Phenotypic and Genotypic Detection of Extended-Spectrum β-Lactamases (ESBL) among *Escherichia coli* Isolated from Symptomatic Female's Genital Tract Infection

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التحري مظهريا وجينيا عن إنزيمات البيتالاكتم واسعة الطيف ضمن الاشرشية القولونية (*Escherichia coli*) المعزولة من اصابات المسلك التناسلي للنساء المصحوبة بالاعراض

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الخلاصة :

تضمنت هده الدراسة 36عزلة سريريه للاشريشية القولونية *(Escherichia coli)* جمعت من نساء (بعمر 18-45 سنة) مصابات بالتهاب المسلك التناسلي المصحوب بالأعراض. تم التحري في هذه العزلات عن إنتاج إنزيمات البيتالاكتم الواسعة الطيف (**ESBL**) مظهريا وجينيا. من الناحية المظهرية، اظهرت معظم العزلات (36/30: 83%) مقاومة للسيفوتاكسيم (CTX) واكثر من نصفها (36/26: 61.1%) كان مقاوما للسيفتازيديم (CAZ). اظهرت فحوصات الغربلة ان كل العزلات (100%) كانت منتجة لـ **ESBL**، في حين كانت النسبة (25: 69.4 %) مع الفحوص التأكيدية. تم التحري كذلك عن انزيمات البيتالاكتم واسعة الطيف جينيا، اذ كانت الانماط الجينية من الـ **ESBL** موجودة في 36/31 (86.1%) من العزلات قيد الدراسة. في هذه الدراسة، جميع الانماط الوراثية الاربعة من **ESBL** كانت موجوده، وأظهرت النتائج ان اكثرها سيادة كان CTX-M (36/26: 72.2%) يتبعها النمط SHV (36/22: 61.1%) ثم النمط OXA (36/7: 19.4%) والنمط TEM (36/1: 2.7%). من مجموع العزلات البالغة 36 هناك 25 منها (69.4%) تمتلك نمطين من جينات الـ **ESBL**. هنالك 17 عزلة ايجابية للنمط SHV من مجموع 22 (77.2%) تكون موجبة للـ CTX-M. وجد ايضا ان 6 عزلات (7/6: 85.7%) ايجابية للنمط الجيني OXA والنمط TEM هي ايضا موجبة للـ CTX-M في حين ان العزلات الموجبة للنمط الاخر من OXA كانت موجبة لـ SHV. معظم العزلات (25/22: 88%) التي لها نمطين من **ESBL** كانت مقاومة من CTX و CAZ . على ما تقدم يمكن الاستنتاج، بان انتاج **ESBL** في حالات التهابات المسلك التناسلي-سيما نوع CTX-M- يمكن ان يساهم (فضلا عن عوامل الضراوة الاخرى) في عملية اختيار بعض العتر للبقاء والتسبب بالامراض وبما ان بكتيريا الاشريشية القولونية موجودة في المسلك البولي التناسلي فانها تسهم في انتقال الاصابات بالبكتيريا لحديثي الولادة. مختبراتنا يجب ان تكون على دراية بوجود مثل هذه الكائنات المنتجة للانزيمات البيتالاكتم واسع الطيف واجراء دراسات لمراقبتها والتحقق منها.

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Abstract

Clinical *E. coli* isolates (36) from women (aged 18-45 years) with symptomatic genital tract infection were detected phenotypically and genotypically for ESBL production. Phenotypically, most (30/36: 83.3%) isolates were resistant to cefotaxime (CTX) and more than half of them (26/36: 72.2%) were resistant to ceftazidime (CAZ). All (100%) and 25 (69.4%) of them were ESBLs producers by screening and confirmatory tests, respectively. Genotypically, ESBL genotypes were detected in 31/36 (86.1%) of isolates. All four ESBL genotypes were found among these isolates with predominance of CTX-M-type (26/36: 72.2%) followed by SHV-type (22/36: 61.1%), OXA-type (7/36: 19.4%), and TEM-type (1/36: 2.7%). Of These isolates, 25/36 (69.4%) had two types of ESBL genes. Seventeen (17/22: 77.2%) of SHV-type positive isolates were CTX-M positive. Six (6/7: 85.7%) of OXA-type and the TEM-type positives were also CTX-M- positive whereas the other OXA-type positive isolate was SHV-type positive. Most (22/25: 88%) isolates with two types of ESBLs were resistant to both CTX and CAZ. It can be concluded that, in female's genital tract infection, ESBL production, especially CTX-M-type, can be added as another factor, in addition to virulence factors, that select for certain strains to survive and cause disease and as vaginal *E. coli* is a reservoir along the fecal-vaginal-urinary/neonatal course of transmission in extraintestinal *E. coli* infections, our clinical

microbiology labs and clinicians need to be aware of the presence of these ESBL-producing organisms and should conduct surveillance studies to ascertain this.

