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Dissmination of TEM and SHV Extended-Spectrum Beta-Lactamases in Clinical Isolates of *Escherichia coli*.

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Abstract

Purpose: To examine the dissemination of *bla*$_{TEM}$ and *bla*$_{SHV}$ genes in *Escherichia coli* isolate from patients with significant bacteriuria.
Methods: During the study period, antibiotic susceptibility patterns were studied in a total of 38 isolates of *E. coli*. The isolates were also examined to determining the presence of *bla*$_{TEM}$ and *bla*$_{SHV}$ genes by PCR assay. Conjugation experiments were performed by using rifampicin resistance *E. coli* MM294 as the recipient.
Results: All isolates were resistance to at least five of the antibiotics tested (multidrug resistance, MDR) and sensitive to imipenem. PCR assay revealed that 57.1% and 14.3% of the isolates carried *bla*$_{TEM}$ and *bla*$_{SHV}$ genes, respectively, additionally 9.5% of the isolates harbored both genes. Conjugation experiments and PCR trials confirmed that the *bla*$_{TEM}$ positive *E. coli* isolates tested were able to transfer this gene in transconjugants.
Conclusions: There was a high occurrence of MDR isolates in patients with significant bacteriuria and the *bla*$_{TEM}$ gene was common among isolates.

Introduction

Urinary tract infection (UTI) is one of the most common infections encountered and treated worldwide. UTI is defined by the presence of organisms in the urinary tract, which is usually sterile [1]. Clinically important infections usually occur due to bacteria, although viruses, fungi and parasites can also cause infections. However, *E. coli* is the most common urinary pathogen isolated from UTIs [2,3].

Treatment of *E. coli* infections was increasingly became difficult because of the multidrug resistance exhibited by the organism. ESBL- producing organisms pose a major problem for clinical therapeutics. ESBLs are defined as β-lactamases capable of hydrolyzing oxyiminocephalosporins and are inhibited by β-lactamase inhibitors [4,5]. Sequence exploration of the ESBLs has allowed them to be grouped into four classes, class A to D [6]. Most ESBLs originate in *E. coli* and *K. pneumoniae* belong to class A which include the TEM- and SHV-type of β-lactamases[7]. These enzymes are commonly located on large, transferable plasmids and the increasing incidence and spread of β-lactam resistance can be attributed to the dissemination of these plasmids [8].

Since, *E. coli* is one of the most common isolates able to produce TEM and SHV β-lactamases, present study carried out to determine the spread of *bla*$_{TEM}$ and *bla*$_{SHV}$ genes among *E. coli* isolated from patients with UTI in Najaf as well as their susceptibility patterns.

Materials and Methods

Bacterial isolates

During the period from December 2006 to March 2007, a total of 38 ampicillin and amoxicillin resistance *E. coli* isolates (β-lactamases resistance) were collected from patients with UTI in three hospitals (Al-Sadr Teaching Hospital, Al-Hakeem Hospital and Al-Zahra Maternity and Children Hospital) in Najaf. Only patients who had pyuria (>10 white blood cells/µl), and significant bacteriuria (>105 CFU/ml) were included in the microbiological analysis. The isolation and identification of *E. coli* strains were performed by minimal standard bacteriological tests, using conventional biochemical tests [9,10].

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Antibiotic susceptibility testing

The isolates were tested for antibiotics susceptibility by disk diffusion technique according to National Committee for Clinical Laboratory Standards guidelines [11]. The following antibiotic disks (drug concentration in µg) were used: ampicillin (10), carbenicillin (100), ceftazidime (30), cefotaxime (30), ceftriaxone (30), cephalothin (30), ciprofloxacin (30), gentamicin (10), imipenem (10), kanamycin (30), nalidixic acid (30), nitrofurantoin (300), trimethoprim (25), chloramphenicol (30) and tobramycin (10) manufactured by Himedia, India. *E. coli* ATCC 25922 used as quality controls.

