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ORIGINAL ARTICLE

Effect of the Different Plasmid-Mediated AmpC Beta-Lactamase Genotypes on the Phenotypic Detection of ESBL in Enterobacteriaceae Isolates

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SUMMARY

Background: **Increasingly, Enterobacteriaceae isolates that are positive for the extended-spectrum-beta-lactamase (ESBL) by phenotypic screening tests yield a negative result when tested with the reference Clinical and Laboratory Standards Institute (CLSI) ESBL confirmatory test. The aim is to determine to what extent the different plasmid AmpC (pAmpC) genotypes could affect the CLSI ESBL confirmatory test for detection of the ESBL phenotype in ESBL/pAmpC co-producers.**

Methods: **A total of 253 Enterobacteriaceae isolates were screened for ESBL and AmpC production according to CLSI guidelines. Out of 186 ESBL and AmpC-screen-positive isolates, 96 isolates were selected for ESBL confirmation by the combined disc diffusion test (CDDT) as well as for detection of the most common ESBL and pAmpC encoding-genes by multiplex PCR.**

Results: **Out of the 96 ESBL/AmpC-screen-positive isolates, all (100%) were positive for at least one of the investigated ESBL genes, and 88 (91.7%) were positive for any of the investigated pAmpC genes. CDDT correctly identified ESBL phenotype more frequently in non-pAmpC carriers than pAmpC carriers (75% vs. 52.3%). CIT alone-containing isolates were associated more with non-confirmed ESBL phenotype rather than confirmed ESBL phenotype (76.2% vs. 30.4%, $p < 0.001$), especially when co-harbored *bla*CTXM alone (76.9% vs. 33.3%, $p < 0.001$) or both *bla*CTXM/*bla*SHV genes (100% vs. 0%). On the other hand, DHA-carrying isolates were more associated with confirmed ESBL phenotype than with non-confirmed ESBL phenotype when co-harboring either *bla*CTXM alone (47.6% vs. 0%, $p < 0.001$) or *bla*CTXM/*bla*SHV genes (100% vs. 0%, $p = 0.022$) or *bla*CTX-M/*bla*TEM genes (100% vs. 0%, $p = 0.03$).**

Conclusions: **For ESBL/pAmpC co-producers of Enterobacteriaceae, CDDT results vary with the type of pAmpC genes and with different ESBL/pAmpC genotype combinations. Therefore, the ESBL-screening test is more sensitive than the CDDT in detecting ESBL phenotype among ESBL/pAmpC coproducers of Enterobacteriaceae.**
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INTRODUCTION

Extended-spectrum-beta-lactamases (ESBLs) and Ampicillinase C (AmpC)-beta-lactamases are major and important resistance mechanisms in Enterobacteriaceae [1,2]. Microbiologists and clinicians are concerned about these antibiotic resistance mechanisms because they cause resistance to a variety of antibiotics, resulting in treatment failure [3].

ESBLs are plasmid-mediated enzymes that hydrolyze penicillins, third generation cephalosporins, and aztreonam, but not cephamycins (cefoxitin and cefotetan). However, they might be inhibited by β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam) [3]. The most prevalent families of ESBLs-encoding genes are blaCTX-M, *bla*SHV, *bla*OXA, and *bla*TEM [4].

AmpC β-lactamases are enzymes that are either chromosomal or plasmid encoded [5]. Plasmid-mediated AmpC (pAmpC) β-lactamases are originated from chromosomes and has been transferred onto plasmids [6]. pAmpC variants are classified into five groups according to their origins: EBC variants (ACT-1 and MIR-1) from *Enterobacter* spp., CIT variants (CMY-2) from *Citrobacter freundii*, FOX and MOX variants from *Aeromonas* spp., DHA variants from *Morganella morgannii* and ACC variants originating from *Hafnia alvei* [7]. pAmpC has been mainly detected in *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Klebsiella oxytoca* (*k. oxytoca*), *Proteus mirabilis* (*P. mirabilis*), and to a lesser extent in *Salmonella* spp. and *Shigella* spp. [8]. AmpC β-lactamases cause antibiotic resistance almost like that conferred by the ESBLs, and also cause resistance to the cephamycins. However, AmpC β-lactamase activity is not inhibited by ESBL inhibitors like clavulanic acid, but is inhibited by boronic acid and cloxacillin [9].

Several phenotypic testing methods have been suggested for routine use to detect ESBL production in gram-negative bacilli. The CLSI confirmatory test uses a β-lactamase inhibitor, commonly clavulanate, in combination with one of the third generation cephalosporins (3GC), such as ceftriaxone, ceftazidime, or cefotaxime [10]. The confirmatory test based on the use of an ESBL inhibitor is best suited for isolates that do not co-express other inhibitor-resistant β-lactamases such as pAmpC [11]. Failure of the routine phenotypic methods to diagnose ESBL in ESBL/pAmpC co-producers can threaten the patients because it leads to treatment failure due to reporting a false susceptibility to cephalosporins [12]. Moreover, these pAmpC-producing organisms become hidden reservoirs for ESBLs, making infection control difficult [13].

In 2018, Nishimura and his colleagues found that the masking effect of pAmpC on the phenotypic confirmation of ESBL producing organisms could vary depending on the ESBL genotype [14]. However, little is known about how frequently the various types of pAmpC genes cause uncertainty about the accuracy of CLSI ESBL confirmatory tests [15]. Therefore, we aimed to investigate the impact of different genotypes of pAmpC on the phenotypic detection of ESBL by the recommended CLSI confirmatory testing method in ESBL/pAmpC co-producers of Enterobacteriaceae.

MATERIALS AND METHODS

Collection of bacterial isolates

A total of 253 non-duplicate bacterial isolates of Enterobacteriaceae were prospectively collected from patients admitted to Mansoura Specialized Medical Hospital with different infections in the period from February 2019 to February 2020. The isolates were retrieved from the routine bacterial cultures of the following samples: blood (n = 90), urine (n = 70), peritoneal fluid (n = 45), wound swab (n = 30), and endotracheal tubes (n = 18).