Key words: ESBL, *E. coli*, female's symptomatic genital tract infection.

Introduction

Escherichia coli is one of the common organisms in the vaginal microflora of pregnant as well as non-pregnant women (1). Vaginal *E. coli* (VEC) may also cause symptomatic infections such as vaginitis or tubo-ovarian abscess and is associated with life threatening neonatal sepsis and meningitis (2). Recently, *E. coli* is one of the predominant microorganisms in cases of aerobic vaginitis (3, 4, 5). Aerobic vaginitis is a term proposed to describe purulent vaginal discharge with predominance of abnormal aerobic flora (3, 6), and its characteristics are different from those of bacterial vaginosis and elicit an important host response and genital complaints are those of a real vaginitis (5, 6, 7).

β-Lactam antibiotics remain the most commonly used antibacterial agents in the present chemotherapeutic armamentarium, and β-lactamases, the enzymes that hydrolyze β-lactam antibiotics are the major cause of resistance to these compounds (8). ESBL enzymes are plasmid-mediated enzymes capable of hydrolyzing and inactivating a wide variety of β-lactams, including third generation cephalosporins, penicillins and monobactams, but have no detectable activity against cephamycines and imipenem (8-10). Until the 2000s, most of the ESBLs were structurally related to the narrow-spectrum TEM- and SHV-type β-lactamases (11). The genetic mutations that give rise to ESBLs broaden the parental resistance pattern to a phenotype that includes resistance to broad-spectrum cephalosporins. Plasmids responsible for ESBL production tend to be large (80 Kb or more in size) and carry resistance to several agents, an important limitation in the design of treatment alternatives (9). Furthermore, in the late 1990s, a novel type of ESBLs, the CTX-M enzymes, emerged worldwide, mostly from *Escherichia coli* (11). The OXA-type enzymes are another growing family of ESBLs and are unique among the ESBLs because they are most often found in *Pseudomonas aeruginosa* rather than in members of the *Enterobacteriaceae* (11, 12).

The incidence of ESBL producing strains of *E. coli* among clinical isolates has been steadily increasing over the past few years resulting in limitation of therapeutic options. The resistant organisms are now a worldwide problem (8-10). These organisms pose a therapeutic challenge, since they are frequently resistant to other kinds of antimicrobial drugs, including aminoglycosides, quinolones, and cotrimoxazole (14). We didn't find (to our knowledge) any study around the world, dealing with ESBL production by vaginal *E. coli*, so that this study was carried out to detect phenotypically and genotypically ESBL production by *E. coli* isolated from non-pregnant women with symptomatic genital tract infection.

Material and Methods

Bacterial isolates and phenotypic screening for ESBL

Thirty six isolates of *E. coli* collected from non-pregnant women (aged 18-45 years) over a 2-year period from May 2008 to June 2010 at Obstetrics and Gynecology Clinics in Al-Kut/Wassit Province/Iraq, were included in this study. The isolates were recovered from high vaginal swabs collected from women with symptomatic genital tract infection and were identified by conventional biochemical tests (15). Cefotaxime (CTX) and ceftazidime (CAZ)

were used for screening for reduced susceptibility to oxyimino-cephalosporins. The presence of ESBLs was confirmed by the double-disk method as recommended by the Clinical and Laboratory Standards Institute (17).

PCR amplification for detection of β-lactamase genes

All isolates were screened for the resistance genes TEM, SHV, CTX-M, and OXA by a multiplex PCR assay using universal primers (Bioneer, Korea) (Table 1), (18, 19, 20). Each isolate was subcultured on trypticase soy agar plates for 24 h at 37°C. From the agar plate, 5 colonies were picked and suspended in 100 µl sterile distilled water. Bacterial suspensions were run for 10 min at 94°C (21) in a DNA thermocycler (MultiGene, Labnet International, Inc., USA) and cell debris were removed by centrifugation (12,000 rpm for 1 min). Five µl of supernatant was used as a template in PCR. PCR amplification reactions were performed in a volume of 50 µl containing 25 µl of KapaTaq 2x Ready Mix (KAPA Biosystems, USA), 25 pmol concentrations of each primer, and 5 µl of DNA template. The cycling parameters were as follows: an initial denaturation at 94°C for 3 min; followed by 35 cycles of 94°C for 30 s, 45°C for 1 min, and 72°C for 1min; and with a final extension at 72°C for 10 min. The amplified PCR products were subjected to electrophoresis at a 2% agarose gel in 0.5X TBE buffer.