PCR amplification

DNA was extracted from bacterial cells by using the Wizard Minipreps DNA kit (Promiga, USA). The DNA was then used as a template in specific PCR for the detection of bla_{TEM}, and bla_{SHV} gene. PCR amplification was performed by using the following primers (Promiga): TEM/F (5'-CGC CGG GTT ATT CTT ATT TGT CGC-3') and TEM/R (5' –TCT TTC CGA TGC CGC CGC CAG TCA-3'); SHV/F (5'-ATG AGT ATT CAA CAT TTC CG-3') and SHV/R (5'-CCA ATG CTT AAT CAG TGA GG-3'). Cycling conditions were as follows: initial denaturation at 94^{o}C for 5 minutes followed by 30 cycles of denaturation at 94^{o}C for 1 minutes (TEM) and 30 second (SHV), annealing at 55^{o}C for 1 minute (TEM) and 68^{o}C for 30 second (SHV), and elongation 72^{o}C at 1 minute (TEM) and 50 seconds (SHV). The final elongation step was extended to10 minutes at 72^{o}C. The PCR products separated on 1.5% agarose gels. Bands visualized with UV-transilluminater, after being stained with ethidium bromide [12].

Transfer of resistance

Mating experiments were performed as previously described [13], with *E. coli* MM294 (rifampicin resistant) as recipient. Exponential cultures of clinical isolates as donor (1 vol) and recipient (2 vol) were inoculated as a spot on brain heart infusion agar, BHIA, (Biolife, Italy). After overnight incubation at 37^{o}C, the bacteria were resuspended, diluted and plated onto BHIA containing rifampicin (256 µg/ml). Transconjugants growing in the selection plates were subjected to PCR analysis.

Results

Among 46 of *E. coli* obtained from patients with significant bacteriuria, 38 (82.6%) isolates identified as β-lactam resistance. All isolates found to be resistant to at least five classes of antibiotics tested, hence they were considered to be MDR. However, the highest resistance rates found for ampicillin, carbenicillin, cephalothin, gentamicin, ciprofloxacin and trimethoprim. Intermediate resistance rates obtained for tobramycin, ceftriaxone, nitrofurantoin and kanamycin. The lowest resistance rates observed for nalidixic acid, cefotaxime, ceftazidime and chloramphenicol. On the other hand, all isolates were susceptible to imipenem, which was the most effective drug (Table 1). Among the isolates, 21 (55.3%) were β-lactamase producers.

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Table (1): Antibiotic resistance pattern of β-lactam resistant *E. coli* isolates (n= 38)

Type of antibiotic	No. (%) of resistant isolates
Ampicillin	38 (100%)
Carbenicillin	36 (94.7%)
Cephalothin	27 (71.1%)
Ciprofloxacin	23 (60.5%)
Trimethoprim	23 (60.5%)
Gentamicin	23 (60.5%)
Tobramycin	22 (57.9%)
Ceftriaxone	21 (55.3%)
Nitrofurantoin	20 (52.6%)
Kanamycin	19 (50%)
Nalidixic acid	(47.4%)18
Cefotaxime	(42.1%)16
Ceftazidime	14 (36.8%)
Chloramphenicol	34.2%) 13 (
Imipenem	0

All 21 β-lactamase producing isolates were tested by PCR, using primers specific to bla_{TEM} and bla_{SHV}. Results revealed that 12 (57.1%) isolates were positive for bla_{TEM} genes, 3 (14.3%) isolates were positive for bla_{SHV} genes, 2 (9.5%) isolates had both bla_{TEM} and bla_{SHV} genes, and 4 (15.4%) isolates had negative results (Table 2).

Table (2): Frequency of TEM and SHV among 21 β-lactamase-producing *E. coli* isolates

Type of β-lactamase	No. (%) PCR positive test
TEM	**12 (57.1)**
SHV	**3 (14.3)**
TEM and SHV	**2 (9.5)**
Negative result	**4 (15.4)**

Table (3) showed antibiotics resistance pattern of original and transconjugation strains by revealing that the transferring of resistance to cephalothin, ceftazidime, ceftriaxone and gentamicin.

