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ROLE OF PLASMIDS IN MEDIATING ANTIBIOTIC RESISTANCE AND EXTENDED – SPECTRUM BETA – LACTAMASE PRODUCTION IN ESCHERICHIA COLI

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دور البلازميدات في إنتاج إنزيم بيتالاكتاميز الواسع المدى لدى بكتيريا الاشيريشيا كولاي

ملخص: أجريت هذه الدراسة بغرض توضيح دور البلازميدات في نقل مقاومة المضادات الحيوية لدى بكتيريا الاشيريشيا كولاي التي تفرز إنزيم البيتالاكتاميز الواسع المدى والتي تم عزلها في عام 2001 من إصابات المسالك البولية المكتسبة من المجتمع في قطاع غزة. و أظهرت نتائج هذه الدراسة أن من بين 300 عزلة للاشيريشيا كولاي وجد أن 11 (3.7%) منها تفرز البيتالاكتاميز الواسع المدى وبعمل دراسة لحساسية هذه العزلات الأخيرة لإثنا عشر مضاداً حيوياً أظهرت مقاومة عالية للمضادات الحيوية خصوصاً الأموكسيسيلين (100%) ، السيفالوسبورين (100%) والجنتاميسين (81.8%). وعند دراسة بلازميدات الإحدى عشر عزلة المنتجة للبيتالاكتاميز الواسع المدى ثبت أن كل هذه العزلات تحوي على بلازميدات ذات أحجام مختلفة. كما أن نتائج نقل البلازميدات من بعض العزلات بطريقة الاقتران أظهرت أن البيتالاكتاميز الواسع المدى ومقاومة بعض المضادات الحيوية مرتبطة بالبلازميد.

الكلمات المفتاحية: البلازميد، إنزيم البيتالاكتاميز الواسع المدى ، الاشيريشيا كولاي

Abstract: This study was carried out in order to investigate the role of plasmids in mediating antimicrobial agents' resistance in extended-spectrum beta-lactamase (ESBL) producing *E. coli* isolates collected from community acquired urinary tract infections in Gaza Strip.

The results of the study showed that, out of 300 *E. coli* isolates collected during the year 2001, 11 (3.7%) of the isolates were ESBL-producers.

When the susceptibility of the ESBL-producers to 12 antimicrobial agents was tested, high resistance especially, to amoxycillin (100.0%), cephalosporins (100.0%) and gentamicin (81.8%) was observed.

Plasmid DNA analysis of the 11 ESBL-producing isolates revealed that, all the ESBL-producers harbored plasmids of different sizes.

The results of plasmid transfer (by conjugation) showed that, all ESBL-producing trsnscojugants and resistance to various antimicrobial agents is plasmid associated.

Key words: Plasmid, ESBL, *Escherichia coli*

Introduction:

Many bacteria acquire resistance to one or more of the antibiotics to which they were formerly susceptible. This resistance emerges as a result of genetic changes and subsequent selection processes. Virtually, genetic changes may be chromosomal or extrachromosomal but the resistance genes are often found on plasmids, which spread easily from one bacterium to another and even from one species of bacterium to another [1/2].

Extended-spectrum beta-lactamases (ESBLs) are bacterial enzymes that confer upon host resistance to various beta lactam drugs. These enzymes are produced mainly by *Klebsiella pneumoniae* and *Escherichia coli* [2/2]. ESBLs arise from mutations to existing plasmid-mediated beta-lactamases, especially TEM and SHV [3/2] and other newly emerging class A enzymes such as CTX-M [4/2].

Emergence of plasmid-encoded ESBLs is a significant evolution in antimicrobial resistance, and it becomes an infection control problem to restrain the spread of this resistance [5/2].

It is known that ESBL producing strains can survive in the hospital environment [6/2]. Boundaries between community and hospital environments are, however, becoming more blurred. This may have consequences for the development of resistance to antimicrobial drugs and spread of resistant strains in the community [7/2].

This study was carried out in order to investigate the role of plasmids in mediating antibiotic resistance and ESBL production in *E. coli* isolates collected from community-acquired UTI female patients.

