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Genetic detection of AmpC beta-lactamase among gram negative isolates "A Single Center Experience"

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Abstract

The misuse of β-Lactam antibiotics results in major problem, microbial resistance against these antibiotics by expression of β-lactamases, facing its use. AmpCs are one of the β-lactamases which confer resistance to penicillins, cephalosporins, cephamycins, and aztreonam, and are not affected by classic β-lactamase inhibitors. Plasmid-mediated AmpC β-lactamases pose a major challenge to infection control because the AmpC gene can be expressed in larger quantities and has a high transmissibility to other bacterial species. This study aimed to detect plasmid mediated AmpC β-lactamases in gram negative isolates in Assiut university hospital. It was performed on 120 cefoxtin resistant isolates obtained from 300-gram negative isolates using the disc diffusion method as a screening test for AmpC production. Since the presence of pAmpC is often associated with the presence of ESBLs, phenotypic detection of ESBL was done using combined disc method and vitek2 compact 15. Phenotypic detection of AmpC was done by disc approximation method and inhibitor-based method using phenyl boronic acid (PBA). Genotypic detection of 5 plasmid mediated AmpC genes families *(MOX, CIT, DHA, EBC, and FOX)* was done by multiplex PCR. Our result showed that *Klebsiella pneumoniae* (62.5%) and *Escherichia coli* (25.8%) were the most frequent isolates. Only 15.8 %, 12.5%, 17.5% resistant isolates to cefoxitin were positive by using disc approximation test, inhibitor-based method using PBA (150 μg/mL), and PBA (600 μg/mL), respectively. Out of the 120 Cefoxitin-resistant isolates, 22 isolates (18.3 %) were positive by multiplex PCR. CIT and MOX were solely detected in 45.5% and 4.5%, respectively. CIT and FOX together were detected in 45.5%, CIT and DHA together in 4.5%. No isolate was positive for EBC gene. Finally, boronic acid test using 600 μg/mL PBA with 30μg ceftazidime, as phenotypic method for detecting AmpC β- lactamases, was ranked very good for marking negative tests.

Keywords: AmpC β-lactamase; beta-lactam resistance; plasmid-mediated AmpC.

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Introduction

The enzymes of extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases are important elements involved in mechanisms of antibiotic resistance in gram negative bacteria. Infections caused by ESBL and/or AmpC-

producing bacteria are of clinical and epidemiological importance and cause high morbidity and mortality in patients.[1]

ESBLs are clavulanate susceptible enzymes that hydrolyze penicillins, extended-spectrum cephalosporins and aztreonam. AmpCs are cephalosporinases that can be differentiated from ESBLs by their ability to hydrolyze cephamycins and other extended-spectrum antibiotics.[2] Unlike ESBLs, AmpC-lactamasas are usually resistant to β-lactamase inhibitors as, clavulanate, sulbactam, and tazobactam, while they are inhibited by novel, non-dependent-lactamase inhibitors as avibactam, relebactam, and vaborbactam.[3]

Detecting AmpC enzymes in organisms that also express ESBLs, which mask the production of AmpC enzyme, are usually difficult.[4] Also, AmpC producing organisms can act as hidden reservoirs for ESBLs.[5] Therefore, the presence of plasmid-mediated AmpC enzymes is often associated with the presence of ESBLs.[6]

AmpC enzyme production is less common than ESBL production in most parts of the world. However, both enzymes may be detected in one strain, which in turn mediates resistance to all β-lactams except carbapenems and cefepime, and is typically associated with multidrug resistance (MDR).[3]

Genes encoding AmpC β-lactamases may be chromosomal (cAmpC) or plasmid-mediated (pAmpC). In Enterobacterales chromosomally encoded AmpC genes are present in *Enterobacter cloacae*, *Serratia marcescens*, *Citrobacter freundii*, *Morganella morganii* and *Escherichia coli*. These genes are often expressed constitutively at low levels.[7] Plasmid-mediated AmpCs are usually constitutively expressed, conferring resistance patterns similar to that of derepressed or overexpressed chromosomal AmpCs.[8] The most important species of the Enterobacterales order that have acquired pAmpCs include *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*, but other important species are also *Klebsiella oxytoca*, *Salmonella enterica* and *Shigella* spp.[9]

