

## Comparison between Multiplex PCR and Phenotypic Detection Methods for Identifying *AmpC* $\beta$ -lactamases Among Clinical Isolates of *Enterobacteriaceae* in Zagazig University Hospitals, Egypt

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### Abstract

No standardized phenotypic methods for the screening and detection of plasmid-mediated *AmpC* enzymes are currently available, which is one of the main problems we are facing nowadays.

**Aim:** This study aimed to evaluate the presence of *AmpC*  $\beta$ -lactamase among *Enterobacteriaceae* isolates separated from patients with nosocomial infections and to detect the most prevalent genetic strains in the separated isolates and evaluation of two phenotypic methods (*AmpC* E test and ceftioxin–cloxacillin double disc synergy test) to detect *AmpC* enzymes.

**Materials and methods:** Total of 1200 gm negative isolates were screened for potential plasmid-mediated *AmpC* enzymes by ceftioxin disc, *AmpC* E test and ceftioxin–cloxacillin double disc synergy tests. The genotypic identification was done using multiplex PCR.

**Results:** The potential *AmpC* producing isolates among all the studied isolates were 4.1% (49/1200) by ceftioxin disc. Plasmid encoded *AmpC* genes were detected by PCR in 28.5% of ceftioxin resistant isolates. The most prevalent *AmpC* genes family were CIT and MOX. The sensitivity of *AmpC* E test and ceftioxin–cloxacillin double disc synergy were 81.3% and 100% respectively and the specificity were 92.3% and 95.9%.

**Keywords:** *AmpC*  $\beta$ -Lactamase; E test; ESBL; Multiplex PCR

### Introduction

The prevalence of multidrug-resistant Gram-negative bacteria has been increased continuously over the past few years and bacterial strains producing *AmpC*  $\beta$ -lactamases and/or extended spectrum  $\beta$ -lactamases (ESBLs) are of particular concern [1].

*AmpC*  $\beta$ -lactamases are clinically significant because they may confer resistance to penicillins, cephalosporins, oxyimino-cephalosporins (e.g., ceftriaxone, cefotaxime, and ceftazidime), cephamycins (e.g., ceftioxin and cefotetan) and monobactams. *AmpC*  $\beta$ -lactamase activity is not affected by the ESBL (expanded-spectrum  $\beta$ -lactamases) inhibitor clavulanic acid, but is inhibited by boronic acid and cloxacillin [2].

In the Ambler structural classification of  $\beta$ -lactamase, *AmpC* enzymes belong to class C [3], while in the functional classification scheme of Bush et al., they were assigned to group 1 [4]. There are no Clinical Laboratory Standards Institute (CLSI) or other approved criteria for *AmpC* detection. Organisms producing enough *AmpC*  $\beta$ -lactamase will typically give a positive ESBL screening test, but fail in the confirmatory tests involving increased sensitivity with clavulanic acid [5]. This phenotype is not, however, specific for an *AmpC* producer, since it can occur with certain complex TEM mutants, OXA-type ESBLs, and carbapenemases and in strains with a high level of TEM-1  $\beta$ -lactamase. Other confirmatory tests are needed [6].

Overexpression of *AmpC*  $\beta$ -lactamases in Gram-negative organisms occurs either by deregulation of the *AmpC* chromosomal gene or by an acquisition of a transferable *AmpC* gene on a plasmid or other transferable element. The transferable *AmpC* gene products are commonly called plasmid-mediated *AmpC*  $\beta$ -lactamases (*PAmpC*) [7].

Some phenotypic tests are available to confirm detection of *PAmpC*. However, these phenotypic tests are not able to differentiate between chromosomal *AmpC* genes and *AmpC* genes that are carried on plasmids [8].

For allowing ease of implementation into the clinical laboratory, Real-time multiplex PCR assay using TaqMan probes for the detection of plasmid-mediated *AmpC*  $\beta$ -lactamase genes has been developed by modifications to a previously designed endpoint *AmpC* multiplex PCR [9].

### Aim of the Study

This study aimed to evaluate the presence of *AmpC*  $\beta$ -lactamase among *Enterobacteriaceae* isolates separated from patients with nosocomial infections and to detect the most prevalent genetic strains in the separated isolates.