The Vitek® 2 system (bioMérieux, Marcy-l'Etoile, France) was used for identification of all isolates up to the species level using gram-negative (GN) cards and for antibiotic susceptibility testing using AST 73 and AST 71 cards. All of the collected isolates were phenotypically screened for ESBL and AmpC production. Thereafter, 96 isolates that were positive for both ESBL and AmpC screening and are known to lack or weakly express chromosomal AmpC (*Klebsiella* spp. and *E. coli*, respectively) were selected for the further investigations.

Phenotypic screening of ESBL and AmpC production

All collected Enterobacteriaceae isolates were screened for production of ESBL and AmpC enzymes by the Kirby-Bauer disc diffusion method following CLSI guidelines [10]. The isolates were identified as ESBL-screen positive if the inhibition zone diameter around any of the following antibiotic discs: ceftazidime (30 μg), ceftriaxone (30 μg), cefotaxime (30 μg), aztreonam (30 μg) or cefpodoxime (10 μg) was ≤ 22 mm, ≤ 25 mm, ≤ 27 mm, ≤ 27 mm, and ≤ 17 mm, respectively. The isolates were identified as AmpC screen positive if the diameter of inhibition zone around the cefoxitin disc (30 μg) was ≤ 18 mm.

Phenotypic confirmation of ESBL production

All ESBL-screen-positive isolates were subjected to the CLSI ESBL confirmatory test (combined disc diffusion method) for phenotypic confirmation of ESBL production. In brief, susceptibility of the tested isolate to cefotaxime (30 μg), cefotaxime/clavulanate (30/10 μg), ceftazidime (30 μg), and ceftazidime/clavulanate (30/10 μg) was performed on Müller-Hinton agar (Merck Co, Germany). The isolate was considered an ESBL-producer if the diameter of the inhibition zone around cefotaxime/clavulanate or ceftazidime/clavulanate discs increased by 5 mm or more when compared to cefotaxime alone or ceftazidime alone, respectively, Figure 4. *E. coli* ATCC 35218 was used as a reference strain [10].

Genotypic detection of ESBL and pAmpC genes

All 96 isolates that tested positive for both ESBL and AmpC screening were subjected to plasmid extraction by the GeneJET™ Plasmid Miniprep Kit (Thermo Scientific, UK) according to the manufacturer's instructions. Thereafter, two multiplex PCR were used for the detection of ESBL and pAmpC encoding genes using the primers listed in Table 1. All the primers used in this study were made by Invitrogen (Thermo Fisher Scientific, UK).

Multiplex PCR for the three most common ESBL gene families was performed in accordance to the previously described protocol [16]. Briefly, the final volume of the reaction was 25 µL consisting of 1 µL (10 picomol) of each forward and reverse primer, 12.5 µL of ready to use master mix (HotStarTaq®Master Mix Kit, QIAGEN GmbH, Germany), 2 µL of the extracted plasmid DNA and 4.5 µL of nuclease free water. The program of PCR on thermal cycler was as follows: 15 minutes of denaturation at 95°C, 35 cycles of amplification (each cycle consisted of 30 seconds of denaturation at 95°C, 30 seconds of annealing the primers at 57°C, and 1 minute for extension of the primer) and the last 10 minutes was for final extension at 72°C.

Multiplex PCR for amplification of family-specific pAmpC genes was done according to protocol of Pérez-Pérez and Hanson [7]. In brief, the total reaction volume was 25 µL; ready to use master mix (12.5 µL), forward and reverse primers of MOX, CIT, DHA (0.6 µM of each), forward and reverse primers of *ACC* and *EBC* (0.5 µM of each) and forward and reverse primers of FOX (0.4 µM), plasmid DNA template (2 µL), and nuclease free water (2.5 µL). Thermal cycler was programed as follows: Initial DNA denaturation at 95°C for 15 minutes, then 35 cycles of DNA denaturation for 30 seconds at 94°C, primer annealing for 30 seconds at 64°C, and primer extension for 1 minute at 72°C, finally, an extension step for 7 minutes at 72°C.

Detection of the resulting amplicons was done by agarose gel electrophoresis (2.5% for pApmC genes and 2% for ESBL genes). Visualization of the separated DNA bands was done by UV transillumination and 100 bp DNA ladder marker (Enzynomics, Korea) was used to determine their sizes, Figure 5 und 6.

Ethical Considerations

This study was approved by the Institutional Research Board, Faculty of Medicine, Mansoura University. Code Number: MS.19.02.479.

Statistical analysis

The collected data were analyzed using Statistical package for Social Science (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY, USA). Non-numerical data were presented as frequency and percentage. The association between two qualitative variables was obtained by chi-squared test. However, Fisher's exact test was used to analyze the association between two qualitative variables when the expected count was less than 5 in more than 20% of cells. A p-value was considered statistically significant if < 0.05.

RESULTS

Phenotypic screening for ESBL and AmpC β-lactamase production

Screening of the collected 253 Enterobacteriaceae isolates for production of ESBL and AmpC enzymes revealed that 8 (3.2%) were screen-negative for both ESBL and AmpC while 245 (96.8%) were ESBL screen-positive (186 isolates were also AmpC-screen positive whereas, 59 were AmpC screen negative). Out of 186 isolates that were screen-positive for both ESBL and AmpC β-lactamases, 96 isolates (60 *E. coli* and 36 *K. pneumoniae*) were selected for further ESBL and plasmid-born AmpC investigations as these types of the organisms are known to lack of or weakly express chromosomal AmpC, Figure 1.