Plasmid-mediated AmpC β-lactamases, including MIR/ACT (associated with the EBC family gene), DHA, MOX, CIT, ACC and FOX are derived from chromosomal AmpC genes of Enterobacteriaceae which display structural and functional similarities to their chromosomal origins.[10]

Previous studies conducted in Egypt reported different frequencies of AmpC genotypes. For instance, a study conducted in three tertiary hospitals in Cairo, Egypt has reported a frequency of AmpC genotypes of 9.7%[11] and another study conducted in Zagazig, Egypt has reported 5.6%[12] and 7.1% in Kafr El-Sheikh has been reported by Khalifa, et al.; 2019[13] and 33.8% in a study by El-Hady and Adel, 2015 in Ain Shams University Hospital.[14]

This variation of frequencies in AmpCs between different areas in Egypt makes examining the frequency of AmpCs in our locality (Assiut, Upper Egypt) important. This may be due to the lack of guidelines for AmpC detection by the Clinical and Laboratory Standards Institute (CLSI). Thus, reliably identifying AmpC-producing organisms is a major challenge in clinical microbiology laboratory.[7] Accurate detection and categorization of drug-resistant bacteria are useful for timely and clinically correct selection of antibiotics. Therefore, the rapid detection of AmpC β-lactamases should be considered. Consequently, this study was conducted to detect plasmid- mediated AmpC β-lactamases in gram negative isolates in Assiut University Hospital.

Materials and Methods

This study was carried out at Microbiology laboratory of Clinical Pathology Department, Assiut University Hospital, Egypt. The study protocol was reviewed and approved by the Ethics Committee of the Faculty of Medicine, Assiut University (IRB no. 17200546; August 7, 2016).

The study included 300-gram negative isolates obtained from different clinical specimens sent to the microbiology laboratory for bacterial culture and identification and for antibiotic sensitivity testing. The isolates were screened for cefoxitin resistance by disc diffusion method using cefoxitin disc according to CLSI guidelines.[15] Isolates showing an inhibition diameter zone <18 mm were considered resistant. Out of these 300 isolates,

120 were cefoxitin resistant. All antibiotic discs used in this study were produced by Oxoid (Cambridge, UK).

The 120 cefoxitin resistant isolates were subjected to the following tests:

ESBL confirmatory test, was performed using combined disc method according to CLSI guidelines.[15]

Briefly, a disc of ceftazidime (30 µg) alone and another disc of ceftazidime in combination with clavulanic acid (30 µg/10 µg) were placed at a distance of 25 mm apart on a Mueller-Hinton agar (MHA) plate inoculated with bacterial suspension (0.5 McFarland turbidity standards) and incubated for 18–24 hrs at a temperature of 37°C. An increase in the inhibition zone diameter of ≥5 mm for a combination disc versus ceftazidime alone was confirmed as ESBL producing bacilli.

Phenotyping of AmpC using disc approximation test

This test was performed as described by Gupta et al., 2014 follows: A 0.50 McFarland bacterial suspension from an overnight blood agar plate was prepared. The surface of a MHA plate was inoculated using this suspension. A 30 µg ceftazidime disc was placed at the center of the plate. 10 µg imipenem, 30 µg cefoxitin, and 20/10 µg amoxicillin-clavulanate discs were placed at a distance of 20 mm from the ceftazidime disc. The plate was inverted and incubated for 18–24 hrs at 35°C. A positive result for AmpC production was considered if blunting or flattening of the zone observed.[16]

Phenotyping of AmpC by Inhibitor based method using phenyl boronic acid (PBA), was performed using two concentrations of PBA.