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Table (3): Antibiotics disk resistant for transconjugants resulted from conjugation between β-lactamase producing isolates and standard strain *E. coli* MM294

Isolate	AMP	CIP	CEF	CTX	CAZ	CRO	CAB	GN
E. coli MM294	S	S	S	S	S	S	S	S
E. coli 10	R	S	R	S	R	R	R	R
E. coli 10 T	R	S	R	S	R	R	R	R
E. coli 30	R	S	R	R	R	R	R	R
E. coli 30T	R	S	R	R	R	R	R	R

R, resistance; S, susceptible; T, transconjugate; AMP, ampicillin; CEF, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; GN, gentamicin; CAB, carbenicillin.

The MIC to β-lactam antibiotics of transconjugants were detected. Table (4) revealed that these transconjugants were able to grow in concentration ≥128µg/ml of ampicillin and amoxicillin. Regarding piperacillin, all the transconjugants were able to grow in concentration less than break point (128 µg/ml). The MIC value of ESBL antibiotics ranged from 16 to 32 µg/ml for cefotaxim, ceftriaxion and ceftazidime, indicating that the resistance isolates are still resistant to these antibiotics as their original isolates.

On the basis of PCR analysis of DNA, results indicate that bla_{TEM} gene in the test isolates were encoded on the transferable plasmid, which was shown transfer in conjugal mating experiments with recipient cells.

Table (4): MICs of β-lactam antibiotics for transconjugants resulted from conjugation between ESBL-producing *E. coli* and standard strain *E. coli* MM294

Isolate	AMP ≥32 µg/ml	AMX ≥32 µg/ml	PIP ≥128 µg/ml	CEF ≥32 µg/ml	CTX ≥64 µg/ml	CAZ ≥32 µg/ml	CRO ≥64 µg/ml
E. coli 10	>128	>128	≥128	>128	16	16	64
E. coli 10T	≥128	≥128	64	≥64	16	16	32
E. coli 30	>128	>128	>128	>128	32	32	64
E. coli 30 T	≥128	≥128	64	46	32	16	32

AMP, ampicillin; AMX, amoxicillin; PIP, piperacillin; CEF, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone.

Discussion

The emergence and dissemination of antibiotics resistance in bacteria has been well documented as a serious problem worldwide [14,15,16]. In the present study, the percentage of isolates demonstrating a MDR phenotypes were extremely high, as compared to rates reported in other countries, in United States it was 7.1% in 2000 [17] and in Slovenia 42% in 2006[18]. Our results showed high rates of resistance in *E. coli* to β-lactam antibiotics including carbenicillin and cephalothin. The mechanism of resistance to β-lactam antibiotics was mainly the production of β-lactamases, which

detected in 55.3% of resistant isolates, whereas 44.7% failed to produce these enzymes. Although the β-lactamases undoubtedly play a major role in the resistance to β-lactam antibiotics, the high ratio of resistance was not only attributable to the production of β-lactamases. The other mechanisms conferring resistance to these compounds is caused by reducing of the activity of β-lactam antibiotics in a resistant cell due to many factors such as; the sensitivity of the antibiotic to β-lactamases, the penetration through the outer membrane, the affinity for the target (PBPs), and the amount of β-lactamase [19,20,21].

From the present study, it appears that imipenem are the drug of choice for serious infections with β-lactam resistance isolates as has been recommended earlie[22,23]. However, these should not be administered as empirical therapy for *E. coli* infections that are not life threatening because their overuse can pose a significant problem [24].

Alternatively, ciprofloxacin, some aminoglycosides and nitrofurantoin may be used if they show in vitro activity against the isolates, leaving far behind the β-lactam- β-lactamase inhibitor combination. The situation may vary from region to region, so institutional local patterns of susceptibility should be used to determine the choice of antibiotics [25].