## Materials and Methods

### Bacterial isolates

This study was done in the period between January 2015 to August 2017 in Clinical Pathology Department Zagazig University Hospital. A total of 1200 non-duplicate clinical isolates were tested. The isolates included in this study were *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Proteus vulgaris* and *Citrobacter koseri*. The clinical isolates were collected from different clinical samples (Pus, sputum, blood, urine). Identification was done by Vitek MS<sup>®</sup> MALDI-TOF mass spectrometry system (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight), which relies on the generation of an organism specific mass spectrum or “protein fingerprint” that is examined against a reference database to provide organism identification.

All gram negative clinical isolates included in this study were subjected to the following:

### MALDI-TOF MS identification

It is a rapid, inexpensive technology used nowadays for identification of most bacterial strains [3]. Four hundred and twenty samples were then analysed using the Vitek MS MALDI-TOF mass spectrometer in linear positive-ion mode, across the mass-to-charge ratio range of 2,000 to 20,000 Da. Each spot was irradiated with 500 laser shots at 50 Hz. Target plates were calibrated and quality controlled both before and after data acquisition by using *Escherichia coli* ATCC8739. A sample containing matrix only (negative control) was assayed for quality control purposes. After the acquisition of spectra, data were transferred from the Vitek MS acquisition to the Vitek MS analysis server and identification results were displayed using Myla v2.4 middleware. The total processing and data analysis time was approximately 20 min for a single isolate. **Data Analysis:** The Vitek MS identification system is based on comparison of the characteristics of the spectra obtained with the Vitek MS v2.0 database. This database was built using spectra for known strains for each claimed species. A single identification is displayed with a confidence value from 60.0 to 99.9.

### Antibiotic susceptibility by Vitek 2 compact

One or two colonies of freshly grown tested organisms were picked up using 10  $\mu$ L plastic loops and dissolved in the saline and well mixed. Optical density of suspension was adjusted to 0.5 McFarland using DensiCHEK<sup>™</sup> Plus. The AST plastic cards (GN71) were used.

### Phenotypic screening test

**Cefoxitin sensitivity test using the Kirby-Bauer disk diffusion method:** Cefoxitin (30 $\mu$ g) was used as screening test for *AmpC*  $\beta$ -lactamase production. According to the CLSI criteria isolates resistant or intermediate to cefoxitin (zone diameter <18 mm) were selected for further processing by phenotypic confirmatory tests and considered as potential P *AmpC* producers.

### Confirmatory phenotypic tests

All the screen positive isolates were subjected to two confirmatory phenotypic tests (*AmpC* E test and Cefoxitin-cloxacillin double

synergy test). *Klebsiella Pneumonia* ATCC-1144<sup>™</sup> (Microbiologics, MediMark, Europe) was used as Positive control strain for *PAMPc*.

***AmpC* E test (BioMérieux SA, France):** E test was performed according to the manufacturer's instructions. The test principle comprises a strip impregnated with a concentration gradient of cefotetan on one half of the strip and cefotetan with cloxacillin on the other half of the strip. MICs of cefotetan alone and cefotetan with cloxacillin were determined as recommended by the manufacturer, where ratios of cefotetan versus cefotetan/cloxacillin of  $\geq 8$  were considered positive for *AmpC*  $\beta$ -lactamase production.

**Cefoxitin-cloxacillin double synergy test (CC-DDS):** *AmpC*  $\beta$ -lactamases, in contrast to ESBLs, hydrolyze broad and extended-spectrum cephalosporins (cephamycins as well as oxyimino- $\beta$ -lactams) and are not inhibited by  $\beta$ -lactamase inhibitors such as Clavulanic acid. For detection of *AmpC* few inhibitors like Boronic Acid, Cloxacillin etc. are employed. The discs of 30  $\mu$ g Cefoxitin containing Cloxacillin 200  $\mu$ g are employed to detect the presence of *AmpC*. A zone diameter difference of  $\geq 4$  mm between Cefoxitin 30  $\mu$ g discs and Cefoxitin-Cloxacillin 30-200  $\mu$ g discs should be interpreted as *AmpC* positive.

### Molecular detection of plasmid-mediated *AmpC* genes by multiplex PCR

Multiplex PCR was considered the gold standard method for detection of *AmpC*  $\beta$ -lactamases. Primers specific for the genes of six different phylogenetic groups (*bla* MOX, *bla* CIT, *bla* DHA, *bla* ACC, *bla* EBC, and *bla* FOX) were used according to Pérez-Pérez and Hanson [9].