Briefly, a 0.50 McFarland bacterial suspension from an overnight blood agar plate was prepared. The surface of a MHA plate was inoculated using this suspension. The first concentration was 150 µg/mL PBA according to Gupta, et al., 2014 as follows: Two 30 µg cefoxitin discs were placed on the inoculated surface of the MHA plate at a distance of 25 mm, center to center. Using sterile tips, 20 µl of 150 µg/mL PBA was dispensed onto one disc.[16] The second concentration was 600 µg/mL PBA, done as described by Elsherif, et al., 2016. Two ceftazidime (30 µg) discs were placed on the inoculated surface of the MHA plate at a distance of 25 mm, center to center. Using sterile tips, 20 µl of 600 µg/mL phenyl PBA was dispensed onto one disc.[17] After overnight incubation, the zone diameter around the antibiotic disc with added PBA and the antibiotic-containing disc alone were compared. An organism that demonstrates a defined increase (≥5-mm) in zone diameter around the antibiotic disc with added boronic acid was considered an AmpC producer.[16,17]

Genotyping of AmpC genes using multiplexed polymerase chain reaction (PCR)

Multiplex PCR was used to differentiate the five plasmid-mediated AmpC specific families (MOX, CIT, DHA, EBC, and FOX). DNA isolation was performed from bacterial isolates using QIAamp DNA Minikit, (Catalog no. 51304, QIAGEN, Germany) according to the manufacturer's instructions.

The PCR reaction mixture was prepared as follows: 25 µL of PCR master mix (Catalog no. 206143, QIAGEN, Germany), 1µL of each primer (2 µM; Catalog no. 10629186, Life Technologies Invitrogen, USA), 12 µL of RNase-free water and 3 µL of DNA template. Total reaction volume was 50 µL. The primer sequence and the target amplicon size are shown in Table 1**.**

E. coli ATCC® 25922 ™ (wild-type) was used as a negative control and no template control was applied in each PCR experiment.

PCR tubes were loaded into the thermal cycler (Veriti thermal cycler, Thermo Fisher Scientific Inc., USA) which was programmed for the following cycling condition: initial denaturation / activation for 5 min at 94°C followed by 35 cycles (denaturation for 30 sec at 94°C, annealing for 30 sec at 65°C, extension for 1 min at 72°C) with a final extension step of 7 min at 72°C.

DNA products were analyzed by 2% agarose gel electrophoresis using agarose (Cat. no. 16500100, Life Technologies Invitrogen, USA) and Tris-Borate-EDTA (TBE) buffer (catalog no. 00390533, Thermo Fisher Scientific Inc., USA). Electrophoresis was performed for 45 min. at

120 volts. The gel was stained with ethidium bromide (catalog no 15585011, Life Technologies Invitrogen, USA), and visualized using UV illumination (Figure 1).

The first PCR product, positive for the CIT gene, was sent for DNA sequencing to evaluate the obtained result. As the sequencing confirmed the obtained result, this DNA sample was used as a known positive control in subsequent PCR experiments.

Statistical methods

The Statistical Package for Social Sciences (SPSS: version 23.0; IBM Corp., New York, USA) was used for data management and analysis. Data were presented as numbers and percentages. Kappa statistics were used for comparison of pAmpC positivity and negativity. Detection rates of the applied phenotypic methods using the PCR method were judged as: values of 0.00 – 0.20 indicated no to slight agreement, 0.21– 0.40 fair agreement, 0.41– 0.60 moderate agreement, 0.61–0.80 good agreement, and 0.81–1.00 almost perfect agreement.

Table 1. Primers used in Multiplexed PCR.[38]

Target(s)	Primers	Sequence (5'to3)	Expected amplicon size(bp)	Gene bank accession no.
MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	MOXMF	GCT GCT CAA GGA GCA CAG GAT	520	D13304
	MOXMR	CAC ATT GAC ATA GGT GTG GTG C		
LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGG CCA GAA CTG ACA GGC AAA	462	X78117
	CITMR	TTT CTC CTG AAC GTG GCT GGC		
DHA-1, DHA-2	DHAMF	AAC TTT CAC AGG TGT GCT GGG T	405	Y16410
	DHAMR	CCG TAC GCA TAC TGG CTT TGC		
MIR-1T ACT-1	EBCMF	TCG GTA AAG CCG ATG TTG CGG	302	M37839
	EBCMR	CTT CCA CTG CGG CTG CCA GTT		
FOX-1 to FOX-5b	FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190	X77455
	FOXMR	CAA AGC GCG TAA CCG GAT TGG		