Phenotypic and genotypic confirmation of ESBL production

Genetic analysis of ESBL genes revealed that all 96 ESBL/AmpC screen-positive isolates contained at least one of genes coding for ESBL. Based on the results of phenotypic ESBL confirmatory test, these 96 ESBL gene positive/ESBL screen positive isolates were categorized into two groups; group of confirmed ESBL phenotype (positive ESBL confirmatory test) that included 52 (54.2%) isolates and group of non-confirmed ESBL phenotype (negative ESBL confirmatory test) that included 44 (45.8%) isolates. A total of 122 ESBL genes were detected in 96 ESBL/AmpC screen-positive isolates. Isolates with single ESBL gene were more frequent than isolates with multiple ESBL genes (75.0% vs. 25%, respectively). The most frequent detected ESBL gene was *bla*CTXM gene (100%) followed by *bla*TEM gene 14/96 (14.6%) and SHV 12/96 (12.5%). *bla*CTX-M gene detected either as a single ESBL gene in 72 (75.0%) isolates or with *bla*TEM in 12 (12.5%) isolates or with *bla*SHV gene in 10 (10.4%) isolates. Only two (2.1%) isolates simultaneously contained all the studied three ESBL genes (CTX-M, SHV, TEM), Figure 2.

Genotypic detection of pAmpC β-lactamase genes

Out of 96 ESBL/AmpC screen positive isolates, multiplex PCR revealed that 88 (91.7%) isolates had one or more pAmpC genes (pAmpC carriers). The most frequent pAmpC genes were *CIT* and *DHA* gene variants; detected in 62 (70.5%) and in 36 (40.9%) isolates, respectively. The less frequent pAmpC genes were *MOX* and *FOX* genes, detected in 6 (6.8%) and 4 (4.5%) isolates, respectively. None of the studied isolates contained either *ACC* or *EBC* genes, Figure 3.

Table 1. Sequence of primers used in multiplex PCR and size of their corresponding amplicons.

Target gene	Primer	Sequence (3'-5')	Amplicon size (bp)
ESBL genes			
***bla*CTX-M universal**	**CTXMF CTXMR**	**CGA TGT GCA GTA CCA GTA A TTA GTG ACC AGA ATC AGC GG**	**585**
***bla*TEM**	**TEMF TEMR**	**ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC**	**1.080**
***bla*SHV**	**SHVF SHVR**	**TTATCTCCCTGTTAGCCACC GATTTGCTGATTTCGCTCGG**	**795**
pAmpC genes			
CIT family LAT-1 to LAT-4, CMY2 to CMY-7, BIL-1	**CITF CITR**	**TGG CCA GAA CTG ACA GGC AAA TTT CTC CTG AAC GTG GCT GGC**	**462**
DHA family DHA-1, DHA-2	**DHAF DHAR**	**AAC TTT CAC AGG TGT GCT GGG T CCG TAC GCA TAC TGG CTT TGC**	**405**
MOX family MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	**MOXF MOXR**	**GCT GCT CAA GGA GCA CAG GAT CAC ATT GAC ATA GGT GTG GTG C**	**520**
FOX family FOX-1 to FOX-5b	**FOXF FOXR**	**AAC ATG GGG TAT CAG GGA GAT G CAA AGC GCG TAA CCG GAT TGG**	**190**
EBC family MIR-1T, ACT-1	**EBCF EBCR**	**TCG GTA AAG CCG ATG TTG CGG CTT CCA CTG CGG CTG CCA GTT**	**302**
ACC family	**ACCF ACCR**	**AAC AGC CTC AGC AGC CGG TTA TTC GCC GCA ATC ATC CCT AGC**	**346**

Table 2. Association between type of ESBL genes and ESBL phenotype confirmation.

ESBL genes	ESBL phenotype No. (%)		p-value
	Confirmed (n = 52)	Non-confirmed (n = 44)	
CTX-M alone	**46 (88.5)**	**26 (59.1)**	**< 0.001**
CTX-M and SHV	**2 (3.8)**	**8 (18.2)**	**< 0.001**
CTX-M and TEM	**4 (7.7)**	**8 (18.2)**	**0.283**
CTX-M, SHV and TEM	**0**	**2 (4.5)**	**0.134**

Table 3. Effect of pAmpC genes carriage on ESBL phenotype confirmation.

ESBL phenotype	pAmpC genes		Total	p-value
	pAmpC carriers No. (%)	Non-pAmpC carriers No. (%)		
Confirmed	**46 (52.3%)**	**6 (75%)**	**52 (54.2%)**	**0.217**
Non-confirmed	**42 (47.7%)**	**2 (25%)**	**44 (45.8%)**	
Total	**88 (100%)**	**8 (100%)**	**96 (100%)**	

Table 4. Effect of different pAmpC genotypes on confirmation of ESBL phenotype in ESBL/AmpC coproducers.

pAmpC genes	ESBL phenotype No. (%)		p-value
	Confirmed (n = 46)	Non-confirmed (n = 42)	
CIT alone	14 (30.4%)	32 (76.2%)	< 0.001
DHA alone	24 (52.2%)	0%	< 0.001
CIT, DHA	8 (17.4%)	4 (9.5%)	0.283
CIT, MOX	0	2 (4.8%)	0.134
MOX, FOX	0	2 (4.8%)	0.134
CIT, MOX, FOX	0	2 (4.8%)	0.134

Table 5. Effect of different combinations of ESBL and pAmpC genotypes on the phenotypic ESBL confirmatory test in ESBL/ pAmpC coproducers.

