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Molecular Detection of Some β-lactamases Genes in Uropathogenic *Escherichia coli*

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Abstract

Around fifty *Escherichia coli* isolates were isolated from sixty midstream urine specimens collected from patients visiting hospitals in Baghdad city. Approximately, 52% of all isolates were identified as extended spectrum beta lactamases (ESBL) producer. Results demonstrated that 92% of these isolates were sensitive to carbapenems. Only four β-lactamase coding genes were detected; bla_{TEM}, bla_{PER}, bla_{VIM} and $bla_{CTX-M-2}$. As a conclusion, this work revealed that local *E. coli* isolates harboured ESBL coding genes which may contribute in its pathogenicity.

Keywords: *Escherichia coli*, ESBL, β-lactamase genes.

التحري الجزيئي عن مورثات البيتالاكتاميز في الممرضة البولية ايشريكيا القولون

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الخلاصة

عزلت خمسون عزلة من الايشريكية القولونية من ستين عينة ادرار وسط المجرى جمعت من مرضى يزورون مستشفيات مدينة بغداد. شخص ما نسبته ٥٢% من العزلات على انها منتجة لانزيمات بيتا- لاكتاميز موسعة الطيف. كما اظهرت النتائج ان ٩٢% من هذه العزلات كان حساسا لمضادات الكاربابينيمات. تم التعرف على اربعة مورثات من مورثات البيتالاكتاميز و هي bla_{TEM} و bla_{PER} و bla_{VIM} و $bla_{CTX-M-2}$. و يمكن ان نخلص الى ان عزلات الايشريكية القولونية المحلية تحمل جينات البيتالاكتاميز موسعة الطيف.

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. *Escherichia 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 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

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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].

The aim of this work is to investigate the problematic ESBL producing *E. coli* in Iraq by identifying some of ESBLs coding genes.

Materials and Methods

Specimens collection

Sixty midstream urine specimens were collected randomly from patients presented with urinary tract infections attending Al-Yarmouk, Al-Numan, and Saint Raphael hospitals in Baghdad for the period from March 2014 to April 2014.

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 reincubated at 37°C for another 24 h. Pink colonies were selected and examined for Gram stainability, cultural, morphological characteristics, and conventional biochemical tests. Identification of *E. coli* isolates was confirmed by automated identification systems such as api 20E and VITEK 2 (Biomérieux, France).

Antibiotic susceptibility test

Kirby-Bauer method was used as described by Morello *et al*. [4] 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 [5]**.**

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 to a Mueller-Hinton plate inoculated with the test organism (*E. coli*). After one hour of inoculation at room temperature, these antibiotic disks are removed and replaced on the same spot by disks containing ceftazidime and aztreonam. Control disks of these two antibiotics are simultaneously placed at least 30 mm from these locations. A positive test is indicated by an increase of zone of inhibition by ≥5 mm for the disks which have replaced the amoxicillin/clavulanate disks compared to the control disks which are 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}, $bla_{\text{OXA-like}}$, $bla_{CTX\text{-M}}$, bla_{PER} and bla_{VIM}, bla_{IMP} and bla_{KPC} genes by polymerase chain reaction (PCR).

DNA was extracted from all *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.* [6]. 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 **[6]**.

id	Primer name	Primer sequence 5'→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, 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 – 4; while those for monoplex PCR were listed in Tables 5-7.

Table 2-Reactants volumes and concentrations used 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 annealing		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*$_{IMP}$ [6]

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*$_{CTX-M-9}$ are 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 are 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	–

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

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 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 the 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 hr on a 1.5% agarose gel containing ethidium bromide. A 100 and 800 bp DNA ladder were used as a size marker [6].

Results and Discussion

Females (n= 29) over numbered males (n= 21) equal a ratio of 1.3:1 which is very expected due to the physiological and anatomical characteristics of females that encourage infection of females over males.

Out of 60, 50 specimens were identified to harbour *E. coli* which is tested by conventional morphological methods and biochemical analysis as well as automated systems represented by Api 20E and VITEK 2.

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 Figure-1. These findings indictate the capacity of these isolates to produce β lactamases.