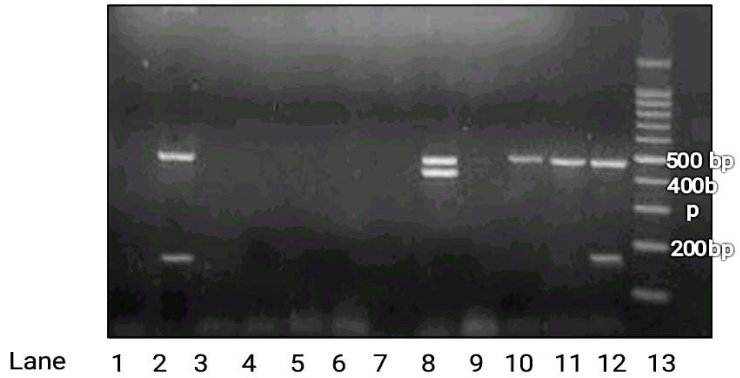

Figure 1. Detection of Plasmid Mediated AmpC Genes by Multiplex PCR. Lane 1: Negative control, Lane 2: Positive control (CIT (462 bp), FOX (190 bp), Lanes 3, 4, 5, 6, 7, and 9: Negative results, Lane 8, CIT (462bp), DHA (405bp), Lane 10, 11: CIT (462bp), Lane 12: CIT (462bp), FOX (190bp), Lane 13: 100bp DNA ladder.

Results

The 120 cefoxitin-resistant isolates were as follows: 75 (62.5%) *Klebsiella pneumoniae* isolates, 31 (25.8%) *E. coli* isolates, 10 (8.3%) *Enterobacter* sp. (including 6 (5%) *Enterobacter cloacae* and 4 (3.3%) *Enterobacter aerogenes*), 3 (2.5%) *Pseudomonas aeruginosa* isolates, and one (0.8%) *Acinetobacter baumannii* isolates.

Results of phenotypic tests for detecting ESBL

Combination Disc Test for ESBL screening was positive in 14 (11.7%) isolates while the Vitek ESBL screening test was positive in 17 (14.2%) isolates (Table 2). Both tests showed a good degree of agreement (*K* degree= 0.67).

Table 2. Agreement between Vitek ESBL and combination disc test.

		Vitek ESBL screening test	
		Positive	Negative
		Count	Count
Combination Disc test	Positive	11	3
	Negative	6	100
K degree		0.67	

Results of phenotypic detection of AmpC β-lactamases and their agreement:

Disc Approximation test was positive in 19 (15.8 %) isolates, whereas in the PBA test 15 (12.5 %) isolates were positive using 150 µg/mL PBA with 30µg cefoxitin. In addition, 21 (17.5 %) isolates were positive using 600 µg/mL PBA with 30µg ceftazidime. These tests showed slight to fair degree of agreement (Table 3 and 4).

Table 3. Agreement between disc approximation test and boronic acid test using 150 µg/mL phenylboronic acid.

		Disc approximation test		Total
		Positive	Negative	
		Count	Count	
Boronic acid test (150µg/mL)	Positive	6	9	15
	Negative	13	92	105
K degree		0.25		

Table 4. Agreement between disc approximation test and boronic acid test using 600 µg/mL phenylboronic acid.

		Disc approximation test		Total
		Positive	Negative	
		Count	Count	
Boronic acid test (600µg/mL)	Positive	9	12	21
	Negative	10	89	99
K degree		0.35		

Results of detection of AmpC gene

Only 22 isolates (18.3 %) were positive by using multiplexed PCR. The distributions of the detected genes are shown in Table 5.

Table 5. Positive results of AmpC genotypes.

Genotype	Count	%
CIT	10	8.3%
FOX + CIT	10	8.3%
CIT + DHA	1	0.8%
MOX	1	0.8%
EBC	0	0%
Total	22	18.3%

Relation of the AmpC gene to microorganisms

Six *Klebsiella Pneumoniae* isolates (8%) were confirmed to be plasmid-mediated AmpC β-lactamase producers using multiplexed PCR (Table 6). Of these, two isolates harbored *bla* CIT gene, two isolates harbored *bla* FOX and CIT genes, one isolate harbored bla MOX gene and one isolate harbored *bla* CIT and DHA genes. Fourteen *E. coli* isolates (45.2%) were confirmed as being plasmid-mediated AmpC β-lactamase producers. Of these, seven isolates harbored *bla* CIT gene and seven isolates harbored *bla* FOX and *bla* CIT genes. Other details are summarized in Table 6. Seventeen isolates (14.2%) were detected as ESBL producers using vitek2 compact 15, among which only two (11.7%) harbored plasmid-mediated AmpC genes.