pAmpC genes	ESBL phenotype No. (%)			p-value
	Confirmed (n = 46)	Non-confirmed (n = 42)	Total (n = 88)	
CTXM-alone containing isolates	42 (91.3%)	26 (61.9%)	68	0.001
CIT	14 (33.3%)	20 (76.9%)	34	< 0.001
DHA	20 (47.6%)	0	20	< 0.001
CIT, DHA	8 (19%)	4 (15.4%)	12	0.756
MOX, CIT	0	2 (3.8%)	2	0.142
CTXM, SHV-containing isolates	2 (4.3%)	8 (19%)	10	0.04
CIT	0	8 (100%)	8	0.022
DHA	2 (100%)	0	2	
CTXM, TEM-containing isolates	2 (4.3%)	6 (14.3%)	8	0.144
CIT	0	2 (33.3%)	2	1
DHA	2 (100%)	0	2	0.03
MOX, FOX	0	2 (33.3%)	2	1
CIT, MOX, FOX	0	2 (33.3%)	2	1
CTXM, TEM, SHV-containing isolates	0	2 (4.8%)	2	0.224
CIT	0	2 (100%)	2	1

Effect of ESBL genotypes on the phenotypic confirmation of ESBL by CDDT

The phenotypic confirmation of ESBL production was significantly associated with isolates containing *bla*-CTX-M alone 46/52 (88.5%). On the other hand, non-confirmation of ESBL phenotype was significantly associated with isolates containing both *bla*CTX-M and *bla*SHV genes 8/44 (18.2%), Table 2.

Effect of the pAmpC genes carriage on the phenotypic confirmation of ESBL

Among 96 genotypically confirmed ESBL isolates, the percentage of isolates confirmed phenotypically as ESBL by CCDT was higher among non-pAmpC carriers than among pAmpC carriers (75% vs. 52.3%, respectively), Table 3.

Effect of various pAmpC genotypes on ESBL confirmatory test results in ESBL/AmpC coproducers

Among pAmpC genes, CIT and DHA gene variants were the only genes having a significant effect on confirmation of ESBL phenotype by the combined disc diffusion method. CIT alone-containing isolates were frequently more associated with non-confirmed ESBL phenotype than confirmed ESBL phenotype (76.2% vs. 30.4%, $p \leq 0.001$). On the other hand, none of the DHA alone-containing isolates was misidentified phenotypically as non-ESBL producer by CDDT, Table 4.

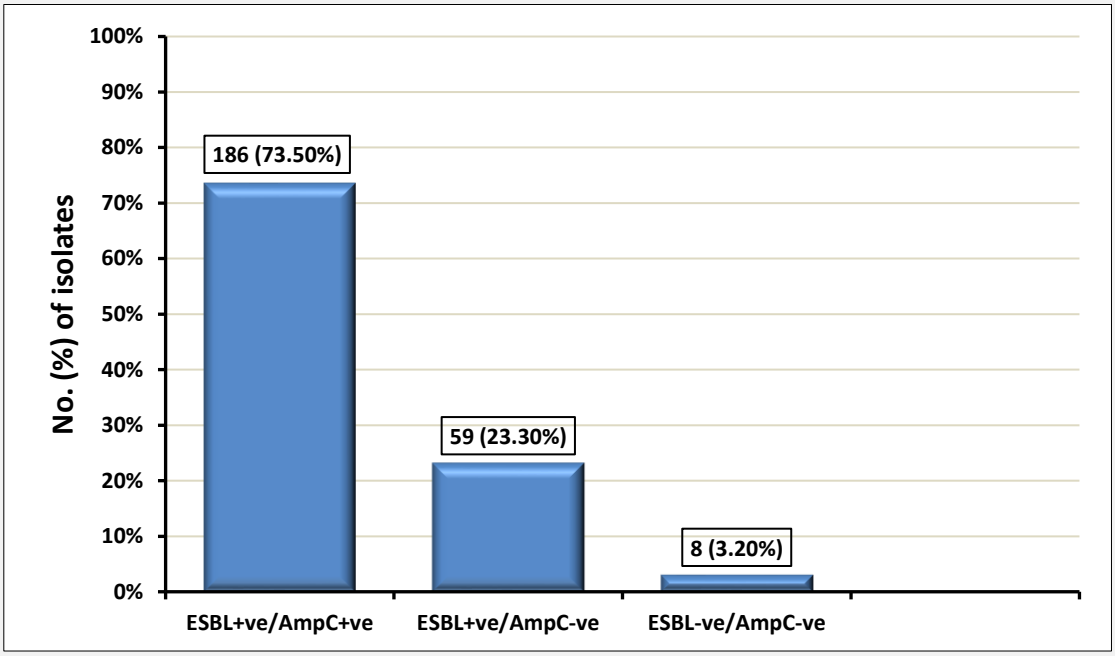

Figure 1. Phenotypic screening of 253 isolates of Enterobacteriaceae for ESBL and AmpC β-lactamase production.