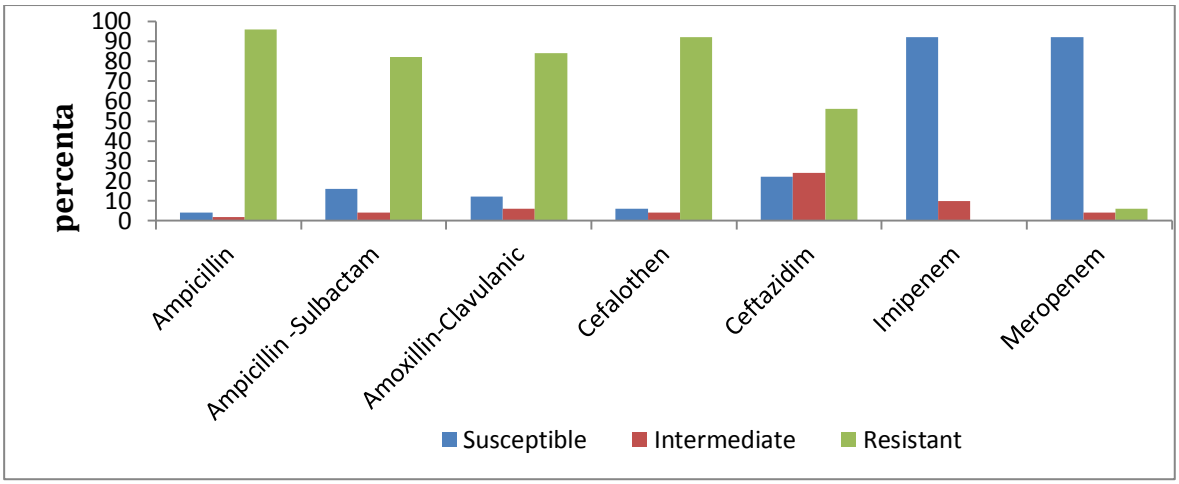

Figure 1-Antibiotic resistance of 50 *E. coli* clinical isolates isolated from 60 patients**.**

About 52% of all 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 Figure-1.

Results are presented in Figures 2, 3, and 4 demonstrated only four β-lactamase genes out of ten β-lactamase genes under investigation were detected; *bla*$_{PER}$ (520bp), *bla*$_{TEM}$ (800bp), *bla*$_{VIM}$ (390bp) and *bla*$_{CTX-M-2}$ (404bp) 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.

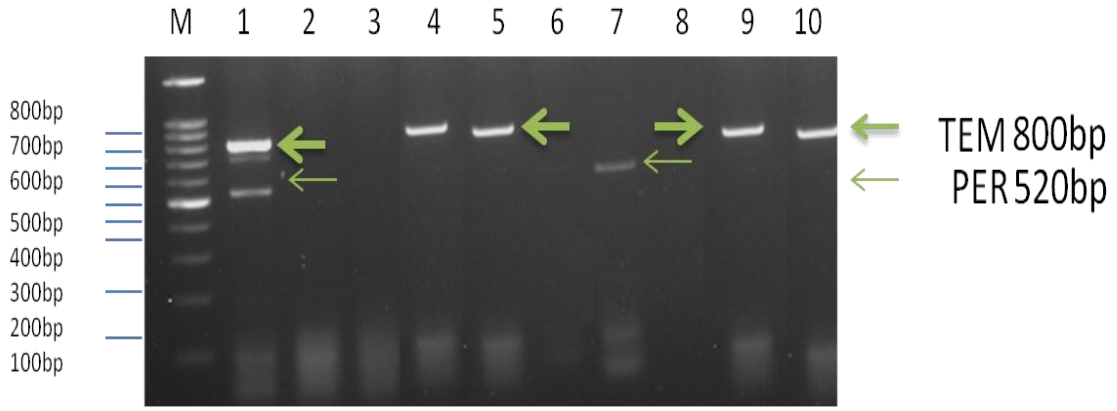

Figure 2- Analysis of the presence of *bla*$_{TEM}$ and *bla*$_{PER}$ among *E. coli* clinical isolates by PCR and run on agarose gel (1.5%) at 5 V/cm for 1 hour, stained with ethidium bromide and visualized on a UV trans illuminator documentation system. Lane M: ladder, lanes 1-10 represent *E. coli*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