Accuracy of phenotypic methods for detecting AmpC β- lactamases using PCR as a gold standard

Disc approximation test was considered a fairly good negative test (specificity of 87.8% and negative predictive value (NPV) of 85.1%), but a poor positive test (sensitivity of 31.8% and positive predictive value (PPV) of 36.8%) (Table 7).

Boronic acid test using 150 µg/mL PBA was also weighed a fairly good negative test (specificity of 87.8% and NPV of 81.9%), but with a very poor positive test (sensitivity of 13.6% and PPV of 20%).

Additionally, boronic acid test using 600 µg/mL PBA with 30 µg ceftazidime was considered a very good negative test (specificity of 91.8% and NPV of 91.8%). However, it was moderately good positive test (sensitivity of 63.6% and PPV of 63.6%).

Table 6. The AmpC gene in relation to microorganisms

		PCR gene				
		CIT	MOX	FOX + CIT	CIT + DHA	No. of positive isolates
	n	Count	Count	Count	Count	
K. Pneumoniae	75	2	1	2	1	6
E. coli	31	7	0	7	0	14
E. cloacae	6	0	0	1	0	1
E. aerogenes	4	0	0	0	0	0
A. baumannii	1	0	0	0	0	0
P. aeruginosa	3	1	0	0	0	1
Total	120	10	1	10	1	22

Table 7. Accuracy of different tests based on PCR as a gold standard.

		Disc approximation test		150 µg/mL PBA		600 µg/mL PBA	
		Positive n=19	Negative n=101	Positive n=15	Negative n=105	Positive n=22	Negative n=98
PCR results	Positive(n=22)	7	15	3	19	14	8
	Negative(n=98)	12	86	12	86	8	90

	Disc approximation test	Boronic acid test (150 µg)	Boronic acid test (600µg)
Sensitivity	31.8%	13.6%	63.6%
Specificity	87.8%	87.8%	91.8%
Positive predictive value	36.8%	20%	63.6%
Negative predictive value	85.1%	81.9%	91.8%
Accuracy	77.5%	74.2%	86.6%

Discussion

In this study, out of 300 gram negative clinical isolates, 120 (40%) were resistant to second generation cephalosporins (cefoxitin) as a screening test for AmpC. The most frequent resistant isolates were *K. pneumoniae* (62.5%) and *E. coli* (25.8%). In previous studies, there was a wide range of proportions of gram-negative isolates resistant to cefoxitin. A study by Wassef et al., 2014[18] reported 5.8% whereas Helmy and Wasfi 2014 [19] reported 18.2%, Chika et al., 2016 [4] 82.4%, Manandhar et al., 2017 [20] 13.2%, Inamdar and Anuradha, 2020[21] 57 %, and Mohd et al., 2016[10] 94.8%. This wide range could be attributed to geographic distribution, sample size and sample type.

Previous studies have suggested the Cefoxitin disc as a useful method in screening for AmpC but it is non-specific[22]. In the present study, only 22 (18.3%) of the 120 cefoxitin-resistant isolates had AmpC genes. Several factors may explain resistance to cefoxitin in AmpC-negative isolates. Firstly, it may arise due to porin channel alterations and mutations as previously reported in *E. coli* and *Klebsiella* spp. Isolates[2]. Secondly, cefoxitin-resistant phenotypes of *E. coli* can result from the overexpression of the chromosomal AmpC gene due to mutations in the promoter or/and attenuator regions resulting in alterations in the permeability of the cell to cefoxitin or a combination of all these factors[8]. Thirdly, cefoxitin is a substrate to active efflux pump in clinical isolates. [2]

ESBL detection in AmpC co-producing bacteria has become problematic for microbiologists, however, their detection would help guide the clinicians to the appropriate antimicrobial therapy. The inhibitor-based confirmatory test approach is most promising for isolates that do not co-produce an inhibitor-resistant β-lactamase such as AmpC.[5]

It has been previously reported that AmpC producing organisms can act as a hidden reservoir for ESBLs.[5] Consequently, in this study we performed a combination disc test which is a CLSI confirmatory test for ESBL using clavulanic acid as a β-lactamase inhibitor with ceftazidime 30 µg. Only 14 (11.7%) of the 120 isolates were positive, and this finding conforms to the results of Vitek2 Compact 15 in which 17 (14.2%) of the 120 isolates were positive. The kappa test indicated a good agreement between these two tests. Therefore, it is possible to use the CLSI confirmatory test of ESBL instead of that performed with Vitek in the routine clinical microbiology laboratory to reduce the cost of using automated detection.