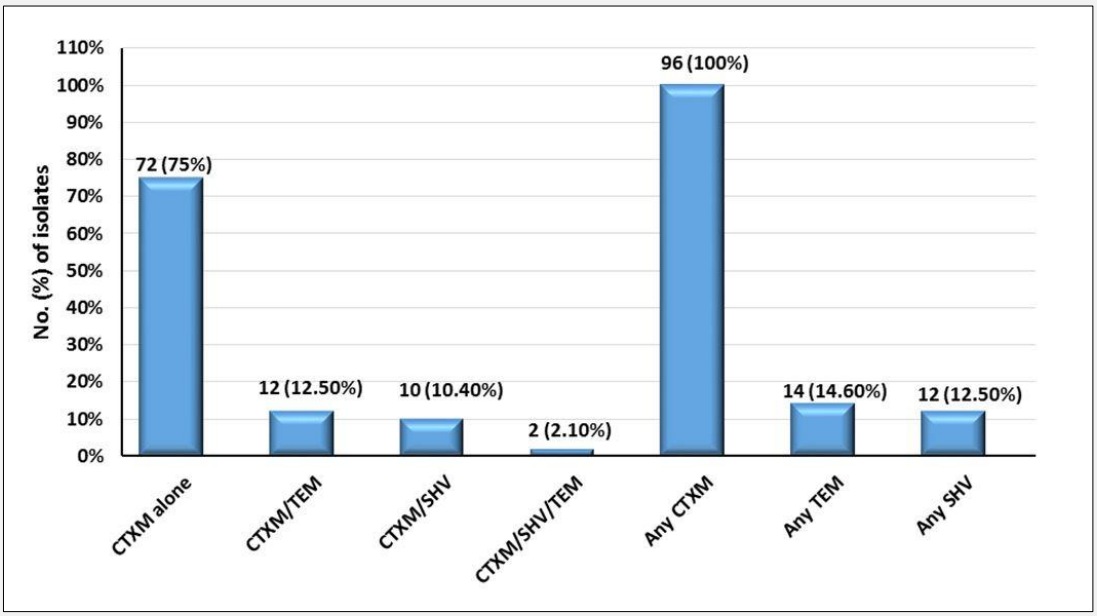

Figure 2. Distribution of ESBL genes in 96 ESBL and/AmpC screen positive isolates.

Any: indicates presence of the gene either alone or in combination with other ESBL genes.

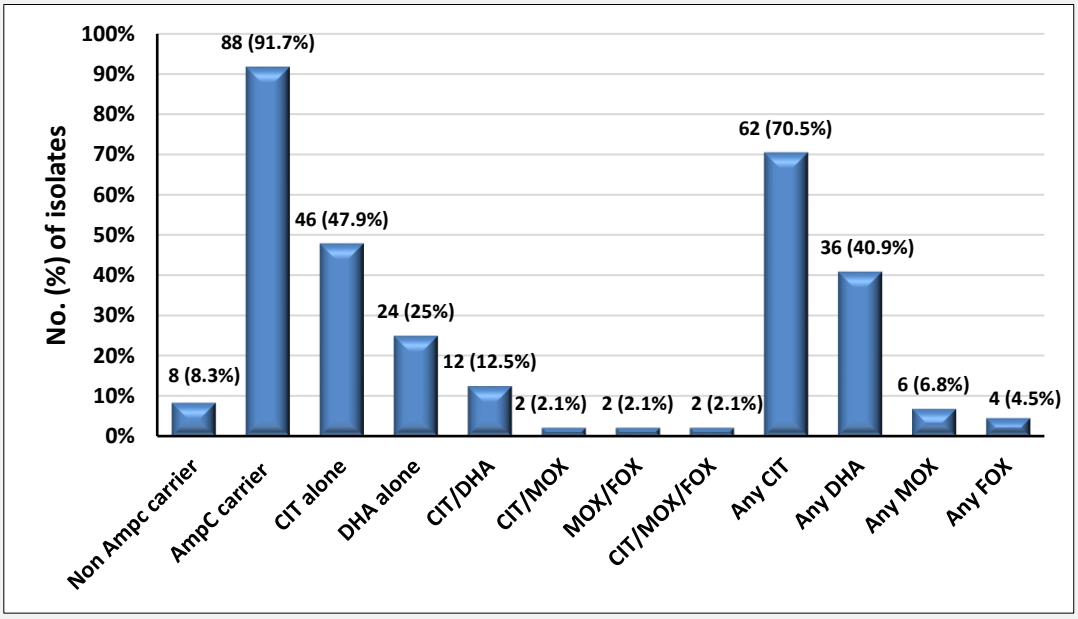

Figure 3. Distribution of pAmpC genes in 96 ESBL and/AmpC screen positive isolates.

Any: indicates presence of the gene either alone or in combination with other ESBL genes.

Combined effect of ESBL and pAmpC genes on the phenotypic confirmation of ESBL in ESBL/pAmpC coproducers

CIT-carrying isolates were significantly more associated with non-confirmed ESBL phenotype than confirmed ESBL phenotype if they also co-harbored either *bla*CTXM gene (76.9% vs. 33.3%, $p < 0.001$) or co-harbored both *bla*CTXM/SHV genes (100% vs. 0%). On the other hand, DHA-carrying isolates were significantly more associated with confirmed ESBL phenotype than with non-confirmed ESBL phenotype if they also co-harbored either *bla*CTXM alone (47.6% vs. 0%, $p < 0.001$) or *bla*CTX-M/SHV genes (100% vs. 0%, $p = 0.022$) or *bla*CTX-M/TEM genes (100% vs. 0%, $p = 0.03$), Table 5.

DISCUSSION

The simultaneous expression of ESBL and pAmpC genes may make it difficult to detect ESBL phenotype by the currently existing confirmatory testing methods [12]. To the best of our knowledge, there is no available data about how variation in pAmpC genotypes could affect the performance of the phenotypic ESBL confirmatory testing that is recommended by the CLSI. Therefore, we selected 96 Enterobacteriaeceae isolates (potentially ESBL and/AmpC co-producers and are known to lack or weakly express chromosomal AmpC) to investigate which type of pAmpC genes could affect the accuracy of the CLSI confirmatory method for phenotypic ESBL detection.

In the present study, screening of Enterobacteriaceae isolates for ESBL production by the recommended CLSI method revealed that the prevalence of potential ESBL producers was 245/253 (96.8%). This is similar to a previous study in our region that reported resistance rates of 97.8%, 100%, 98.6%, and 98.5% to ceftazidime, cefotaxime, ceftriaxone, and aztreonam, respectively, among *Enterobacteriaceae* isolates [17].