PCR was performed using thermal cycler (Gene Amp PCR system 2400, Roche) with cycling condition of initial denaturation step at 95°C for 5 min followed by 30 cycles of Denaturation at 94°C for 45sec. Annealing at 62°C for 45 sec. Extension at 72°C for 1 min and final extension at 72°C for 5 min. Cyclic repetition resulted in exponential amplification of the DNA that lied between the two oligopeptides used. Sequences of the primers used in PCR are seen in table 1.

Famil y	Target (s)	Primer	Sequence (5' to 3')	Amplicon Size (bp)
MOX	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	MOXMF	GCT GCT CAA GGA GCA CAG GAT	520
		MOXMR	CAC ATT GAC ATA GGT GTG GTG C	
CIT	LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGG CCA GAA CTG ACA GGC AAA	462
		CITMR	TTT CTC CTG AAC GTG GCT GGC	
DHA	DHA-1, DHA-2	DHAMF	AAC TTT CAC AGG TGT GCT GGG T	405
		DHAMR	CCG TAC GCA TAC TGG CTT TGC	
EBC	MIR-1T, ACT-1	EBCMF	TCG GTA AAG CCG ATG TTG CGG	302
		EBCMR	CTT CCA CTG CGG CTG CCA GTT	

FOX	FOX-1 to FOX-5b	FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190
		FOXMR	CAA AGC GCG TAA CCG GAT TGG	
ACC	ACC	ACCMF	AAC AGC CTC AGC AGC CGG TTA	346
		ACCMR	TTC GCC GCA ATC ATC CCT AGC	

**Table 1:** Sequences of primers used in multiplex PCR.

PCR products were separated on a 2% agarose gel. 3 µl of loading dye was added to 6 µl (ladder) 100bp DNA ladder (Invitrogen, life technologies), The PCR marker was also loaded into one of the wells. The amplified products were then visualized with transilluminator.

## Results

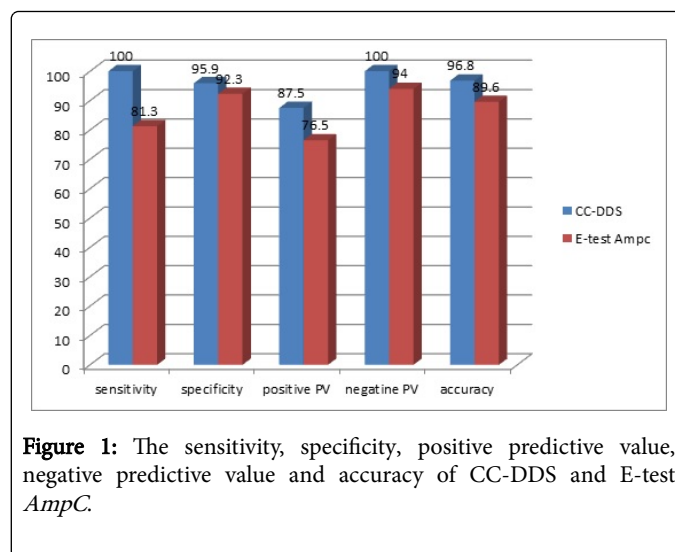
This study was carried out on gram negative clinical isolates recovered from the microbiology laboratory of Zagazig University hospitals during the time period from January 2015 to August 2017.

All gram negative clinical isolates were screened for ceftioxin susceptibility as an indicator for *AmpC* production by Kirby Bauer disc diffusion test disc. Isolates with inhibition zone diameter ≤ 18 mm were considered potential *AmpC* producers. Only 49 (4.1%) were ceftioxin resistant. The prevalence of ceftioxin resistant strains was 3.7% (24/657) in *E.coli*, 4.5% (21/465) in *K. pneumoniae* and 8.9% (4/45) in *Proteus mirabilis*. The occurrence rate of ceftioxin resistant strains was high in *Proteus mirabilis* followed by *K. pneumoniae* then *E.coli* as shown in Table 2.