ESBL detection may be masked by high levels of AmpC production. Moreover, clavulanate may act as an inducer of high level AmpC resulting in false negativity in ESBL detection by increasing resistance to screening drugs.[5]

Regarding phenotypic tests, phenotypic methods are needed to detect pAmpC positivity in laboratories due to lack of availability and high cost of molecular methods. In this study, the disc approximation and the boronic acid tests were used as phenotypic detection of AmpCs from 120 cefoxitin resistant isolates. The disc approximation test was positive in only 19 isolates (15.8 %) and had 77.5% accuracy, 87.8% specificity, 85.1% NPV, 31.8% sensitivity, and 36.8% PPV.

Boronic acid test using cefoxitin and 150 µg/mL PBA was positive in 15 isolates (12.5%) which had 74.2% accuracy and was considered a fairly good negative test with 87.8% specificity and 81.9% NPV but had low sensitivity (13.6%) and PPV (20%). Boronic acid test using ceftazidime and 600 µg/mL PBA was positive in 21 isolates (17.5 %) and had 86.6% accuracy and was considered a very good negative test with 91.8% specificity, 91.8% NPV, 63.6% sensitivity and 63.6% PPV. Such data indicated that all were good negative tests, but their sensitivity was low, except for the test using 600 µg/mL PBA.

False positive results encountered in phenotypic tests may be explained by several factors. First, the presence of more AmpC *β*-lactamase genes is possible, which continue to expand beyond those contained in the six families genes covered by PCR.[23] Second, phenotypic tests could not differentiate between positive results due to upregulation of chromosomally mediated AmpC *β*-lactamases and those due to genes that are carried on plasmids.[24] Alternatively, false negative results may be because the genes are detected using PCR but are not effectively phenotypically expressed.[18]

Moreover, the boronic acid tests showed a high rate of false-positive results, which could be due to the ability of PBA to inhibit not only AmpC enzymes but also the *K. pneumoniae* carbapenemase enzyme.[25] Only 15/120 (12.5 %) were positive by using 150 µg/mL PBA with 30µg cefoxitin and only 21/120 (17.5 %) positive by using 600 µg/mL PBA with 30µg ceftazidime. However, in studies using different concentrations of PBA, different results were reported. Wassef et al., 2014, reported 56.5% of isolates were positive in the tests using 150 µg/mL PBA with 30µg cefoxitin.[18] Another study reported that 67% of isolates were positive in test using ceftazidime 30µg with 400 µg/mL PBA

with a sensitivity of 68%, specificity of 50%, PPV of 70%, NPV of 48%.[26] However, a study by Elsherif et al., 2016, found that 84.6% of isolates were positive tests (using 600 µg/mL phenylboronic acid with 30µg ceftazidime) with a sensitivity of 84.67%, specificity of 100%, PPV of 100% and NPV of 50%.[17] Another study, used 30µg cefoxitin and 400µg phenylboronic acid, reported that 66.6% of isolates were positive with a sensitivity of 65.2%, specificity of 73.9%, PPV of 94.4% and NPV of 20 %.[19] A study by Barua et al., 2013, showed that PBA method was 100% sensitive and 96% specific using 30 µg of cefotetan and 400µg/mL of PBA in detecting AmpC producers; hence, they recommended this test as it is easier to perform and sensitive, which is contrary to our results.[27] The sample size, sample type and drug used, PBA concentration and geographic distribution could contribute to this variation between the two studies. A recent study by Inamdar and Anuradha, 2020, reported that Cefoxitin Cloxacillin -double disc synergy test (CC-DDS) method had a better detection rate than other phenotypic confirmatory methods and recommended the CC-DDS method for routine AmpC detection when needed as it is easy to perform.[21]

In this study, only 19/120 isolates (15.8%) were approximation disc test positive. However, previous studies have reported higher results: 35.5% in a study by Wassef et al., 2014[18], 35.3% in a study by Chika et al., 2016[4], 33.8% in a study by El-Hady and Adel, 2015[14], and 25.2 % in a study by Tan et al., 2009[24] but all showed poor sensitivity results despite such variations.

