Detection of Plasmid-Mediated AmpC Beta-Lactamases in Clinically Significant Bacterial Isolates in a Research Institute Hospital in Egypt

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Abstract: Plasmid-mediated AmpC beta-lactamases (pAmpCs) are cephalosporinases that hydrolyze cephamycins as well as other extended-spectrum cephalosporins and are poorly inhibited by clavulanic acid. Although reported with increasing frequency, their true rate of occurrence in *Enterobacteriaceae* remains unknown and there are no standardized diagnostic tests recommended for their detection. **Aim**: the present study was designed to investigate these issues among cephalosporin-resistant isolates of *Echerichia coli, Klebsiella spp.* and *Proteus mirabilis* and to assess the performance characteristics of 3 phenotypic tests, using different inhibitors, compared to the PCR, for their rapid and accurate detection. **Methods:** Sixty out of 178 (33.7%) of enterobacterial clinical isolates from Theodor Bilharz Research Institute (TBRI), Cairo, Egypt resistant to third generation cephalosporins and cephamycins were included in the study. Bacterial species were identified using API E20. AmpC-producers were phenotypically detected using cloxacillin and aminophenyl boronic acid (APB)-based tests as well as AmpC disc test. ESBL-producers were detected using modified double disc synergy test (MDDST). *AmpC* genes clusters: (*bla* $_{ACC}$, *bla* $_{EBC}$, *bla* $_{FOX}$, *bla* $_{CMY}$, *bla* $_{MOX}$ and *bla* $_{DHA}$) and ESBL gene clusters *(bla* $_{TEM}$,*bla* $_{SHV}$ and *bla* $_{CTX-M}$) were amplified using PCR. **Results**: *AmpC* genes were detected in 28.3% (17/60), ESBLs in 53.3% (32/60) of test isolates and 10 isolates produced both ESBLs and pAmpC enzymes (16.7%). Cloxacillin disc potentiation test (DPT) was more sensitive (82.4%) and specific (95.3%) in detecting pAmpC enzymes than the APB-based tests and then the AmpC disc test (76.5% and 86 % respectively). All disc tests used in the study accurately detected pAmpC-positive isolates in ESBL-negative isolates (sensitivity 100%) but the sensitivity decreased by the co-presence of ESBL enzymes in the same isolate (60% by the APB test and AmpC disc tests; 70% by the cloxacillin DPT). All studied isolates remained sensitive to imipenem. **Conclusion:** This study reveals high prevalence of pAmpC and ESBL enzymes among bacterial isolates from our hospital. ESBL production may mask the phenotypic detection of pAmpC enzymes. Cloxacillin DPT is a simple and reliable method for detection of pAmpCs. MDDST and cloxacillin agar dilution tests may serve as reliable confirmatory tests for detection of ESBLs in AmpC-positive isolates. Imipenem remains the best treatment option in treating serious infections caused by pAmpC-producing isolates even in case of co-production of ESBL enzymes.

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1. Background

Extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamases (AmpCs) are important mechanisms of resistance among *Enterobacteriaceae*. Infections caused by ESBL and /or AmpC- positive bacteria are of particular clinical and epidemiological importance and cause higher patient morbidity and mortality (**Livermore, 2012 and Maina *et al.*, 2012**). ESBLs are typically plasmid-mediated, clavulanate-susceptible enzymes that hydrolyze penicillins, extended-spectrum cephalosporins and aztreonam. AmpCs are cephalosporinases that are poorly inhibited by clavulanic acid. They can be differentiated from ESBLs by their ability to hydrolyze cephamycins as well as other extended-spectrum cephalosporins. AmpCs were presumed to be chromosomally-mediated but since the late 1980s they have disseminated on plasmids and now represent a substantial clinical threat. The emergence and spread of pAmpCs in *Enterobacteriaceae* has made their detection clinically relevant, particularly in bacterial species naturally lacking chromosomal cephalosporinase namely in *E.coli, Klebsiella spp.* and *Proteus mirabilis*. Detection a pAmpC in a strain with a coexisting ESBL is even more challenging ***(Philippon et al., 2002)***. Moreover, AmpCs can interfere with ESBL detection particularly when using the current CLSI ESBL confirmatory tests. Resulting

failures to detect ESBLs can endanger patients because false susceptibility to cephalosporins may be reported ***(Thomson, 2001)***. This warrants the need for an alternative ESBL confirmatory test of greater accuracy for AmpC-producing isolates ***(Munier et al., 2010)***.

At present, there are no guidelines or standardized phenotypic methods recommended for detection of AmpCs. Although several methods have been described, including disc synergy assays using Amp-C inhibitors, a specific E test format for AmpC testing and a three dimensional test. However, these methods are expensive, tedious and have not been systematically compared ***(Kanamori et al., 2012 and Sabia et al., 2012)***.

