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Phenotypic and Genotypic Patterns of Beta-Lactam Resistance among *Escherichia coli* Clinical Isolates

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A B S T R A C T

The aim of the present study was to determine the phenotypic resistance pattern to β-lactam antibiotics among *Escherichia coli* clinical isolates and to identify the genetic determinants responsible for β-lactam antibiotic resistance. Forty *Escherichia coli* isolates were included in this study. Identification of tested strains was confirmed using MALDI-TOF/MS. Phenotypic and genotypic β-lactamase patterns were investigated. Twelve isolates were resistant to carbapenems, while most of them exhibited resistance to the one or more of the third and fourth generation cephalosporins (ESC) (35 out of 40). Phenotypically, the production of extended-spectrum beta-lactamase (ESBL), metallo-β-lactamases (MBL), and carbapenemases was detected in 31, 8, and 15 isolates, respectively. Genotypically, ESBL and carbapenemase encoding genes were detected in 34 and 19 isolates, respectively. The results of the current study indicate the high prevalence of β-lactam resistance among *Escherichia coli* isolates.

Introduction

Bacterial resistance is a major concern for physicians because resistant bacteria, particularly *Staphylococci*, *Pseudomonas* species, *Escherichia coli* and *Enterococci*, are becoming commonplace in healthcare institutions. Clinically important bacteria are characterized not only by single drug resistance, but also by multidrug resistance (MDR) (Odonkor and Addo, 2011).

Escherichia coli species can lead to a wide range of disease states, notably pneumonia, urinary tract infections (UTIs), septicemia, and soft tissue infections (Adekunle, 2012). Treatment varies depending on the site of infections and it involves the use of a variety of antibiotics. Beta-lactams (β-lactam), especially third-generation cephalosporins and carbapenems may be used as monotherapy or combination therapy with aminoglycosides for treatment of susceptible isolates (Podschun and Ullmann, 1998).

Infections caused by ESBL-producing *Escherichia coli* have been described in the hospital setting, although during the last decade these organisms have begun to disseminate into the community, becoming an

emerging public health problem. ESBLs are enzymes commonly associated with TEM and SHV. Moreover, CTX-M-type ESBLs have emerged within the community, particularly among *E. coli* and *K. pneumoniae* isolated from UTIs, with a widespread prevalence and multidrug resistance in many countries worldwide(Nathisuwan *et al.,* 2001). Infection with carbapenem-resistant *Enterobacteria-ceae* (CRE) or carbapenemase-producing *Enterobacteriaceae* is emerging as an important challenge in health-care settings.

The concern is that carbapenem is often used as a drug of last resort when battling resistant bacterial strains. Furthermore, new slight mutations could result in infections for which there is very little, if anything, healthcare professionals can do to treat patients with resistant organisms (Bradford, 2001). Many clinical laboratories have problems in rapid diagnosis and identification of *Escherichia coli* infection and also in detection of the various β-lactamases produced by these isolates. Confusion exists about the importance of these resistance mechanisms, optimal test methods, and appropriate reporting conventions. In view of need of cheap and easy methods for the diagnosis of various β-lactamases in basic microbiological laboratories, a prospective study should be carried out in order to determine resistance mechanism by various β-lactamases in *Escherichia coli* clinical isolates using various phenotypic and genotypic methods.

Materials and Methods

All culture media and antibiotic disks used in this study were purchased from Oxoid (Cambridge, UK).

Collection of clinical isolates

A total of 40 *Escherichia coli* isolates were obtained from different clinical samples submitted to the Microbiology Department, Medical Research Institute, Alexandria University. The identification of *Escherichia coli* isolates was confirmed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF/MS; Bruker, Billerica, MA, USA).