Materials and Methods

The study was approved officially by the Palestinian Ministry of Health. All participants were given an informed consent in Arabic. The subjects were handled in terms of code numbers, without personal data or names to ensure confidentiality. All processing steps of the study samples and records were dealt with in a way to ensure privacy and accuracy.

Three hundred *E. coli* isolates collected from outpatient females with clinical evidence of community-acquired urinary tract infections (UTI) during the year 2001 in the Gaza Strip were investigated for ESBL production.

Resistance to cefpodoxime disks (10 μg) has been studied as a screening method for ESBL production by disk diffusion technique, using sensitivity breakpoint of $\leq$ 21 mm [8/3].

The Epsilometer test (E-test) method was used for confirmation of ESBL production (AB Biodisk, Solna, Sweden). The strips were used according to the manufacturer's instructions. A ratio of MIC ceftazidime, ceftazidime-clavulanic acid and MIC cefotaxime, cefotaxime-clavulanic acid of $\geq$ 8 was considered to be ESBL-positive [9/3].

In order to investigate the role of plasmids in mediating antibiotic resistance in *E. coli* the 11 ESBL-producing isolates were analyzed for the presence of plasmid DNA.

Plasmid DNA minipreps were prepared from *E. coli* suspensions following the alkaline lysis procedure developed by Birnboim and Doly (1979) and the minipreps were purified by GFX^{TM} Micro Plasmid Prep Kit (Sigma). The plasmids were visualized and documented according to standard procedures [10/3].

Conjugal transfer of plasmids was achieved by the method of Rotimi *et al* [10/3] using a drug sensitive recipient (which was made nalidixic acid resistant).

Results

Eleven (3.7%) of the isolates were found to be resistant to cefpodoxime according to the disk diffusion test and thus were classified as putative ESBL-producers.

By calculating the ratio of MIC ceftazidime, ceftazidime-clavulanic acid and cefotaxime, cefotaxime-clavulanic acid, the results confirmed that the 11 isolates have ratios greater than 8 and were thus considered ESBL- producers.

When the susceptibility of the ESBL-producers to 12 antimicrobial agents was tested, high resistance especially, to amoxycillin (100.0%), cephalosporins (100.0%) and gentamicin (81.8%) was observed. All the isolates, however, were completely sensitive to amoxycillin-clavulanic acid and nitrofurantoin (Table 1).

Table (1): Susceptibility of ESBL-producers to the selected antimicrobials.

Antimicrobial agent	**Sensitive % (n)***	**Resistant % (n)**
AMX	0.0 (0)	100.0 (11)
CF	0.0 (0)	100.0 (11)
CMX	0.0 (0)	100.0 (11)
CXT	0.0 (0)	100.0 (11)
SXT	18.2 (2)	81.8 (9)
GM	18.2 (2)	81.8 (9)
NA	36.4 (4)	63.6 (7)
CIP	45.5 (5)	54.5 (6)
TE	54.5 (6)	45.5 (5)
AN	90.9 (10)	9.1 (1)
ACM	100.0 (11)	0.0 (0)
NTFN	100.0 (11)	0.0 (0)

*No.=number of isolates

AMX, Amoxycillin; SXT, Cotrimoxazole; TE, Tetracycline; NA, Nalidixic acid; CIP, Ciprofloxacin; GM, Gentamicin; CF, Cephalexin; CMX, Cefuroxime; CXT, Cefotaxime; ACM, Amoxycillin-clavulanic acid; NTFN, Nitrofurantoin; AN, Amikacin.

The analysis of plasmid DNA profiles of the 11 ESBL-producing isolates revealed that, all the ESBL-producers harbored plasmids with different molecular weights (Table 2).

Table (2): Molecular weight of plasmids encountered in the 11 ESBL-producing *E. coli* isolates.

