_{TEM} confers resistance to CAZ, which in turn, render this isolate cells not to be in need to form biofilm to protect themselves. In contrast, sensitive isolate which lack gene responsible for CAZ resistance, showed formation of moderate biofilm, and this can be referred to that in the absence of resistance gene, bacteria tend to form biofilm.

Effect of antibiotic stress on slime layer production

These results could be attributed to *E. coli* N 11 resistant isolate traded off resistant genes by those coding for production of slime layer in order to overcome environmental stress comprised by antibiotic challenge. This explanation can be more convenient by noticing that *E. coli* S isolate exhibited strong ability to produce slime layer (**Fig**. **5, c**). Likewise, on CRB, similar phenomenon was noticed in **Fig**. 6.

Another possible explanations are that the antibiotic may influence the assembly of carbohydrate and protein molecules that composed the extracellular matrix which known as slime layer, or that antibiotic suppress this layer production to avoid decrease in metabolic activities of the cells because it was previously found that over-production of component exo-polysaccharides leads to a decrease in metabolic activities of the cells as it can be a barrier to the penetration and diffusion of the nutrients and oxygen [18].

Effect of pH and ceftazidime stress on *E. coli* growth

The present results showed that low pH slightly shortened the initial phase in addition to the synergy effect of sub MIC of CAZ which affect bacterial processes and led to reduce bacterial number. Whereas, high pH value effect may lead to faster rates of decline of bacterial cells as well as the effect of CAZ sub MIC. N 11 isolate showed absence of survival capacity in both pH values 6 and 8 in spite of resistance gene in its own genome, which in turn, can be referred to the effect of medium acidity and alkalinity because when this isolate was tested in other experiments and was challenged with antibiotic sub MIC stress alone, there was some colonies which were hardly capable to survive in pH 7, but when acid and alkaline stress was added to the medium, their capability to survive was eliminated. S strain which lack the resistance gene was also affected by the acidity of the medium but in low rate when compared to N 11 isolate.

Effect of temperature and ceftazidime stress on *E. coli* growth

These current results which revealed that the viable count at low temperature was high when compared with that at high temperature can be explained by that when an organism is grown below its optimum and above its minimum temperature, it grows much more slowly because the metabolic enzymes are performing inadequately for maximum growth and the plasma membrane becomes rigid and unable to transport nutrients effectively.

In general, such differences in physiological processes between resistant and sensitive isolates could be attributed to that acquisition of resistant genes (in this case beta lactamases coding genes) applies additional metabolic activities represented by synthesizing new proteins and RNA; which, in turn, will affect the energetics of the cell and rendering them less fitted.

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Conflict of interest

The authors declare that they have no conflict of interests.

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