Species	No. (%) of isolates	No. (%) of ceftioxin resistant isolates in each species
<i>Escherichia coli</i>	657 (54.8)	24 (3.7)
<i>Klebsiella pneumoniae</i>	465 (38.8)	21 (4.5)
<i>Klebsiella oxytoca</i>	15 (1.2)	0
<i>Proteus mirabilis</i>	45 (3.7)	4 (8.9)
<i>Proteus vulgaris</i>	13 (1.08)	0
<i>Citrobacter koseri</i>	5 (0.4)	0
Total	1200(100)	49 (4.1%)

**Table 2:** Distribution of gram negative isolates among different species and prevalence of ceftioxin resistant isolates in each species.

Figure 1 shows that CC-DDS test has higher sensitivity, specificity, positive predictive value, negative predictive value and accuracy than E-test *AmpC*.



**Figure 1:** The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of CC-DDS and E-test *AmpC*.

### Occurrence rate of *PampC* genotype

Multiplex PCR detected *PampC* in 14 of the 49 potential *PampC*-producers (28.5%), which included *E. coli* (=7), *K. pneumoniae* (=5) and *Proteus mirabilis* (=2). The prevalence of *PampC* production among all tested isolates was 1.2%.

Based on species, 1.06% (7/657) of *E. coli*, 1.07 (5/465) of *K. pneumoniae* and 4.4% (2/45) of *Proteus mirabilis* isolates. Additionally, the detected *bla* CIT genes were predominately present in *E.coli* (5/7), *K. pneumoniae* (3/5). All these data are shown in Table 3.

Organism	<i>PampC</i> negative	CIT positive	MOX positive	DHA positive	<i>PampC</i> genotypes N (%)
<i>Kleb P.</i> (n=21)	16	3	1	1	5 (23.8%)
<i>E.coli</i> (n=24)	17	5	1	1	7 (29.2%)
<i>Proteus M.</i> (n=4)	2	1	1	0	2 (50%)
Total (n=49)	35 (71.4%)	9 (18.4%)	3 (6.1%)	2 (4.1%)	14 (28.5%)

**Table 3:** Distribution of *PampC* genes within studied isolates.

Figure 2 shows *PampC* genes detected by multiplex PCR, the most predominant gene was *bla* CIT gene 18.4% (9/49) followed by *bla* MOX 6.1% (3/49) then *bla* DHA 4.1% (2/49). No genes belonging to the ACC, FOX or EBC family were detected. The amplicon size was 462bp (CIT), 405bp (DHA) and 520bp (MOX). Figures 3 and 4 show gel-electrophoresis for *PampC* genes.

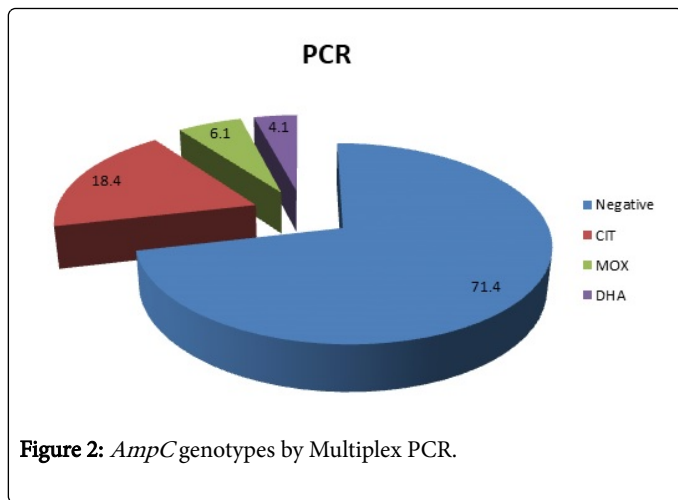


Figure 2: *AmpC* genotypes by Multiplex PCR.