ESBL-producers	Plasmid Molecular Weight (kb)							
	3.9	**4.4**	**5.1**	**23.1**	**34.6**	**42.7**	**60.2**	**70.8**
A	-	-	-	+	-	-	-	-
B	-	-	-	+	-	-	-	+
C	-	-	-	-	-	+	-	+
D	-	-	-	+	-	-	-	+
E	-	-	-	-	-	+	-	-
F	-	-	-	-	-	+	-	-
G	-	+	+	+	-	-	+	+
H	-	-	-	+	-	-	-	+
I	-	-	-	+	-	-	-	-
J	+	-	-	+	+	+	-	+
K	-	-	+	+	-	-	+	+

+ = Plasmid present

- = No plasmid

The 70.8 kb plasmid was evident in 7 (46.7%) of the tested isolates. While, the 23.1 kb plasmid was the most common and was encountered in 9 (60.0%) of the isolates. Six (40.0%) of the isolates possessed both plasmids (70.8, 23.1 kb). Altogether, there was 7 plasmid patterns among the 11 isolates (Table 3).

As depicted in Tables (2 and 3), the results showed that, isolates E and F had the same plasmid profiles (42.7 kb) and the same resistance patterns with the exception that, isolate E had an additional resistance to gentamicin.

Furthermore, isolates B, D and H had the same plasmid profiles. Where, isolates B and D had the same resistance patterns, while, isolate H had no resistance to cotrimoxazole, nalidixic acid or ciprofloxacin.

Table (3), summarizes the 7 plasmid profiles found in the 11 ESBL producing *E. coli* isolates, where, 5 (33.3%) of the isolates harbored one plasmid, 4 (26.7%) harbored two, whereas, 4 (26.7%) contained three or more plasmids of varying sizes.

Table (3): Correlation between plasmid profiles and resistance patterns.

Palsmid profile	Isolate	Resistance patterns
70.8, 60.2, 23.1, 5.1, 4.4	G	AMX, SXT, GM, CF, CMX, CXT
70.8, 42.7, 34.6, 23.1, 3.9	J	AMX, CF, CMX, CXT
70.8, 60.2, 23.1, 5.1	K	AMX, SXT, GM, CF, CMX, CXT

70.8, 42.7	C	AMX, SXT, TE, NA, GM, CF, CMX, CXT, AN
70.8, 23.1	B, D, H	B, D ; AMX, SXT, NA, CIP, GM, CF, CMX, CXT H; AMX, GM, CF, CMX, CXT
42.7	E, F	E; AMX, SXT, TE, NA, CIP, GM, CF, CMX, CXT F; AMX, SXT, TE, NA, CIP, CF, CMX, CXT
23.1	A, I	AMX, SXT, TE, NA, CIP, GM, CF, CMX, CXT

For studying plasmid transfer by conjugation, four multiresistant isolates (G, H, J and K) were used as donors. Resistance to antimicrobial agents' was successfully transferred from the donors to a plasmid-free recipient (which was made nalidixic acid resistant). Transfer of amoxycillin, cephalexin, cefuroxime and cefotaxime resistance was achieved in the fourth-transconjugants (L/G, L/H, L/J and L/K). Moreover, transconjugants L/H and L/K gained resistance to gentamicin (Table 4).

Table (4): The effect of conjugal plasmid transfer on susceptibility patterns.

Isolate	Antimicrobial Agent											
	AMX	**ACM**	**CF**	**CMX**	**CXT**	**NA**	**CIP**	**GM**	**AN**	**TE**	**SXT**	**NTFN**
Donors												
G	R*	S	R	R	R	S	S	R	S	S	R	S
H	R	S	R	R	R	S	S	R	S	S	S	S
J	R	S	R	R	R	S	S	S	S	S	S	S
K	R	S	R	R	R	S	S	R	S	S	R	S
Recipient												
L	S	S	S	S	S	R	S	S	S	S	S	S
Transconjugants												
L/G	**R**	S	**R**	**R**	**R**	R	S	S	S	S	S	S
L/H	**R**	S	**R**	**R**	**R**	R	S	**R**	S	S	S	S
L/J	**R**	S	**R**	**R**	**R**	R	S	S	S	S	S	S
L/K	**R**	S	**R**	**R**	**R**	R	S	**R**	S	S	S	S

*Enlarged bold letters illustrate changes on susceptibility pattern conferred upon the recipient.
R=Resistant
S=Sensitive

When the transconjugants were examined for ESBL production, the results showed that all of them had acquired the ability to produce the enzyme. Table 5 illustrates the results of analysis of plasmid DNA in ESBL-producing transconjugants, which reveal that the four transconjugants (L/G, L/H, L/J and L/K) had gained certain plasmids.