Among 96 ESBL screen-positive isolates, the CCDT was able to confirm ESBL phenotype in 54.2%, which is comparable to those reported by Poulou et al. [18] and Rizi et al. [19], 65.4% and 55.8%, respectively. On the other hand, the prevalence of ESBL-producing isolates in this study is considered much greater than that reported in several previous Egyptian studies, 17% by Fam et al. [20] and 38.8% by Shash et al. [21]. This variation in the prevalence rates of ESBL producers could be related to differences in either the investigated species, geographical regions, infection control measures, or different regimens of the empiric antibiotic. Moreover, in some countries, selective pressure produced by the abuse of cephalosporins could lead to an increase in the emergence rates of ESBL producers [22]. In this study, the percentage of phenotypically confirmed

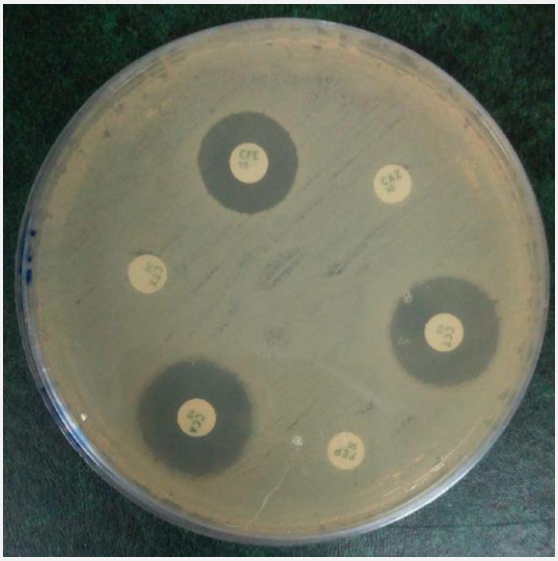

Figure 4. Combined disc diffusion test for confirmation of ESBL phenotype.

CAZ: Ceftazidim, CCA: Ceftazidim/Clavulinic, CTX: Cefotaxime, CCT: Cefotaxime/Clavulinic, FEP: Cefepim, CPE: Cefepim/Calvulinic An ESBL producer isolate showing difference between the inhibition zone around CCT and CTX or between CCA and CAZ discs ≥ 5mm diameter.

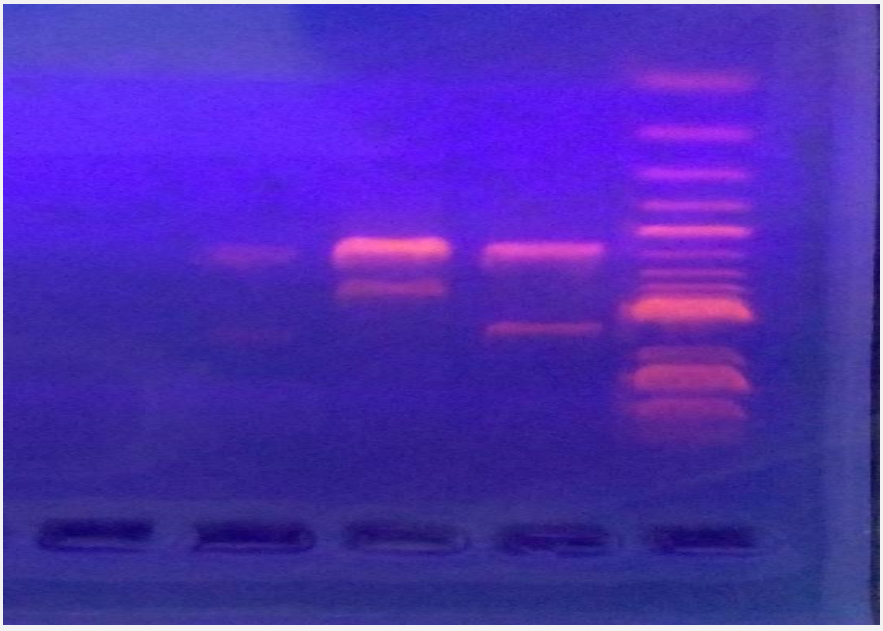

Figure 5. Agarose gel electrophoresis of ESBL genes.

From left to right; lane 1: Negative control, lane 2: *bla*CTXM and *bla*TEM, lane 3: *bla*CTXM and *bla*SHV, lane 4: *bla*CTXM and *bla*TEM and lane 5: ladder (100 bp).

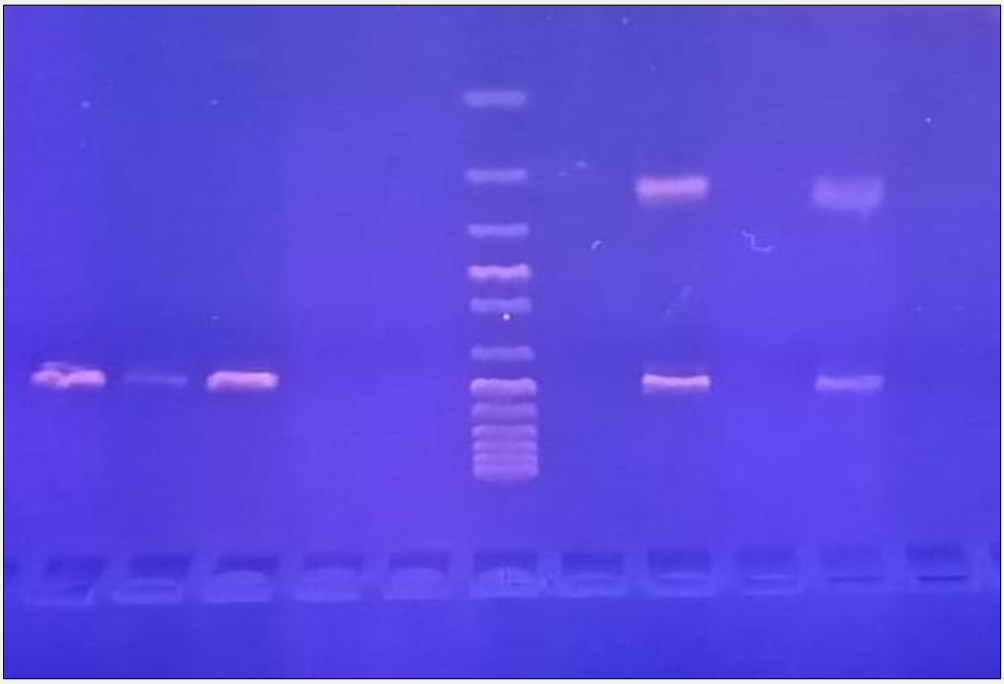

Figure 6. Agarose gel electrophoresis of pAmpC genes.