This prospective study was designed and conducted at Theodor Bilharz Research Institute in Cairo to (a) determine the prevalence of ESBLs and AmpC-producers among bacterial isolates with reduced susceptibility to extended spectrum cephalosporines and cephamycins of *E. coli, Klebsiellae*, and *Proteus mirabilis* (b) to evaluate the efficacy of the modified double disc synergy test (MDDST) -and cloxacillin agar dilution tests as confirmatory ESBL tests in AmpC–positive isolates and (c) to assess the performance characteristics of 3 phenotypic tests compared to the PCR as a gold standard test for the rapid and accurate detection of AmpCs in ESBL-positive and ESBL- negative isolates.

2. Materials and Methods

I. Bacterial Isolates: 178 *Enterobacteriacea* isolates causing infections were prospectively collected for a period of 6 months, from July 2008 to December 2008, from hospitalized patients or patients attending the outpatient clinic of Microbiology Department of Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. Sixty isolates of which were selected according to the following selection criteria: (i) species that are known to lack chromosomal *ampC* (*Klebsiella* spp. and *Proteus mirabilis*) or minimally express chromosomal AmpC enzyme (*E. coli*); (ii) isolates showing decreased susceptibility to cephamycin cefoxitin (FOX) (iii) resistance to third generation cephalosporins (e.g. ceftazidime) with or without inhibition by clavulanic acid (i.e. ESBL-positive or ESBL-negative respectively) ***(CLSI, 2009)***. Bacterial species identification was confirmed by using the analytical profile index for *Enerobacteriaceae* (API-20E) (Bio-Mérieux SA, France).

II. Screening for Beta -Lactam Resistance and Antibiotic Susceptibility testing :

ESBLs were detected simultaneous with antibiotic susceptibility testing by using the modified double-disc test (MDDST) ***(Pitout et al., 2003)***. Briefly, an amoxicillin-clavulanate disc (AMC, 30 µg/10 µg) was placed in the centre and cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), cefepime (FEP, 30 µg), aztreonam (ATM, 30 µg), cefoxitin disc (FOX, 30 µg), piperacillin/tazobactam (TZP; 100/10µg), ampicillin (AMP;10 µg) and cephalothin (CL; 30 µg) (Bio-Rad, France) were placed at a distance of 30 mm center to center. The TZP disc was put in proximity with the FEP to detect ESBLs in AmpC-positive isolates. Appearance of a clear extension of the edge of the inhibition zone of any of the discs: CAZ, CTX, FEP or ATM towards the AMC disc or between the FEP and TZP discs indicated ESBL -production by the isolate. AmpC β-lactamase presence was suspected by resistance to CTX or CAZ, resistance to both AMC and FOX, and absence of any enhancement of the inhibitory zone towards the AMC. Susceptibility to amikacin and ciprofloxacin was also tested according to CLSI guidelines ***(CLSI, 2009).***

III-Phenotypic tests for Detection of AmpC

1- Cloxacillin agar dilution test (CAD*):*

Bacterial suspensions were adjusted to 0.5 McFarland turbidity standard and were inoculated simultaneously on two MHA plates (Bio-Rad, France): one containing cloxacillin (200µg/ml) (Orbénine IV 1g/5ml, France) and the second without cloxacillan. The same set of antibiotic discs: cefotaxime (CTX; 30µg), ceftazidime (CAZ;30µg), cefepime (FEP ; 30µg), aztreonam (ATM;30µg), amoxicillin- clavulanicacid (AMC; 30/10µg). Piperacillin / tazobactam (TZP; 100/10µg), cefoxitin (FOX; 30 µg) and imipenem (IPM; 10 µg) (Bio-Rad, France) was applied to both MHA plates. Zones of inhibition around antibiotic discs as well as synergy between antibiotics were compared in the two sets of agar plates. The presence of pAmpC enzymes was suspected if the zone diameters of any of the β-lactam antibiotic discs increased by 5mm or more in the cloxacillin agar plate compared to the cloxacillin-free agar plate. ESBL-positive isolates were simultaneously confirmed by appearance of synergy between TZP and FEP or between AMC and CAZ/ CTX/ ATM or FEP.