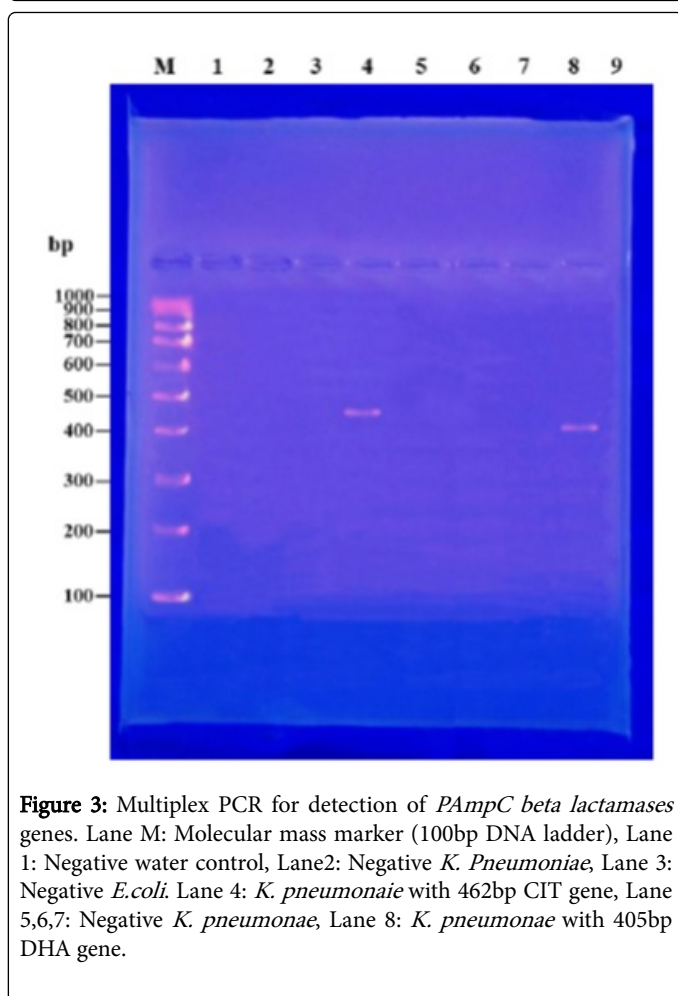


Figure 3: Multiplex PCR for detection of *PAmpC beta lactamases* genes. Lane M: Molecular mass marker (100bp DNA ladder), Lane 1: Negative water control, Lane2: Negative *K. Pneumoniae*, Lane 3: Negative *E.coli*. Lane 4: *K. pneumoniae* with 462bp CIT gene, Lane 5,6,7: Negative *K. pneumoniae*, Lane 8: *K. pneumoniae* with 405bp DHA gene.

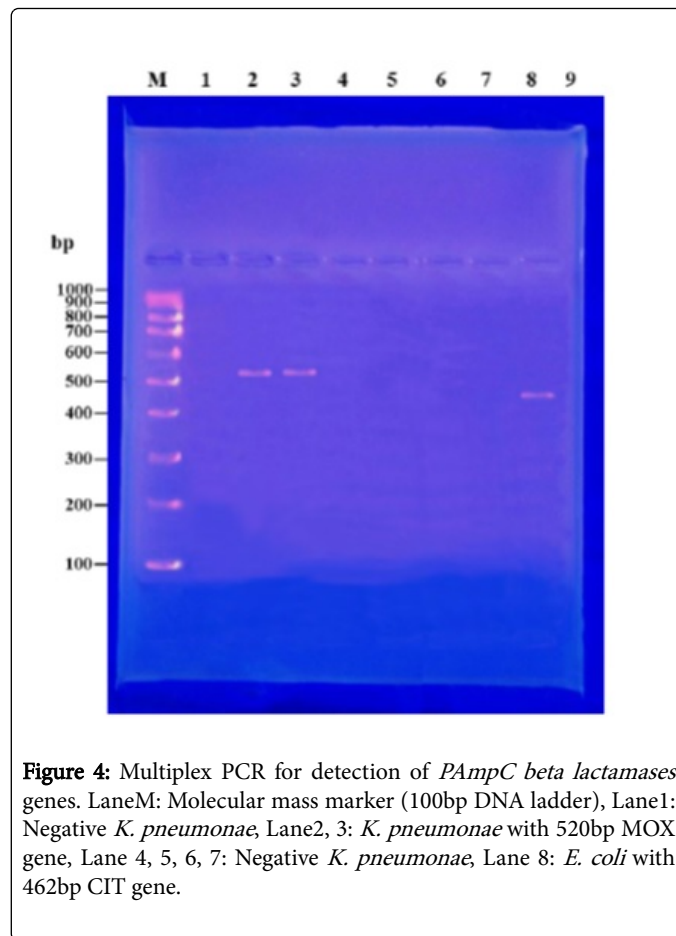


Figure 4: Multiplex PCR for detection of *PAmpC beta lactamases* genes. LaneM: Molecular mass marker (100bp DNA ladder), Lane1: Negative *K. pneumoniae*, Lane2, 3: *K. pneumoniae* with 520bp MOX gene, Lane 4, 5, 6, 7: Negative *K. pneumoniae*, Lane 8: *E. coli* with 462bp CIT gene.