Antimicrobial susceptibility

Kirby–Bauer method (Jacoby, 2009) was used for the antimicrobial susceptibility testing of *Escherichia coli* strains on Mueller–Hinton agar plates. Beta-lactam antibiotics were chosen according to the CLSI recommendations (Clinical and Laboratory Standards Institute, 2017). The disks used were cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), cefoxitin (FOX), cefepime (FEP), tazobactam/ piperacillin (TZP), aztreonam (ATM), imipenem (IPM), and meropenem (MEM). The sizes of the inhibition zones were interpreted according to CLSIand the organisms were reported as sensitive, intermediate, or resistant to the agents that have been tested(Clinical and Laboratory Standards Institute, 2017).

β-lactamases phenotypic characterization

Strains that were found to be resistant to CAZ and/or FOX were further screened for the presence of extended-spectrum beta-lactamase (ESBL) hyperproduction, whereas those resistant to IPM and/or MEM were further screened for the presence of carbapenemases and MBL.

Screening for ESBL production

Combined disk test

Escherichia coli isolates resistant to CAZ and/or CTX were investigated for ESBL production using combined disk method. Mueller Hinton agar (MHA) was inoculated

with standard inoculum (0.5 McFarland) of the test isolate. It was tested for ceftazidime (30 µg) and ceftazidime- clavulanic acid (30 µg/10 µg). An increase in zone diameter of ≥ 5 mm in the presence of clavulanic acid than ceftazidime alone was interpreted as ESBL producer(Bauer *et al.,* 1966).

Chromogenic medium chromID ESBL

Each test isolate was inoculated onto ChromID ESBL and incubated for 24 hours at 37°C. The presence of green, brownish-green colonies of β-glucosidase-producing *Escherichia coli*isolates indicates potential ESBL producers(Glupczynski *et al.,* 2007).

Screening for carbapenemase production

Escherichia coli strains resistant to one or more of the carbapenems were screened for carbapenemase production by modified Hodge test (MHT) using carbapenem-susceptible *Escherichia coli* as the indicator organism (Huang *et al.,* 2010). Mueller–Hinton agar plate was inoculated with 0.5 McFarland suspension of the indicator strain. Then, two disks containing IPM (10 µg) and MEM (10 µg) were placed on the agar plate away from each other. Heavy inoculum of the test strain was streaked onto the Mueller–Hinton agar plate in a straight line from the edge of one disk to the plate periphery. Carbapenemase production induces a cloverleaf-shaped indentation of growth of the indicator strain after overnight incubation (Huang *et al.,* 2010).

Screening for MBL production

Overnight broth of the carbapenem resistant test strains was prepared at a 0.5 McFarland standard and spread on MHA plate using cotton swab. Disks of imipenem (10µg) alone and imipenem in combination with EDTA were placed on the plate at a distance of 4-5 cm from each other. The inhibition zones displayed around the IPM (Oxoid, UK) and the IPM-EDTA (EIP) disks were compared after 16 hours of incubation at 37°C. The difference of ≥7mm between the inhibition zone diameter of the EIP disk and that of IPM only disk was considered to be a positive for the presence of MBLs (Coudron, 2005).

Detection of β-lactamase encoding genes

Genotypic detection of different β-lactamase genes belonging to ESBL, carbapenemases and MBL was performed using polymerase chain reaction (PCR). All primers used in this study are listed in Supplementary Tables 1-3. The primers were purchased from Biosearch Technologies (Novato, CA, USA). The PCR Master mixMyTaq HS Red Mix was supplied by BioLine (London, UK). PCR amplification of the extracted DNA was carried out on Veriti Thermal Cycler (Applied Biosystems, CA, USA). Bacterial DNA was extracted by boiling method; shortly 3–4 colonies were suspended in sterile Tris-EDTA buffer to make a heavy suspension. The suspension was incubated in a boiling water bath for 15 min followed by rapid cooling on ice and centrifugation. The supernatant was used as a DNA template. PCR was performed in a total volume of 25 µl including 12.5 µl 2X MyTaq HS Red Mix, 10 picomoles of each primer, and 0.5 µl DNA extract. A negative control was prepared by the addition of the same contents to the tube without DNA extract.