2- Cloxacillin Disc Tests (Clox DST and Clox DPT):

AmpC-production was detected by testing the effects of cloxacillin as inhibitor on zone diameters of third generation cephalosporines. Disc synergy tests DST (DST) and Disc potentation tests (DPT) were performed as described by ***Yagi et al.(2005)***. Concentrations used for cloxacillin (Orbénine IV 1g/5ml, France) was 200 µg ***(Tan et al., 2009).*** The two disc tests were simultaneously performed for each isolate on the same plate by dividing the MHA plate into two halves; one for the DST and the other for the DPT. Cloxacillin was added to a sterile blank disc CAZ and CTX discs were placed on either side of the

disc with 2 cm center to centre distance to test for synergy. The inhibitor was added to the CAZ disc to test for potentiation. Synergy between inhibitor containing disks clox (2µl) and either CAZ or CTX disks in DST and an increase in the zone diameter of ≥5 mm in the CAZ-inhibitor disc compared to the CAZ disc alone indicated Amp-C positive ***(Yagi et al., 2005 and Tan et al., 2009).***

3- Boronic acid Disc Tests (APB-DST and APB-PT):

AmpC-production was also detected by using boronic acid as inhibitor in same methodology described for cloxacillin disc tests as described by ***Yagi et al. (2005)***. Concentration used for aminophenyl boronic acid hemisulfate salt (APB) (Sigma-Aldrich, USA) was 400 µg. Dimethylsulfoxide (DMSO) (Sigma-Aldrich, USA) was used to dissolve APB to reach the final desired concentration ***(Song et al., 2007 and Tan et al., 2009).***

4-AmpC Disc Test:

The AmpC disc test was performed as described by ***Singhal et al. (2005).*** Briefly, 0.5 McFarland suspension of ATCC *E.coli* 25922 (supplied by US Naval Medical Research Unit 3, NAMRU-3, Abbassia, Cairo) was inoculated on the surface of Mueller-Hinton agar plate. A 30 µg cefoxitin disc (Bio-Rad, France) was placed on the inoculated surface of the agar. A sterile plain disc inoculated with several colonies of the test organism was placed beside the cefoxitin disc almost touching it. After overnight incubation at 37°C, the plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (positive result), or the absence of a distortion, indicating no significant inactivation of cefoxitin (negative result).

IV. Molecular Methods for detection of *bla* $_{ampC}$ and *bla* $_{ESBL}$:

Bacterial DNA was extracted using DNA QIAmp DNA mini kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Genes encoding β-lactamase were amplified using previously described primers except for the MOX cluster, where a primer pair was designed in our study for optimal PCR amplification of all members of this cluster (Table 1).

Table 1: Primers for detection of *bla*$_{ESBL}$ and different plasmid-mediated AmpC β-lactamase clusters

Amplified gene	Forward primer (5-3')	Reverse primer (5-3')	Reference
Bla $_{TEM}$	TGAAGACGAAAGGGCCTCCTG	TAATCAGTGAGGCACCTATCTC	***Chen et al., 2004.***
Bla $_{SHV}$	CACTCAAGGATGTATTGTG	TTAGCGTTGCCAGTGCTCG	***Chen et al., 2004.***
Bla $_{CTXM}$	GGTTAAAAAATCACTGCGTC	TTGGTGACGATTTTAGCCGC	***Leflon-Guibout et al., 2004***
Primer Name	**Targets**	**Sequence (5'-3')**	**Reference**
ACC-F	ACC	AAC AGC CTC AGC AGC CGG TTA	***Pérez-Pérez and Hanson, 2002.***
ACC-R	ACC	TTC GCC GCA ATC ATC CCT AGC	***Pérez-Pérez and Hanson, 2002.***
EBC-F	MIR-1T and ACT-1	TCG GTA AAG CCG ATG TTG CGG	***Pérez-Pérez and Hanson, 2002.***
EBC-R	MIR-1T and ACT-1	CTT CCA CTG CGG CTG CCA GTT	***Pérez-Pérez and Hanson, 2002.***
FOX-F	FOX-1 to FOX-5b	AAC ATG GGG TAT CAG GGA GAT G	***Pérez-Pérez and Hanson, 2002.***
FOX-R	FOX-1 to FOX-5b	CAA AGC GCG TAA CCG GAT TGG	***Pérez-Pérez and Hanson, 2002.***
CMY-F	LAT-1to LAT-4,CMY-2 to CMY-7 and BIL-1	TGG CCG TTG CCG TTA TCT AC	***Chen et al., 2004.***
CMY-R	LAT-1to LAT-4,CMY-2 to CMY-7 and BIL-1	CCC GTT TTA TGC ACC CAT GA	***Chen et al., 2004.***
MOX-F	MOX-1and MOX-2, CMY-1 and CMY-8 to CMY-11	GCT CAA GGA GCA CAG GAT	**designed as part of this study ***
MOX-R2	MOX-1and MOX-2, CMY-1 and CMY-8 to CMY-11	CAG GAT GGC GTG GGC CGC	**designed as part of this study ****
DHA-F	DHA-1 and DHA -2	GCC GGT CAC TGA AAA TAC AC	***Chen et al., 2004.***
DHA-R	DHA-1 and DHA-2	TAC GGC TGA ACC TGG TTG TC	***Chen et al., 2004.***