Our study revealed that 57.1% of β-lactamases producing *E. coli* gave PCR products with TEM-specific primers. The TEM β-lactamases spread worldwide and it is known to be found in many Enterobacteriaceae. However, *E. coli* shows reduced susceptibility to first and second generation cephalosporins by the production of plasmid-mediated, TEM β-lactamase. Since 1980s, the emergence of resistance to third generation cephalosporins has been reported in strains of *E. coli* [26,27]. In recent local study, Al-Hilali found that 18 of the 22 enteropathogenic *E. coli* yielded amplification products with TEM-PCR specific primers [28].

The results in this study also showed that 14.3% out of the 21 β-lactamase-producing *E. coli* isolates positive with bla_{SHV}. Most *E. coli* isolates have chromosomally or plasmid-mediated SHV-1 β-lactamase, which is a narrow-spectrum β-lactamase with activity against penicillins. More than 50 variants of SHV which are important worldwide and currently recognized on the basis of unique combination of amino acid replacement [29]. SHV-2 and SHV-5 (plasmid-mediated β-lactamases) enzymes have been recorded in at least five countries, with the latter type widespread in Greece. In one study, in Thailand reported that the frequency of bla_{SHV} gene was 8% of the confirmed ESBL producing *E. coli* isolates [30].

Among the *E. coli* isolates produced β-lactamase, 19.0% isolates were unable to yield amplification products with TEM and SHV-PCR specific primers. Perhaps, these isolates may be original negative for ESBLs, or may be harbored another ESBLs genes.

E. coli have been an important source of transferable antibiotic resistance [31]. PCR confirmed that *E. coli* isolates were able to transfer the bla_{TEM} gene in transconjugants. *bla*-genes are usually carried by large and transferable plasmids. Thus the plasmid localization of the genetic determinants facilitates their horizontal spread in bacterial populations, particularly by means of conjugation, accumulation of resistance genes results in strains that contain multiresistant plasmids [32].

For this reason, such ESBL-producing multiresistant isolates pose a serious therapeutic problem in hospital setting. Our study has shown the spreading of multidrug resistant and *bla*-genes harbored *E. coli* isolates among patients with significant bacteriuria. Hence, it is suggested that, routine diagnosis of ESBL-producing isolates should be done in hospitals in Najaf and more importantly,

avoiding misuse and overuse of antibiotics may reverse the undesired effects of multidrug resistant and ESBL-producing bacteria.

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انتشار إنزيمات البيتالاكتاميز واسعة الطيف نوع TEM وSHV في العزلات ألسريريه لبكتريا *Escherichia coli*

الخلاصة

هدف الدراسة: التحري عن مدى انتشار جينات bla_{SHV} و bla_{TEM} بين عزلات *E. coli* المعزولة من المرضىالمصابين بالبيله البوليه.

طرائق العمل: تم دراسة أنماط حساسية 38 عزلة *E. coli* للمضادات الحيوية المختبرة. وقد اجري التحري عن وجود جينات bla_{SHV} و bla_{TEM} باستعمال تقنيه PCR كما تم اختبار اقتران البكتريا باستخدام العزلة *E. coli* MM294المقاومة للرفامبسين كمستلم للمورثتين bla_{TEM} و bla_{SHV}.

النتائج: وجد أن كل عزلات *E. coli* كانت مقاومه إلى ما لا يقل عن خمسه أنواع من المضادات الحيوية (متعددة المقاومة) إلا أنها كانت حساسة للمضاد Imipenem. اظهر استعمال تقنيه PCR أن 57.1% و 14.3% من العزلات تحمل المورثين bla_{TEM} و bla_{SHV} على التوالي، فضلا عن ان 9.5% من هذه العزلات كانت تحمل المورثتين bla_{TEM} و bla_{SHV} معا. أكد اختبار الاقتران وتقنيه PCR أن عزلات *E. coli* التي تحمل المورثة bla_{TEM} قادرة على نقلها الى البكتريا المقترنة.

الاستنتاجات: توجد نسبه عاليه من العزلات متعددة المقاومة للمضادات الحيوية بين المرضى الذين لديهم بيلا بولية. يعد bla_{TEM} المورث السائد بين هذه العزلات.