Results and Discussion

The aim of the present study was to determine the phenotypic resistance pattern to β-lactam antibiotics among *Escherichia coli* clinical isolates and to identify the genetic determinants responsible for β-lactam antibiotic resistance. The phenotypic methods of β-lactamase detection were to be compared with genotypic techniques in an attempt to

select an easy, cheap and reproducible technique for the detection of these enzymes to be used in clinical laboratories.

The identification of 40 isolates included in this study as *Escherichia coli* was confirmed using MALDI-TOF/MS. Thirteen isolates included in this study were obtained from urine (32.5%) followed by 11 from sputum (27.5%). Concerning the remaining 16 isolates, 7 (17.5%) and 9 (22.5%) isolates were isolated from wound and nasal swabs, respectively.

Antibiotic susceptibility testing using Kirby–Bauer method showed that out of the 40 isolates a total of 33 (82.5%) and 8 (20%) isolates were resistant to third generation cephalosporin and carbapenem, respectively. The detailed results for the disk diffusion test are shown in Table 1.

In a previous survey, a significant increase in the ESBL rate was reported from all parts of the world. The actual magnitude of problem posed by ESBL producers is not known as routine susceptibility testing fails to detect all ESBL producers (Kaftandzieva *et al.*, 2011). *K. pneumoniae* and *E. coli* remain the major ESBL-producing organisms isolated worldwide (Sarojamma and Ramakrishna, 2011)which are recommended to be routinely tested and reported by the CLSI. Prevalence of ESBLs varies from an institute to another. Previous studies have reported ESBL production varying from 4 to 80% (Jacoby and Munoz-Price, 2005; Shahlol AM *et al.*, 2015).

The first phenotypic test performed for ESBL detection in this study was the CDT. Phenotypic detection of ESBL was carried out using combined disk method and chromID ESBL agar; 30 (75%) and 33 (82.5%) *Escherichia coli* isolates showed ESBL production, respectively.

The CLSI guidelines recommended the CDT as the most trusted phenotypic test for determination of ESBL presence (Clinical and Laboratory Standards Institute, 2017). This technically simple method is considered as an inexpensive alternative for the DDST in the detection of ESBL producers (Abdel-Hady *et al.*, 2008). Expectedly, the CDT that lacks the optimal disk spacing problem shows higher sensitivity than the DSST. This could be observed in our study, whereas30 (75%) of the isolates scored a positive result showing an enhancement of ≥ 5 mm in the zone of inhibition when clavulanic acid was added to the CAZ disk. Similar incidence rate was reported by Dalela *et al.*, (Dalela, 2012).

In the present study all clinical isolates were screened for ESBL producers using chromID ESBL agar, which showed positive results with 33 (82.5%) of the tested isolates. Using PCR as the gold standard, this test showed a sensitivity of 97.1%. This is in partial agreement with Grohs *et al.*, (Rodriguez-Bano and Pascual, 2008) who declared a higher sensitivity of this chromogenic agar (97.5%). (Grohs is correct reference)

The results of the detection of blaTEM, blaSHV, blaCTX-M, blaCTX-M9 and blaOXA-1 genes among 40 *Escherichia coli* strains are shown in Table 2. A high prevalence of ESBL-production by Enterobacteriaceae (78.4%) was also reported by Alsultan *et al.*, (Grohs *et al.*, 2013). (Alsultan is correct reference) Comparing our results with other studies in Egypt, it was found that our work showed the highest level of ESBL resistance (Afifi, 2013; Alsultan *et al.*, 2013; Abdallah *et al.*, 2015) (remove Alsultan) Possibly, the high prevalence in the Middle East, especially Egypt, is related to the uncontrolled use of antibiotics in these countries, where many drugs are still available over the counter.