*Accession numbers; CMY-10_Kp_AF381618,CMY-11_Ec_AF381626,MOX-1_Kp_D13304,CMY-8_Kp_AF167990, CMY-9_Ec_AB061794 from GenBank; **Accession numbers. MOX-2_Kp_AJ276453,CMY-1_Kp_X92508, CMY-10_Kp_AF381618, CMY-11_Ec_AF381626,MOX-1_Kp_D13304, CMY-8_Kp_AF167990,CMY-9_Ec_AB061794 from GenBank. Tm used for both primers was 55˚ C and amplicon size was expected at 1000 bp.

Statistical Methods:

Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages). A probability value (P value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel 2007 (Microsoft Corporation, NY., USA), SPSS

(Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows and Quick Calcs online calculators for scientists (Graph pad software Inc., San Diego, CA, USA).

3. Results

1-Isolates and specimens collected:

During the study period 60/ 178 (33.7%) clinical enterobacterial isolates fulfilled the selection criteria and were tested for pAmpCs. *E. coli* was the major species collected (34/60; 57%), followed by *Klebsiella* spp. (23/60; 38%) then *Proteus mirabilis* representing only 5% of studied isolates. The majority of isolates were recovered from urine specimens (38/60; 63%). The remaining isolates were isolated from pus (10/60; 17%), sputum (7/60; 12%) and body fluids (including blood and ascitic fluid cultures) (5/60; 8%). Most of the specimens were collected from patients admitted to nephrology, urology, surgery and gastroenterology wards (37/60; 62%), while 22% were from patients admitted to ICU (13/60) and 16% from patients attending outpatient clinic (10/60).

2. Detection of *bla* $_{ampC}$ and *bla* $_{ESBL}$ genes by PCR:

AmpC genes was detected in 28.3% (17/60) and ESBL genes in 53.3% (32/60) of the isolates included in the study (p<0.05). Ten of the 60 isolates (16.7 %) produced both ESBL and AmpC enzymes (Table 2). CMY genes were detected in 13 of 17 AmpC-positive isoalates (76.5%) and genes belonging to the DHA family were found in 4 isolates (23.5%) (Figure 1a, b). No *ampC* genes belonging to the ACC, FOX, MOX or EBC families were detected.PCR reactions using bla_{TEM} and bla_{SHV} and bla_{CTX-M} primers showed that all ESBL isolates were positive for CTX-M but negative for TEM and SHV-genes (data not shown).

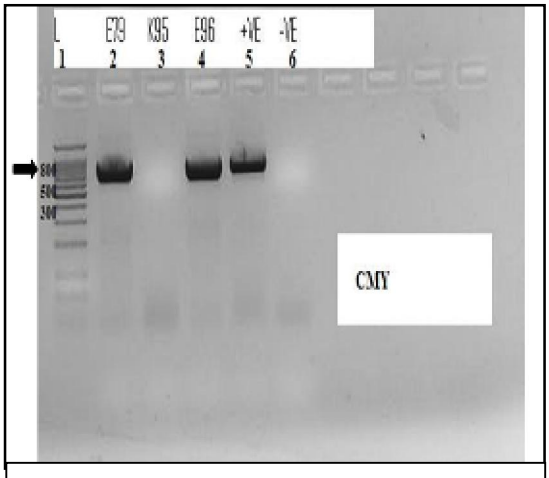

Figure 1a: Agarose gel electrophoresis of CMY PCR reactions for different isolates lane1:100bp DNA ladder lane2, 4: CMY positive *E.coli*, lane3: CMY-negative *Klebsiella* isolate an, lane 5: positive control and lane 6: negative control.

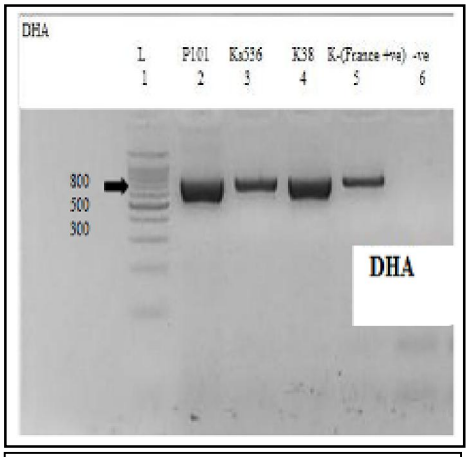

Figure 1b: Agarose gel electrophoresis of DHA PCR reactions for different isolates Lane 1:100bp DNA ladder: Lanes 2, 3, 4: DHA-positive Proteus and Klebsiellae isolates Lane, 5: positive control, Lane 6: negative control.