From left to right; lane 1, 2, 3: CIT, lane 4, 5: negative, lane 6: ladder (50 - 1,000 bp), lane 7: negative, lane 8, 10: MOX and FOX, lane 9, 11: negative.

ESBL is considered low when compared to Ramadan et al. [23] who reported 82.8% as a prevalence rate of ESBL phenotype. This could be explained by the fact that a high proportion (91.7%) of our studied isolates co-harboring pAmpC genes, which might lead to an underestimation of ESBL phenotype detection by the confirmatory methods [13].

Multiplex PCR detection of ESBL genes showed that all 96 ESBL-screen positive isolates had one or more of the ESBL coding genes. However, the combined disc method, as a phenotypic confirmatory test for ESBL production, confirmed the ESBL phenotype in only 52 (54.2%) isolates indicating that the phenotypic screening methods were more sensitive than confirmatory methods for detecting ESBL in ESB/pAmpC co-producers.

The *bla*CTXM gene was the most frequently detected ESBL gene (100%). This finding is in line with several regional and international studies that reported a high prevalence of the *bla*CTXM gene among Enterobacteriaceae in Egypt [20,24], in Burkina Faso [25], in Iran [26], and in Japan [27].

Twenty-four (25%) of the investigated 96 isolates harbored *bla*CTXM gene in combination with the *bla*TEM and/or *bla*SHV genes. Similarly, the co-carriage of multiple ESBL genes in the same isolate was detected previously in Egypt [24] and other countries; Burkina Faso [25], and Iran [26]. In this study, 186 (73.5%) of 253 isolates of Enterobacteriaceae were identified as potentials of AmpC B-lactamase producers based on their insusceptibilities to cefoxitin (30 mg). This is in accordance with a recent study in Egypt that reported a high prevalence of cefoxitin resistance in Enterobacteriaceae: 86.1% among *Klebsiella spp.* and 53.4% among *E. coli* [28]. In Iran, Rizi et al. (2020) also showed that 68.4% of the studied Enterobacteriaceae were potential of AmpC producers [19]. However, in a prior study in Egypt conducted by Wassef et al., the prevalence rate of cefoxitin resistance was low (5.8%) [29]. Prevalence rates of cefoxitin resistance as well as the type of acquired pAmpCs detected could vary according to many factors such as the geographical location, the species studied, the sample size, and the study period [30]. Therefore, comparing the prevalence rates of acquired AmpCs genotypes across different studies is difficult.

Multiplex PCR of pAmpC encoding genes showed that 88 (91.7%) of 96 cefoxitin resistant isolates contained one or more of pAmpC β-lactamase gene. This agrees with a previous study in Egypt, which reported 88.46% of cefoxitin resistant isolates tested positive for the

pAmpC genes by PCR assay [31]. However, this prevalence of pAmpC gene carriers was higher than that reported in Egypt by Fam et al., who found pAmpC prevalence to be 28.3% [32]. The high prevalence of pAmpC producers could be explained by many reasons, such as specimens being collected from hospitalized patients where it is likely that they had previously been exposed to cephalosporin therapy, either empirically or following the antibiotic policy of the hospital, as previously reported in an Egyptian study [33]. Additionally, the widespread use of oxyimino-cephalosporins is one of the driving forces behind the rise in AmpC-production as a result of selective pressure [34].

It was not surprising to find in this study that not all cefoxitin resistant isolates were AmpC β-lactamase gene carriers. Similarly, Fam et al. and Yilmaz et al. found that not all cefoxitin resistant isolates are AmpC β-lactamase carriers [32,35]. This is due to the fact that AmpC β-lactamase production is not the only mechanism of cefoxitin resistance; other enzymatic mechanisms such as metallo-beta-lactamase or non-enzymatic mechanisms such as mutation of porin channels may also be involved [36].

The most prevalent pAmpC genes were CIT and DHA gene variants (70.5% and 40.9%, respectively). This is in agreement with two studies carried out in Egypt, by Fam et al. and Hosny and Kashif, which detected CMY in 76.5% and in 60%, respectively, and DHA-1 in 23.5% and in 40%, respectively [32,37]. FOX gene was detected in a small percentage (4.5%) of the studied isolates. However, our findings contradict other studies carried out by Barwa et al. and Wassef et al., where the FOX family had the highest prevalence rate [38,29]. No ACC or EBC family genes were detected in this study, which is similar to Fam et al. and Robatjazi et al. [32,39]. Absence of ACC family genes could be explained by the fact that cefoxitin inhibits the enzymes of this family and all investigated isolates in the current study were cefoxitin resistant [40]. This is consistent with the findings of Tan and his colleagues, who discovered that using cephamycin resistance as a screening test for detection of the ACC family of enzymes is less sensitive [9].

Investigating the effect of ESBL genotype on confirmation of ESBL phenotype by CDDT revealed that confirmation of the ESBL phenotype was significantly associated with isolates carrying *bla*CTXM alone 46/52 (88.5%), p-value < 0.001. Similarly, Hassan et al. reported that the presence of any *bla*CTXM was significantly associated with a positive ESBL confirmatory test ($p < 0.001$) [17].