Escherichia coli strains were phenotypically tested for the presence of carbapenemases using MHT, where 10 out of 40 strains were positive. MHT has been used extensively as a phenotypic method for the detection of carbapenemase activity (Birgy *et al.,* 2012), and it is the only carbapenemase detection method recommended by the CLSI for screening purposes. However, there are various shortcomings with MHT. The assay cannot distinguish the type of carbapenemase involved. Most importantly, false-positive results have been observed with isolates producing CTX-M-type ESBLs or increased amounts of AmpC β-lactamases (cephalosporinases) (Miriagou *et al.,* 2010; Tzouvelekis *et al.,* 2012).

Eight *Escherichia coli* strains resistant to IPM and/or MEM were phenotypically tested for the presence of MBL using combined disk method. All 8 strains were positive determined using IPM–EDTA combined disk method. The CDT is the most commonly used format of MBL detection assays. In this test, the β-lactam disk is potentiated with an inhibitor, and the diameter of its inhibition zone is then compared with that of the β-lactam disk alone. An increase in the inhibition zone diameter above a predefined cut-off value indicates MBL activity (Marchiaro *et al.,* 2005). This test shows high sensitivity even with isolates with low carbapenem resistance levels (Senda *et al.,* 1996; Tzouvelekis *et al.,* 2012). Clinical isolates tested by the IPM/ IPM-EDTA disk method for MBL production may give poor results, perhaps due to involvement of other resistance mechanisms that may interfere with the test (Hrabak *et al.,* 2014).

The results in the present study showed that out of 40 *Escherichia coli* tested, 8 (20%) isolates were MBL producers.Yadav and Sharma (2017) (Yadav and Sharma) reported that 7.04% of the carbapenemase producing *Enterobacteriaceae* were found to be MBL producers.

Molecular developments make genotypic detection more readily available and cost effective for diagnostic laboratories to identify different types of ESBLs (Sundsfjord *et al.,* 2004). Previous studies (Thabit *et al.,* 2011; Abdallah *et al.,* 2015) have described various molecular approaches for the rapid screening of ESBL-positive organisms for the presence of different ESBL genes.

In the present work, conventional and multiplex PCR were used in the detection of ESBL-producing clinical strains. Generally, ESBL encoding genes were observed among 34 (85%) isolates. Concerning conventional PCR, $bla_{\text{OXA-1}}$ and $bla_{\text{CTX-9}}$ were detected in 26 (65) and 21 (52.5%) isolates, respectively. On the other hand, multiplex PCR revealed 34 (85%) isolates harbouring bla_{SHV}, followed by $bla_{\text{CTX-M}}$ and bla_{TEM} in 30 (75%) and 17 (42.5%) isolates, respectively. Multiplex PCR gave the same results as conventional PCR for bla_{TEM}, $bla_{\text{CTX-M}}$ and bla_{SHV} gene separately. This means that by using multiplex PCR, time, chemicals and cost could be saved. Our results were relatively higher than those reported by most of the previously published studies. For instance, (Paniagua *et al.,* 2010)stated that the distribution of the ESBL enzymes was as follows: $bla_{\text{CTX-M-9}}$ group (40%); $bla_{\text{CTX-M-1}}$ group (26.6%); bla_{SHV}-type (29%); and bla_{TEM}-type (4.4%).

Furthermore, the prevalence of ESBLs in the present study varies from others because of the differences in infection control practices between hospitals or due to differences in the use of cephalosporins (Wayne, 2008). On the other hand, the high occurrence of ESBL producers in the current work probably relates to rampant and inadvertent use of third-generation cephalosporins. Over the counter availability could be another cause when

patients resort to self-medication(Begum and Damle, 2015).

Several Multiplex PCR assays for carbapenemase genes have been described (Poirel *et al.,* 2011; Swayne *et al.,* 2011; Monteiro *et al.,* 2012) but require real-time PCR facilities or rely on amplicon detection by gel electrophoresis and might therefore not be convenient for all laboratories (Birgy *et al.,* 2012).