In this study, the sensitivity and specificity of all three phenotypic methods were inadequate in detecting pAmpC positivity, as they can lead to false positive and false negative results. However, boronic acid test using 600µg/mL PBA can be used as a good negative test to exclude the presence of AmpC producers.

Plasmid-mediated AmpC β-lactamases pose a big challenge to infection control because the AmpC gene can be expressed in larger amounts and has high transmissibility to other bacterial species.[10] The results of this study showed that 22 (18.3%) of the 120 isolates had AmpC genes. This frequency was lower than that reported in several previous studies, 25.38% in a study by Mitu et al., 2019, in Bangladesh[28]; 27.5% in a study by Nishimura et al., 2018, in Nagazaky, Japan[29]; 34.2% in a study by Mohd Khari et al., 2016 in Malaysia which was done on enterobacter spp[10] and 33.8% in a study by El-Hady and Adel, 2015.[14] However, the frequency of isolates harboring AmpCs in this study was higher than that reported in other studies in Egypt. A study conducted in three tertiary hospitals in Cairo, Egypt, reported a 9.7% frequency rate[11], another study conducted in Zagazig, Egypt, reported a frequency rate of 5.6%[12], and in Kafr El-Sheikh, Khalifa et al., 2019, reported a frequency rate of 7.1%.[13] Low frequency rates were reported in previous studies: 14% in Iran[30], a frequency rate of 9% has been reported by four studies (Pascual et al.; 2016, Zhou et al.; 2017, Kazemian et al.; 2019 and Ribeiro et al 2019[31-34]), and a frequency rate of less than 1% was reported in a study on *E. coli* strains in North and Eastern Europe.[35]

In this study CIT gene was the most frequent gene detected with 45.5% alone and 45.5 with FOX gene and 4.5% with DHA gene. So, the total CIT gene detected in current study was 95.5%.

In previous studies, according to the isolates harboring AmpC genes a study by Wassef et al., 2014, reported that 40.9% belonged to each of the MOX and the FOX families, 13.6% belonged to the EBC family, and 4.5% to the CIT family.[18] In another study by Rizi et al., 2020, the most frequent genotype of plasmid mediated AmpC was CMY 38 %, followed by FOX 29%, MIR 14 %, MOX 8 % and DHA 6 %.[7] Another study by Caliskan et al., 2019, has reported that 18% of isolates harbored the DHA gene,10% the FOX gene, 8% the CIT gene,13% the EBC gene, 3% the MOX gene and 46% had mixed genes.[26] In a study by Fam et al., 2013, reported that 76.5% of isolates harbored the CIT gene and 23.5% the DHA gene.[36] A study in China has reported that 93.2% of isolates harbored the DHA gene and 6.8% the CMY gene.[37] Such variations Could be attributed to geographic distribution, sample size and sample type.

In conclusion, the phenotypic methods of detecting AmpC β-lactamase production is of limited values as suggested by the low

agreement as detected using PCR, however the test using 600 μg/mL PBA could possibly act as a good negative test. Approximately 18.3% of the isolates were AmpC β-lactamase producer and the most frequently detected gene was CIT gene. The finding that all PCR positive *E. coli* isolates, accounting for approximately 50% of all *E. coli* isolates, harbored the CIT gene is of special interest.

Author Contributions

HGR, MZA and AMA proposed and designed the study. HGR and NOSK applied for the research fund. NOSK performed the laboratory work. TTHE designed and interpreted genotyping experiments. AMA, TTHE and NOSK interpreted the laboratory test results and analyzed the data. AMA and NOSK wrote the paper draft. HGR, MZA and TTHE revised and edited the paper.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Ethics Committee of the Faculty of Medicine, Assiut University (IRB no. 17200546; August 7, 2016).

Informed consent

A signed consent form was obtained from each study participant.

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