Statistical analysis

The x^2 test was used for statistical comparison of groups; values < 0.05 were regarded as significant (22).

Table 1. Nucleotide sequences of PCR primers used to amplify four ESBLs

Gene		Primer sequence(5'-3')	Amplicon size (bp)	Reference (s)
bla$_{TEM}$	F	AAACGCTGGTGAAAGTA	822	18, 19
	R	AGCGATCTGTCTAT		
bla$_{SHV}$	F	ATGCGTTATATTCGCCTGTG	753	18, 19
	R	TGCTTTGTTATTCGGGCCAA		
bla$_{CTX-M}$	F	CGCTTTGCGATGTGCAG	550	18, 19
	R	ACCGCGATATCGTTGGT		
bla$_{OXA}$	F	ATATCTCTACTGTTGCATCTCC	619	20
	R	AAACCCTTCAAACCATCC		

Results

Thirty six *E. coli* isolates from females with symptomatic genital tract infection, were surveyed phenotypically and genotypically for ESBL production. Phenotypically, most (30/36: 83.3%) isolates were resistant to CTX and more than half of them (26/36: 72.2%) were resistant to CAZ. All (100%) and 25 (69.4%) of them were ESBLs producers when tested by screening and confirmatory tests, respectively (Table 2).

Table 2: Phenotypic and Genotypic detection of ESBL production by 36 *E. coli* isolated from female's symptomatic genital tract infection

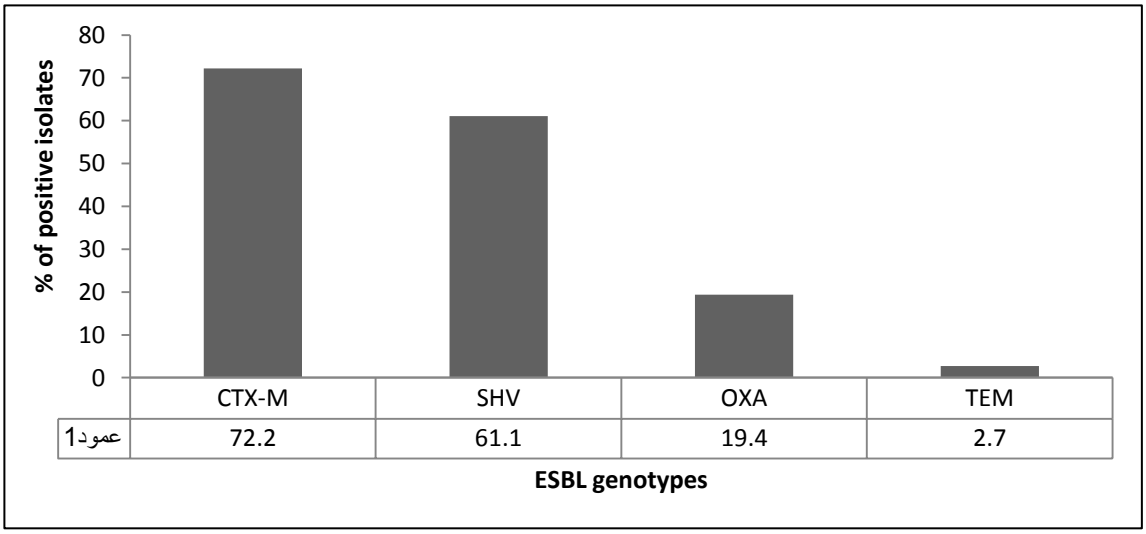
Characteristics	**No. (%) of positive *E. coli***

		isolates
Resistance to:	CTX	30 (83.3): R = 26; I = 4
	CAZ	26 (72.2): R = 25; I = 1
Phenotypic detection of ESBLs:	ESBL screening test	36 (100)
	ESBL confirmatory test (DDST)	25 (69.4)
Genotypic detection of ESBLs (*bla* genotype):	CTX-M	26 (72.2)
	SHV	22 (61.1)
	TEM	1 (2.7)
	OXA	7 (19.4)

CTX: cefotaxime; CAZ: ceftazidime; R: resistant; I: intermediate resistant; DDST: double disc synergy test; ESBL: extended spectrum β-lactamase.