Additionally, we demonstrated that the non-confirmed ESBL phenotype was significantly associated with isolates containing both *bla*CTX-M and *bla*SHV genes (8/44, 18.2%, $p = 0.022$). This disagrees with Hassan et al. who reported that the presence of any *bla*SHV ($p = 0.03$), or any combination of *bla*CTX-M and *bla*SHV ($p = 0.023$), resulted in a positive ESBL confirmatory test in the majority of cases [17]. These disparities could be due to the inclusion of isolates with different combinations of ß-lactamases in the different studies. In the current study, it is noted that all ten CTXM/SHV containing isolates in the current study were also pAmpC co-carriers; 80% were co-harboring *CIT* gene variant and were misidentified as ESBL non-producers while the other 20% were co-harboring *DHA* gene variant and were accurately identified as ESBL producers. Therefore, it seems that the type of pAmpC gene could affect the performance of the CDDT for detection of ESBL phenotype among *bla*CTXM/SHV containing isolates. Investigation of the effect of co-carriage of pAmpC on detecting ESBL phenotype using the recommended CLSI method (CDDT) revealed that the ESBL confirmatory test is able to correctly identify ESBL phenotype more among non-pAmpC carriers than among pAmpC carriers (75% vs. 52.3%, respectively). This is in line with Nishimura et al. who found that non-pAmpC carriers had a statistically significant greater prevalence of the correctly identified ESBL phenotype than pAmpC carriers (88.8% vs. 69.2%, respectively) [14]. In fact, in 2007, SENTRY Asia-Pacific data raised concerns about the prevalence of ESBL screen-positive/confirmatory testing-negative among *E. coli* isolates co-harboring pAmpC genes; up to 75% of non-confirmed isolates were found to harbor pAmpC genes [41]. Similarly, we found that out of 44 isolates with non-confirmed ESBL, 42 (95.5%) were pAmpC carriers and 2 (4.5%) were non-pAmpC carriers. The pAmpC β-lactamases are resistant to β-lactamase inhibitors like clavulanic acid. Thus, the increase in the inhibition zone diameter around clavulante/cephalosprine disc in the CCDT by ESBL producers would be completely masked by pAmpC enzymes resulting in a non-confirmed ESBL phenotype in ESBL/pAmpC co-producers. Furthermore, clavulanate may act as an inducer of high-level pAmpC, resulting in false negativity in ESBL detection by increasing resistance to the screening drugs [13].

Investigation of how the performance of the CDDT for detection of the ESBL phenotype could vary with the type of the co-existing AmpC genes revealed that unidentification of ESBL phenotype was significantly associated with isolates that contained only the CIT gene variant of pAmpC (CIT alone-containing isolates were detected among 32/42 (76.2%) of isolates with non-confirmed ESBL phenotype). Similarly, Deshpande et al. detected CIT genes in 50.0% of the isolates despite a negative ESBL confirmatory test [42]. Likewise, Bell et al. found that a significant proportion of the phenotypic ESBL non-confirmation was linked to the presence of pAmpC enzymes of the CIT type [41]. On the other hand, the phenotypic confirmation of ESBL by CDDT was significantly associated with isolates containing only the DHA type of pAmpC enzymes (DHA alone-containing isolates were detected among 24/46 (52.2%) of isolates with confirmed ESBL phenotype). This is consistent with the findings of Pérez-Llarena et al. who discovered a DHA variant that could be suppressed

slightly better with clavulanic acid and sulbactam [43]. However, Bell et al. found that the phenotypic ESBL non-confirmation was associated with pAmpC enzymes of the DHA type [41]. Besides the different variants of CIT or DHA types that could be present in the isolates of the different studies, these discrepancies could also be attributed to the different prevalence rates of the different types of AmpC genes in different studies as well as distinct combinations of pAmpC and ESBL genotypes. The latter explanation was evident in the present study; we found that the DHA variant gene was significantly associated with phenotypic ESBL confirmation only when it co-existed with *bla*CTXM alone or with *bla*CTX/SHV or *bla*CTX/TEM containing isolates. Similarly, the present study found that the masking effect of the CIT gene on the ESBL phenotype could vary according to the type of ESBL gene co-existing with it. We found that the CIT gene was only significantly associated with non-confirmation of the ESBL phenotype when it co-existed with either *bla*CTXM alone (76.9%) or with both *bla*CTXM and *bla*SHV (100%). However, not all isolates co-harboring CIT alone were not identified as ESBL producers by CDDT; 33.3% of CIT/CTXM containing isolates were detected as phenotypic ESBL. Therefore, these 33.3% of CIT/*bla*CTXM-containing isolates that were correctly identified as phenotypic ESBL may have variants of CIT or *bla*CTXM that differ from those present in the 76.9% of CIT/CTX-containing isolates that were not identified as phenotypic ESBL.

Limitation of the study

The sample size is small, particularly for the non-pAmpC carriers. In addition, detection of ESBL or pAmpC genes was not followed by a sequence analysis to determine which variant of these genes could misidentify the ESBL phenotype by the recommended CLSI confirmatory method.

CONCLUSION

The performance of the phenotypic ESBL confirmatory method (CDDT) in ESBL/pAmpC co-producers varied not only with the type of pAmpC gene but also with different combinations between ESBL and pAmpC genotype. It could correctly confirm the ESBL phenotype in isolates co-harboring DHA type of pAmpC enzymes especially if combined with *bla*CTXM alone or with *bla*CTX/SHV or *bla*CTX/TEM. However, CDDT could not correctly detect the ESBL phenotype in isolates co-harboring only CIT gene variant of pAmpC especially if combined with *bla*CTXM alone or with both *bla*CTXM and *bla* SHV. Therefore, in ESBL/pAmpc co-producers, the screening tests for detecting the ESBL phenotype could be more sensitive than the confirmatory tests. Our study represents a good platform for future research to investigate the impact of different types of pAmpC genotypes on the performance of CLSI ESBL confirmatory method using a larger sample size and performing sequence analysis of the detected pAmpC and ESBL genes to detect which variant of these genes could mask the ESBL phenotype detection.

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Declaration of Interest:

There was no conflict of interest.

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