## Discussion

*Enterobacteriaceae* producing plasmid-mediated *AmpC beta-lactamases (PAmpC)* have become a major therapeutic challenge. The detection of *AmpC*-producing *Klebsiella spp.*, *Escherichia coli*, *P. mirabilis* and *Salmonella spp.* is of significant clinical relevance, since *AmpC* producers may appear susceptible to expanded-spectrum cephalosporins when initially tested, therefore underestimation occurred. This may lead to inappropriate antimicrobial choice and therapeutic failure [10].

The prevalence of  $\beta$ -lactamase producers differs from a year to another and is totally different from a hospital to another. The variation in the results between different studies might be due to differences in the time of collection of isolates and differences in the way and designs of studying.

In our study cefoxitin susceptibility was used as screen test for *AmpC*. This was agreed by Gupta et al. and Wassef et al. [11,12]. Potential screening tools were used by many studies as reduced susceptibility to cephamycins and/or ceftazidime [13] reduced susceptibility to cefoxitin and to third generation cephalosporins [14] a positive ESBL screening test [15]. Taneja et al., [16] reported using both piperacillin disc and piperacillin-tazobactam disc as useful screening procedure for detection of *AmpC* production as *AmpC beta-lactamase* producers are more susceptible to tazobactam as compared to clavulanic acid.

Only 4.1% (49/1200) of the screened gram negative isolates show cefoxitin resistance. This prevalence rate was near to that reported by an Egyptian study previously conducted at Cairo University hospital [12], where cefoxitin prevalence rate was 5.8%, but it was lower than [17], where prevalence rate was 18.2%. This difference in prevalence rate could be explained by difference in type and sample size of the screened population as their study was conducted on specific group of patients having urinary tract infections.

In Turkey, a study reported significantly higher prevalence rate of 46.5% [18], another study by Japoni et al. [19] reported a rate of 46% in Iran, while a study done in China reported 16.9% [20]. The geographical distribution, selection criteria and sample size of screened population could contribute to this variation between these studies.

In the present study 3.7% of *E. coli* isolates and 4.5% of *Klebsiella pneumoniae* isolates were found to be resistant to cefoxitin. Another study conducted in India documented higher prevalence of cefoxitin resistance being 72% of *Klebsiella* and 62.5% of *E. coli* isolates [21]. 28.4% of *E. coli* and 32.1% of *K. pneumoniae* positive isolates for presumptive *AmpC* producers by cefoxitin screen test was reported by Barwa et al. [22].

The most frequent cefoxitin resistant isolates were *E. Coli* {24/49 (49%)} followed by *Klebsiella pneumoniae* {21/49 (42.8%)}, then *Proteus mirabilis* {4/49 (8.2%)}. This agreed with Fam et al. [23], who reported that *E. coli* is the most frequent specie among cefoxitin resistant isolates followed by *Klebsiella* then *P. mirabilis* (57%, 38% and 5% respectively).

Another study done by Al Hardy and Adel [24] donated that from a 148 total isolates, 50 (33.8%) were *AmpC*  $\beta$ -lactamase producers. The isolates were {23/50 (46%) *K. pneumoniae*, 16/50 (32%) *E. coli*, 11/50 (22%) *P. mirabilis*}. Cefoxitin resistant isolates were commonly isolated from blood and sputum samples (30% and 28.6%). This is in contrast with what reported by Fam et al. [23] that 63% of resistant isolates were isolated from urine.

The CC-DDS test had sensitivity 100% and specificity 95.9%. This comes in agreement with other studies conducted by Thean et al. [25] and Polsfuss et al. [26] having 95% sensitivity in both studies, while the specificity of CC-DDS in the first study was 100% and 97.2% respectively in the second study.

In the present study CC-DDS had higher sensitivity and specificity than E-test *AmpC*. This result was agreed by Polsfuss et al. [26], they have documented that CC-DDS had higher sensitivity than E-test *AmpC* (97.2% vs. 77.4%) and the specificity was 100% for both methods. The higher specificity in the later study could be explained: firstly the later study assessed validity of E test to detect *AmpC* activity including plasmid-mediated *AmpC*  $\beta$ -lactamases and chromosomal *AmpC*, while in the present study validity of E test to detect *PAMPc* only. Secondly: E test was used to screen both cefoxitin resistant and cefoxitin susceptible isolates, while the present study was conducted on cefoxitin resistant isolates. Thirdly, difference in sample size and species distribution.