There was a trend towards significant association of the bacterial species with the type of β-lactamase gene detected: in *E. coli* species 64.7% were ESBL-positive while in *Klebsiella* spp, ESBLs and AmpCs were equally found in 10 isolates but 8 of 10 isolates harbored both genes. AmpC was significantly more prevalent in *Klebsiella spp*. than in *E. coli* (43.5% versus 17.6%) (p<0.05) (Table2).

Table 2: Prevalence and distribution of bla_{ESBL} and bla_{ampC} genes in bacterial species

Isolates	bla_{ESBL} + N (%)	bla_{ampC} + N (%)	bla_{ESBL} + bla_{ampC} + N (%)
***E. coli* (N=34)**	22 (64.7)	6 (17.6)	2(5)
***Klebsiellae* spp (N=23)**	10 (43.5)	10 (43.5) *	8(26)
***Proteus mirabilis* (N=3)**	0	1 (33)	-
Total (N=60)	32 (53.3)*	17 (28.3)	10 (16.7)

** P< 0.05

3- Antibiotic Susceptibility and Beta-Lactamase screening test

All isolates carrying *bla* $_{CTX-M}$ gene detected by PCR were successfully detected by the MDDST used for screening for beta-lactam resistance. Isolates showed synergy between FEP and either TZP or AMC (Figure 2).

Figure 2: Modified double disc synergy test showing synergy between TZP and FEP in an ESBL-positive/AmpC positive isolate.

The overall resistance to aminopenicillins and to third generation cephalosporins was exclusively high in AmpC-positive isolates, reaching 100 % for AMP, CTX and CAZ. Ten of 17 (58.8%) of the AmpC-positive isolates were resistant to fourth generation cephalosporin (FEP); and they all harbored. an ESBL enzyme of the CTX-M15. The overall resistance rate of Amp C-positive and AmpC negative isolates to CIP was 83.3% (94% and 79% respectively). In the AmpC positive group, isolates with concomitant ESBL production were constantly resistant to CIP except in one *E.coli* isolate (E875). The aminoglycoside amikacin (AK) was the most effective, after imipenem, for both AmpC-positive and AmpC-negative isolates as 50% of the isolates were susceptible to it. It was noted that among the *E. coli* isolates, resistance to AK was significantly higher in AmpC-negative than AmpC- positive groups (P = 0.05). None of the studied isolates were resistant to IPM.

4- Phenotypic tests

The cloxacillin agar dilution test detected 64.7% (12/17) of the PCR ampC-positive isolates by comparison of the zones of inhibition around beta-lactam antibiotics in clox agar plate to the M-H agar plate (sensitivity and specificity of 70.6% and 93 % respectively). Phenotypic detection of ESBL was simultaneously evident on M-H agar plates without cloxacillin by detection of synergy between FEP and TZP. All 10 ESBL-positive/Amp-C positive isolates were successfully detected by this method denoting that MDDST is a good confirmatory test for detection of ESBLs co-producing AmpCs (Figure 3).

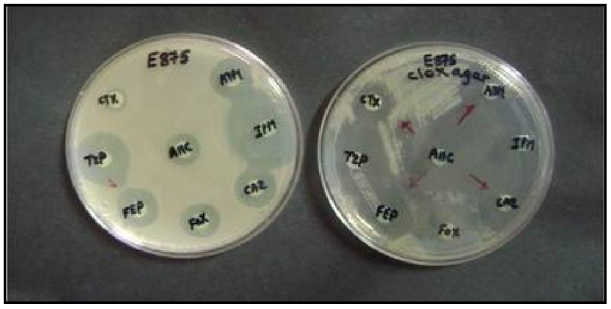

Figure 3: ESBL and AmpC-producing E. coli isolate (E875) detected by cloxacillin agar dilution test Left: MH agar without cloxacillin showing synergy between TZP and FEP indicating ESBL production in AmpC positive isolate, Right: MH agar with cloxacillin (200µg/ml) showing enlargement in the zones of inhibition around beta-lactam indicating AmpC production

Analysis of performance characteristics of phenotypic tests used for detection of pAmpCs among the 17 *bla* $_{AmpC}$ positive isolates showed that cloxacillin disc tests were the most sensitive (82.4%) followed by boronic acid DST and AmpC disc test (76.5% both). Cloxacillin DPT was the most specific (95%). On comparing the inhibitory effect of cloxacillin and boronic acid on AmpC enzymes using the synergy (DST) or potentiation (DPT) effects; it was noted that cloxacillin was a better inhibitor particularly among AmpC-positive *Klebsiellae* spp. All AmpC-positive *E. coli* isolates were successfully detected by both inhibitory agents (Table 3) (Figures 4, 5).