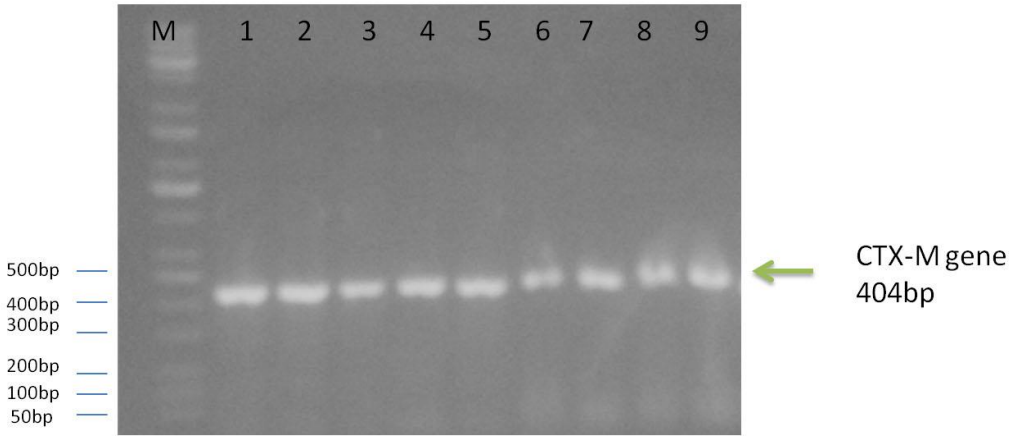

Figure 3- Analysis of the presence of *bla*$_{CTX-M-2}$ among *E. coli* clinical isolates by PCR and run on agarose gel (1.5%) at 5 V/cm for 1 hour, stained with ethidium bromide and visualized on a UV trans illuminator documentation system. Lane M: ladder, lanes 1-9 represent *E. coli* isolates 3, 26,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

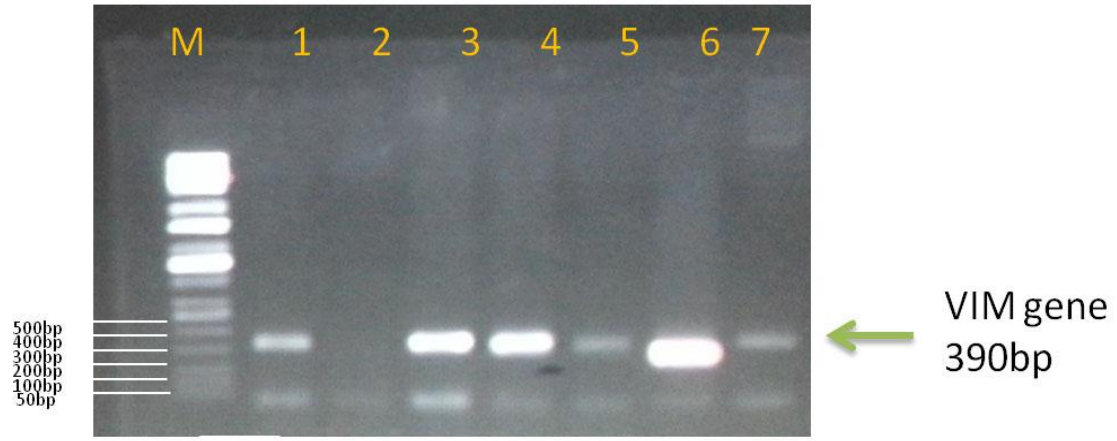

Figure 4- Analysis of the presence of *bla*$_{VIM}$ among *E. coli* clinical isolates by PCR and run on agarose gel (1.5%) at 5 V/cm for 1 hour, stained with ethidium bromide and visualized on a UV transilluminator documentation system. Lane M: ladder, lanes 1-7 represent *E. coli* isolates 9, 10, 11,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

In a local study done by Aziz [7], the result revealed that the most common ESBL in *E. coli* were $bla_{\text{CTX-M}}$ (CTX-M-14 and CTX-M-15) and bla_{TEM}; which represented 18 and 11% of total isolates tested, respectively. Likewise, Auda [8] and Jabbar [9] stated that $bla_{\text{CTX-M-2}}$ is the most frequent beta lactamase gene in *E. coli* isolates.

In conclusion, ESBL *E. coli* local isolates are found to exist relatively at a high level among clinical isolates derived from UTI patients.

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