Table (5): Molecular weight of plasmids gained upon conjugal transfer.

	Plasmid Molecular Weight (kb)		
Transconjugants			
L/G	23.1	-	-
L/ H	23.1	70.8	-
L/J	23.1	34.6	42.7
L/ K	23.1	-	-
Recipient			
L	-	-	-

Discussion

The results of this study confirmed that, out of 300 *E. coli* isolates, 11 (3.7%) of the isolates were ESBL-producers. Lower ratios, however, have been reported by many investigators, worldwide. For instance, Stobberingh *et al.* [9/5] and De Champs *et al.* [4/5] noted that, ESBL-producing *E. coli* ranged from 0.1 to 1.5%. However, other investigators have reported higher ratios. For example, in Greece, Vatopoulos *et al* [11/5], in Korea, Pai *et al* [3/5], in New York, Saurina *et al* [12/5] and in some USA hospitals Kaye *et al* [8/5] indicated that, ESBL-producing isolates ranged from 4.0 to 40.0%.

Similarities and differences in data concerning antimicrobial resistance may be due to the fact that they may have been collected on different periods. Also, the investigated target populations may have various sociodemographical, socioeconomical, socioepidemiological and clinical parameters.

Table (1) represents the resistance rate of the ESBL-producers to twelve antimicrobials. Most of the isolates showed co-resistance to non beta-lactam antimicrobials, especially to cotrimoxazole, gentamicin, nalidixic acid and ciprofloxacin. They, however, were completely sensitive to amoxycillin-clavulanic acid and nitrofurantoin. In this regard, we can conclude that, for *E. coli* isolates, amoxycillin-clavulanic acid, nitrofurantoin and amikacin are still effective treatment options.

Regarding plasmid analysis, the results in Table 3 revealed that, isolates G and K had the same resistance patterns (AMX, SXT, GM, CF, CMX, CXT) and plasmid profiles (70.8, 60.2, 23.1 and 5.1 kb). With the exception that, isolate G has an extra plasmid with a molecular weight of 4.4 kb. However, a similar plasmid (4.4 kb) was also found in an isolate which was sensitive to all tested antimicrobial agents. Consequently, this plasmid may have no relation to antimicrobial agents' resistance, at least, for the tested antimicrobial agents.

On the other hand, the plasmid 23.1 kb was detected among most of the tested ESBL-producing isolates and in all of the transconjugants, therefore, it is

suggested that this plasmid is involved in ESBL production and multiple drug resistance.

Plasmids were also evident in extracts prepared from all the resistant donors (G, H, J and K) and transconjugants that had received resistance markers (L/G, L/H, L/J and L/K) but not in the sensitive recipient (L). The plasmids gained by the transconjugants corresponded to certain plasmids of the donors. Where, the plasmid profiles of the transconjugants were represented by (23.1 kb), (70.8, 23.1 kb), (42.7, 34.6, 23.1) and (23.1 kb), respectively. These results also prove that the 23.1 kb plasmid is important in mediating ESBL production and multiple drug resistance.

As a result of mating experiments, we can conclude that, the transfer of amoxycillin, cephalexin, cefuroxime, cefotaxime and gentamicin resistance is important. Beta-lactam antibiotics are commonly used as drugs of choice for the treatment of UTIs. Consequently, the spread of beta-lactam antibiotic resistance may limit the choice of antimicrobial therapy of severe infections. Similarly, the use of long-term gentamicin therapy may select for the spread of multiple resistance and it may provide a reservoir of antimicrobial agents' resistance in the community.

This study clearly shows that, infection with these highly resistant isolates, which is usually confined to hospitals, is now being acquired in the community. The results also, revealed that, ESBL production and resistance to at least some of the antimicrobial agents used, are plasmid mediated and that some of those plasmids are transferable.

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