*PAMPc* positive isolates in the present study were sensitive to meropenem, imipenem, and Tigecycline 89.5%, 84.2% and 68.4%. These results disagreed with other study conducted by Bedenić et al. [27] reported higher susceptibility to Tigecycline and carbapenems (100%). Another study by Manoharan et al. [21] reported higher sensitivity 99% for Tigecycline and 97% for imipenem and meropenem. 100% efficacy of meropenem against *AmpC*  $\beta$

*lactamase* producing bacteria was also reported in a study by Gupta et al., Hassan et al. and Mohamudha et al. [11,28,29].

Lower susceptibility rates were detected in our study. This could be attributed to firstly, empirical usage of these antibiotics in the treatment of nosocomial infection in our hospital that could potentially produce resistant strains. Secondly, presence of other mechanisms associated with *AmpC* production such as multidrug efflux pumps and porin loss. This agreed with Weifeng et al. and Matsumura et al. [30,31] that documented the role of porin loss coupled with *AmpC* enzymes in conferring resistance of *K. pneumoniae* to Carbapenems and cefoxitin. Another possibility is the association of PABs with carbapenamases. This was agreed by Dodaiah and Anjaneya [32], who reported co-existence of *AmpC*  $\beta$ -lactamase and carbapenamases in 6.52% gram negative isolates in their study.

In the present study 14 of 49 (28.5%) cefoxitin resistant isolates were confirmed to possess *PAMPc* gene by multiplex PCR. Our result showed agreement with Egyptian studies previously conducted at Cairo University Hospital [12,23], where *PAMPc* prevalence were 26% and 28.3% respectively. This result came in agreement with another study Japoni-Nejad et al. [19] that reported 19% prevalence of *PAMPc* genes. Barwa et al., [22] reported higher prevalence (60%) this might be attributed to using different amplification primer sets for detection of *PAMPc* in the latter study. Different result was detected in another study Reuland et al. [33], reported lower prevalence of *PAMPc* genes (3.9%) this might be attributed to difference in selection criteria of the studied isolates as their study was conducted on highly resistant gram negative isolates.

In the present study, multiplex PCR revealed that CIT is the most predominant gene (64.3%) followed by MOX (21.4%) and DHA (14.3%). CIT-type enzymes appear to be prevalent in China [20], India [34], Turkey [18] and Tunisia [35]. In Egypt, these results agreed with studies carried by [17,23,36] in, which CIT showed the highest prevalence rate (86.9%, 76.5% and 60% respectively).

In another study conducted by Wassef et al. [12], the FOX family showed the highest prevalence rate. A study done by Akinyemi et al. [37] in Nigeria revealed the highest occurrence (43.8%) of *AmpC* Fox genes in *S. typhi* strains followed by *S. typhimurium* (25%).

A study carried out by Al Hardy and Ala [24] reported 30.4% *AmpC*  $\beta$ -lactamase producers detected by FOX group genes, 56.5% by CIT group genes (including CMY-2) and 73.9% by MOX group genes (including CYM-1) in the clinical isolates of *Enterobacteriaceae*.

## Conclusion

Isolates of *E. coli*, *K. pneumoniae*, and *Proteus mirabilis* showed the occurrence of plasmid mediated *AmpC*  $\beta$ -lactamase which is an alarm of increasing the probability of dissemination of these plasmid mediated resistance genes within our hospital. Identification of *AmpC* types may aid in control of hospital infection and help the physician to prescribe the most appropriate antibiotic.

Regarding phenotypic confirmatory tests, CC-DDS showed higher sensitivity and specificity than E-test *AmpC*, additionally E-test is costly compared to the former to be used by clinical laboratories for routine screening procedure. Thus, CC-DDS is a more suitable routine confirmatory procedure for early detection of *PAMPc*-producing bacteria. Better understanding of the genetic relatedness and the molecular epidemiology of this resistance mechanism is done by

sequencing and typing of the strains. The multiplex PCR revealed that CIT and MOX are the most predominant genes detected in our selected isolates.

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