Table.1 Resistance of the 40 Escherichia coli isolates to different β-lactam antibiotics

Antibiotic	Resistant		Sensitive	
	Number	percentage	number	percentage
Cefotaxime (CTX)	33	82.5	7	7.5
Ceftazidime (CAZ)	31	77.5	9	22.5
Ceftrixone (CRO)	28	70	12	30
Cefepime (FEP)	28	70	12	30
Cefoxitin (FOX)	15	37.5	25	62.5
Aztreonam (ATM)	24	60	16	40
Tazobactam/Piperacillin (TZP)	20	50	10	50
Imipenem (IPM)	8	20	32	80
Meropenem (MEM)	8	20	32	80

Table.2 ESBL gene detection among 40 Escherichia coli isolates

	Positive Results		Negative Results	
	No.	(%)	No.	(%)
***bla*$_{\text{CTX-M9}}$**	21	52.5	19	47.5
***bla*$_{\text{CTX-M}}$**	30	75	10	25
***bla*$_{\text{SHV}}$**	34	85	6	15
***bla*$_{\text{TEM}}$**	17	42.5	23	57.5
***bla*$_{\text{OXA-1}}$**	26	65	14	35

Table.3 Genotypic detection of carbapenemases among tested Escherichia coli isolates

	Positive Results		Negative Results	
	No.	(%)	No.	(%)
***bla*$_{\text{NDM}}$**	13	32.5	27	67.5
***bla*$_{\text{VIM}}$**	0	0	40	100
***bla*$_{\text{IMP}}$**	2	5	38	95
***bla*$_{\text{KPC}}$**	4	10	36	90
***bla*$_{\text{OXA-48}}$**	19	47.5	21	52.5

Four (4%) isolates were *bla*$_{\text{KPC}}$ positive, while 2 (5%), 19(47.5%) and 13 (32.5%) and isolates marked positive results with *bla*$_{\text{IMP,}}$ *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM}}$, respectively (Table 3). The fact that *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP}}$ were absent by multiplex PCR may be due to other variants

of the diverse *bla*$_{VIM}$ and *bla*$_{IMP}$ family. Molecular assays for carbapenemase detection can only detect known carbapenemase genes, while new variants of known carbapenemases might be missed (Kaase *et al.*, 2012). Therefore, phenotypic tests like the modified Hodge test and chromogenic agar still play a role in carbapenemase detection and can additionally be used to identify strains that need molecular testing in order to reduce costs.

Concerning the second multiplex PCR, it was shown that 13 (32.5%) and 19 (47.5%) isolates yielded amplified products for *bla*$_{NDM}$ and *bla*$_{OXA-48}$, respectively. KPC was included in the second multiplex reaction, but all tested isolates showed negative result. The presence of this gene was speculated, therefore further investigation was done using conventional PCR for *bla*$_{KPC}$ showing amplified product with 4 (10%) isolates. In comparison with our results, Govindaswamy *et al.*, (2019)stated that 61.7% and 10.6% were bla$_{NDM-1}$ and bla$_{KPC}$ positive, respectively, while the prevalence of bla$_{VIM}$, bla$_{IMP}$ and bla$_{OXA-48}$ was 30.8%, 2.1% and 5.3%, respectively.

In conclusion, ESBL production is the most common mechanism of resistance to β-lactams among *Escherichia coli*. Production of β-lactamases is in continuous and rapid increase worldwide among *Escherichia coli* isolates showing multiple antibiotic resistance. Therefore, we recommend the introduction of systemic screening for β-lactamase producers in the routine diagnostic laboratories is an important issue both for the diagnosis and surveillance purposes. In addition, accurate local periodic reports of the resistance pattern as well as establishment of national central laboratories for data collection and nationwide surveillance studies for β-lactamase emergence is important and will help in the assessment of the actual prevalence of β-lactamases in Egypt.

Conflict of Interest

The authors declare no conflict of interest.

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