Genotypically, ESBL genotypes were detected in 31/36 (86.1%) of isolates. All four ESBL genotypes were found among these isolates (Fig. 2) with predominance of CTX-M-type (26/36: 72.2%) followed by SHV-type (22/36: 61.1%), OXA-type (7/36: 19.4%), and TEM-type (1/36: 2.7%). Of These isolates, 25/36 (69.4%) had two types of ESBL genes. Seventeen (17/22: 77.2%) of SHV-type positive isolates were CTX-M positive. Six (6/7: 85.7%) of OXA-type and the TEM-type positives were also CTX-M- positive whereas the other OXA-type positive isolate was SHV-type positive. Most (22/25: 88%) isolates with two types of ESBLs were resistant to both CTX and CAZ except three isolates: one with genotype of CTX-M and TEM which was resistant to CTX and sensitive to CAZ, the second with genotype of SHV and OXA, which had intermediate resistance to CTX and was sensitive to CAZ, and the third isolate with CTX-M and SHV, which had intermediate resistance to both antibiotics. Among ESBL-positive isolates, 26/31 (83.8%). had CTX-M-type and 22/31 (70.9%) had SHV-type ESBL.

Fig. 2: Percent distribution of four ESBL genotypes among *E. coli* isolated from women with symptomatic genital tract infection.



Discussion

This study was designed to detect phenotypically and genotypically ESBLs' distribution among clinical *E. coli* isolated from non-pregnant women with symptomatic genital tract infection. Most isolates were resistant to CTX

and about half of them were resistant to CAZ. All of CAZ resistant isolates were also resistant to CTX. This indicated the widespread resistance to broad spectrum cephalosporins in our community as a result of extensive use of these antibiotics for treatment. Concentrated use of third-generation cephalosporins in Iraq may be the most prominent risk factor for emergence of ESBL-producing pathogens (23). High rate of ESBL production by *E. coli* may be due to the selective pressure imposed by extensive use of antimicrobials (24). The over-reliance on antibiotics, and insufficient application of infection control measures and improved hygiene, has eroded the effectiveness of older, inexpensive agents and threatens the efficacy of recently introduced ones (8).

The members of Enterobacteriaceae possess many mechanisms of resistance to β-lactam antibiotics such as loss of porin, efflux pumps, etc. However, β-lactamases are the most common and clinically significant mechanism of resistance to β-lactam antibiotics among this bacterial group (8). Four types of ESBLs were detected genotypically in this study, namely: TEM-, SHV-, CTX-M-, and OXA-type. Genotypically, 86.1% of this study included isolates were ESBL producers. CTX-M-type ESBL was the most common, followed by SHV- and OXA-type while TEM-type was rare. This result is consistent with the present situation in most parts of the world. During the last 2 decades, most of the ESBL found in *E. coli* and, in general, in gram-negative bacilli, has been of TEM or SHV lineage. Recently TEM and SHV types have been replaced by CTX-M-type ESBL (11, 25). CTX-M β-lactamases have spread among Enterobacteriaceae in most parts of the world (26- 28). In the Middle East area, reports pointed out that CTX-M is the predominant ESBL in *E. coli* (23, 29-31). Among the different ESBLs, particular attention should be paid to the worldwide increasing prevalence of the CTX-M types. These enzymes are prevalent not only in nosocomial environment, but also in the community setting (32-34). Antibiotic selective pressure probably contributes to the increasing prevalence of cefotaxime and ceftriaxone hydrolyzing CTX-M β-lactamases in clinical setting (35-36). This high distribution of CTX-M-type ESBLs among this study's isolates explains the high rate of resistance to CTX in comparison to CAZ since CTX-M β-lactamases, in contrast to most TEM and SHV ESBLs, preferentially hydrolyze cefotaxime over ceftazidime (37-38), but point mutations around the active site of some enzymes belonging to the CTX-M-1 and CTX-M-9 groups have increased their ability to hydrolyse ceftazidime significantly (39-40).

ESBL confirmatory test results were not correlated with genotyping results (Table 2), as 80.6% (25/31) of ESBL genotype positive isolates by PCR, were positive by confirmatory test. This can be explained by several ways each of which needs further investigation. Extended-spectrum β-lactamases (ESBLs) are generally sensitive to inhibition by clavulanic acid, though resistant variants have been reported. Combinations of β-lactamase inhibitors and penicillins have led to selection of the phenotype that resists inhibition of β-lactamase (8). The effectiveness of inhibitor may be reduced in the presence of multiple ESBLs in the bacteria (41). ESBL testing in AmpC-producing species of Enterobacteriaceae is an unresolved issue in the field of ESBL testing (42). Another possibility is the possession of more than one resistance mechanism (8).

It can be concluded that, in female's genital tract infection, ESBL production, especially CTX-M-type, can be added as another factor, in addition to virulence factors, that select for certain strains to survive and cause disease and as vaginal *Escherichia coli* is a reservoir along the fecal-vaginal-urinary/neonatal course of transmission in extraintestinal *E. coli* infections, our clinical microbiology labs and clinicians need to be aware of the presence of ESBL-producing organisms and should conduct surveillance studies to ascertain this.

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