Table 3: Comparison of sensitivities, specificities and predictive values of phenotypic tests for Detection of AmpC-positive isolates

	Cloxacillin disc tests		Boronic acid disc tests		AmpC disc test
	DST	DPT	DST	DPT	
Sensitivity	82.4%	82.4%	76.5%	64.7%	76.5%
Specificity	93%	95.3%	86%	88.4%	86%
PPV	82.4%	87.5%	68.4%	68.8%	68.4%
NPV	93%	93.2%	90.2%	86.4%	90.2%

Comparison of statistical data for the disc tests in ESBL-positive and ESBL-negative groups showed that all disc tests were absolutely sensitive (100%) for detection of AmpCs in ESBL-negative isolates. In ESBL-positive isolates, the sensitivity ranged from 60 to 70%. Specificities of all phenotypic tests were higher in ESBL-positive than in ESBL-negative isolates (Table 4).

Table 4: Comparison of sensitivities, specificities and predictive values of all disc tests for detection of AmpC-positive isolates among ESBL-positive and ESBL-negative

	Cloxacillin disc tests*		APB-disc tests*		AmpC-disc tests	
	ESBL-Positive	ESBL-Negative	ESBL-Positive	ESBL-Negative	ESBL-Positive	ESBL-Negative
Sensitivity	70%	100%**	60%	100%**	60%	100%**
Specificity	100%	90.5%	90.9%	80.9%	90.9%	81%
PPV	100%	77.8%	80%	63.6%	75%	63.6%
NPV	88%	100%	83.3%	100%	83.3%	100%

*Taking highest values of both formats (DDST and DPT).

**Absence of ESBL in the isolate increases the sensitivity of phenotypic tests for detection of AmpC β-lactamase

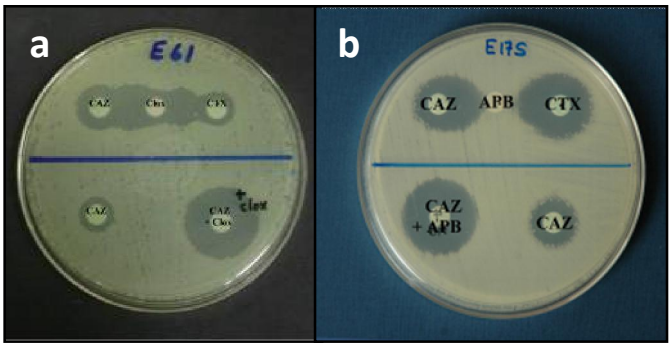

Figure 4 (a, b): Clox disc tests and APB disc tests in Amp-C positive isolates. Synergy between inhibitor \containing discs clox (2µl) /APB and both CAZ and CTX discs in DST (upper halves). Increase in zone diameter by 5 mm or more in CAZ+cloxacillin/APB compared to CAZ only in DPT (lower halves).

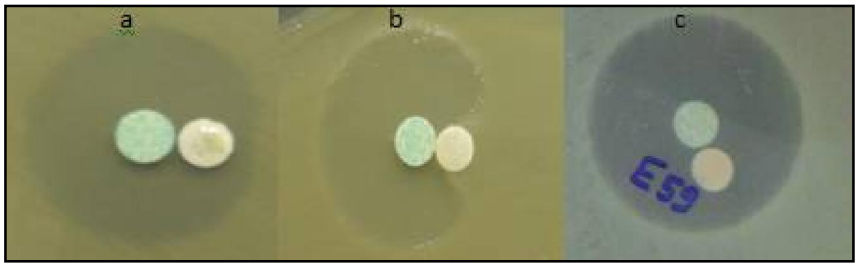

Figure 5 a,b,c: AmpC disc test results: a: isolate showing positive result in the form of flattening, b: isolate showing positive result in the form of indentation and c: isolate showing negative result

4. Discussion

The importance of detecting AmpC-producing isolates is highlighted by data showing high clinical failure rates when AmpC-producing strains of *K.pneumoniae* are treated with cephalosporin agents or the subsequent development of antibiotic resistance in such strains ***(Jacoby, 2009)***. By antibiotic susceptibility testing it might be difficult to distinguish ESBL-producing organisms from plasmid AmpC-producing organisms because of their similar resistance**.** Distinguishing between AmpC- and ESBL-producing organisms has epidemiological significance and has therapeutic importance as well ***(Park et al., 2009)***. In Egypt, one study assessed AmpC production among *Enterobacteriaceae* ***(El-Hefnawy et al., 2008)*** however molecular techniques were not attempted. So, little is known about the genetic background of AmpC-producing isolates in Egypt.

Results of the present study showed that the prevalence of *ampC* genes in 60/178 isolates of the selected species was 28.3% whereas ESBL-producers were detected in 53.3%. AmpC prevalence was lower than that reported by ***El-Hefnawy et al. (2008)*** from Egypt (34% in 50 *K. pneumoniae* and *E. coli* isolates),

but it was equivocal with another study from Singapore (26% in 255 clinical isolates of *E. coli*, *Klebsiella* spp. and *P. mirabilis*). In the first study, molecular techniques were not attempted and they used one phenotypic test, the three dimensional test (TDT), while in the second study both TDT and the multiplex PCR were used ***(Tan et al., 2008).*** These results were much higher than those reported in other parts of the world; in Spain (0.43%) ***(Mata et al., 2009)***, in China (2.79%) ***(Li et al., 2008)*** [22] and in USA (3.3%) ***(Moland et al., 2006).*** This may be due to the differences in the study population and the epidemiological differences in various geographic regions.

The high prevalence of AmpC producers in our study could be explained by 3 points: First, the prevalence of ESBL-production was high (53.3%) and many of our *ampC*-positive isolates were also ESBL-producers (10/17). Previous Egyptian studies have also reported the high prevalence of ESBLs in Egypt; 38% ***(El-Kholy et al., 2003)***, 61% ***(Al-Agamy et al., 2006)*** and 65.8% ***(Fam and El-Damarawy, 2008).*** It has been recently found that infection with pAmpC-producer is frequently associated with a high co-presence rate of ESBLs ***(Park et al., 2009).***

Second, molecular analysis for the ESBL type confirmed the identification of CTX-M in all our ESBL-positive isolates. In another recent Egyptian study by ***Fam et al. (2011),*** the production of CTX-M15 enzyme has been exclusively identified in all their ESBL-positive isolates (83/520). Interestingly, increasing frequency of co-existence of bla_{ampC} with bla_{CTX-M} has also been reported ***(Shahid et al., 2009).***

Third, as 84% of our specimens were collected from inpatients and from patients admitted to ICU, it could be expected that they have been exposed to previous cephalosporin therapy whether empirically or according to the hospital antibiotic policy or due to unjustified courses as reported in a previous Egyptian study ***by El-Kholy et al.(2003).*** Knowing that selective-pressure produced by the extensive use of oxyimino-cephalosporins are among the driving forces of increasing the prevalence of AmpC- production ***(Park et al., 2009)***, the high rate of AmpC production could be anticipated.

It is noteworthy to mention that in our isolates, *ampC* genes were significantly more pervasive in *Klebsiella* spp. than in *E. coli* (43.4% versus 17.6%).This finding was similar to that found in Singapore (30% and 23% respectively) ***(Tan et al., 2008)***. But both rates were much higher than that reported in China (4.3% in *Klebsiella* spp. and 1.9% in *E. coli*) ***(Li et al., 2008).***

In our study 10/60 (16.7%) of the isolates were both AmpC and ESBL-positive by molecular detection methods. This rate was higher than that reported from western parts of the world: 7/117 (6%) in Spain ***(Mata et al., 2009)***, 4/81 (4.9%) in Minnesota in USA ***(Kohner et al., 2009).*** Interestingly, 80% of our ESBL-positive *Klebsiella* spp. was *ampC*-positive. This rate is comparable with that reported in India as ***Vandana and Honnavar (2009)*** detected AmpC in 39/52 (75%) of ESBL-producing isolates of *K.pneumoniae.*

As both ESBL and AmpC β-lactamases may co-exist, therefore their detection is difficult because they mask each other. In the present study, all 10 ESBL-producers in AmpC-positive isolates were successfully identified by using the MDDST, in which FEP and TZP were utilized in approximation, implying that the use of this phenotypic method could overcome the masking effect of AmpC on phenotypic detection of ESBLs. Similar observation has been reported by ***Khan et al, (2008)*** and ***Fam and El-Damarawy (2008).***

Several geographical-related clusters of pAmpC enzymes have been described ***(Odeh et al., 2002).*** These include a North American cluster (MIR-1 and ACT-1), a Central and South American cluster (FOX-1 and FOX-2) and an Asian cluster (CMY-2, CMY-2b, LAT-1, and LAT-2) ***(Shahid et al., 2009).*** Polymerase chain reaction of pAmpC-positive isolates using different primers of pAmpC clusters revealed that CMY was the most predominant gene (70.5%) followed by DHA (23.5%). These results were in agreement with 2 studies from Spain and Singapore in which CMY-2 was found in 66.7% and 79% and DHA-1 in the 25.6% and 21% respectively ***(Tan et al., 2008; Mata et al., 2009).***

Use of a cefoxitin disk is useful in screening for AmpC but it is non-specific. In our study only 17/60 of cefoxitin-resistant isolates had *ampC* genes. Several factors may explain resistance to cefoxitin in the AmpC-negative isolates: First, it may arise due to porin channel alterations and mutations as previously reported in *E. coli* and *Klebsiella* spp. isolates ***(Philippon et al., 2002; Jacoby, 2009)***. Second, cefoxitin-resistance phenotype in *E. coli* can result from over expression 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 ***(Mulvey et al., 2005).*** Third, cefoxitin has been demostrated as a substrate to active efflux pump in clinical isolates ***(Pages et al., 2009).***

In the current study, APB, cloxacillin and AmpC disc test were evaluated for detection of AmpC enzymes versus PCR. Our results showed that cloxacillin was a better inhibitor of AmpC enzymes than boronic acid as it gave higher detection rates both in the DDST and in the DPT. Cloxacillin agar dilution test also gave good results (sensitivity 70.6% and

specificity 93%) but higher sensitivity and specificity were obtained on using ceftazidime disks impregnated with cloxacillin (82.4% and 95.3% respectively). Similar results were reported by ***Tan et al. (2009)***, who compared the use of cloxacillin agar dilution test, boronic acid and cloxacillin disk tests and found that the best results were obtained by using cefpodoxime disk with added cloxacillin (specificity 92%, sensitivity 83%).

False-positive results encountered in cloxacillin and APB-based tests may be explained by the possibility of presence of more AmpC β-lactamase genes that continue to expand beyond those contained in the six families genes covered by PCR ***(Pitout et al., 2009)***. On the other hand, false-negative results may be explained by the fact that the gene may be detected by PCR but is not effectively phenotypically expressed. Boronic acid tests failed to detect many AmpC producers (sensitivity 76.5%) and as reported in previous studies, they were mainly of the DHA1-type ***(Coudron, 2005; Yagi et al., 2005; Pitout et al., 2009; Vandana and Honnavar, 2009).***

Though AmpC disk test did not give the best result in our study, (sensitivity 76% and specificity 86%), it was a simple, convenient and required no special inhibitors. The same remark was also encountered by previous investigators in Brazil ***(Dias et al., 2008)***. This was emphasized by ***Philippon et al. (2002)***, who reported that AmpC disk test was associated with a high rate of false-negative results, which limits its use**.**

An important observation in the current study is that the sensitivity of the phenotypic tests was negatively influenced by the co-presence of ESBL enzyme in the test isolate. In ESBL- positive isolates, the sensitivity of cloxacillin and APB disk tests were 70% and 60% respectively but both tests were absolutely sensitive (100%) in ESBL-negative isolates.Similar finding has been reported by ***Brenwald et al. (2005)*** who noted that the activity of ESBL masked the inhibitory effect of the boronic acid utilized in their study.

In our study resistance to FEP was high in both AmpC-positive (88.2%) and in AmpC-negative isolates (83.7%). This could be explained by the co-presence of ESBL enzyme of the CTX-M type in 10/17 of our AmpC-positive and in 22/43 of the AmpC-negative isolates. High resistance to FEP in AmpC-positive isolates has been also reported lately in Iran (73.5%) and China (57%). ***(Li et al., 2008; Khorvash et al., 2009).*** The high rate of resistance to this drug in our AmpC-negative isolates (86%) could be related to the presence of ESBL enzyme in 51% of those isolates

Resistance to amoxicillin/clavulanate was high among AmpC-positive as well as among AmpC-negative isolates (100% and 97.6% respectively). That was expected among AmpC-positive isolates as AmpC enzymes are known to be poorly inhibited by clavulanic acid ***(Decré et al., 2002)***, whereas tazobactam partially restores susceptibility to piperacillin ***(Philippon et al., 2002)***. Resistance among AmpC-negative isolates was also remarkably high. Apart from the 22 ESBL-producing isolates, most of the non-ESBLs were also resistant to AMC. This may be related to the possibility of emergence of new β-lactamases in these isolates ***(Okesola and Makanjuola, 2009).*** Though the AmpC-positive isolates in our study showed in vitro relatively higher susceptibility to piperacillin/tazobactum combination (59%), however it should not be considered for treatment of infections due to these organisms ***(Yang and Guglielmo, 2007 ; Taneja et al., 2008).***

In the current study we tested the aminoglycoside amikain (AK) and the fluoroquinolone ciprofloxacin (CIP), as they are commonly used in our hospital setting. Our AmpC-positive isolates showed high resistance to CIP (94.1%) and to AK (41.2%). Our results were higher than those reported from Spain, (51.3% and 36.7% respectively) ***(Mata et al., 2009).*** This may be due to the increased use of these agents for common infections such as in urinary and respiratory tract infections ***(Karlowsky et al., 2003).***

Fortunately all our studied isolates retained susceptibility to imipenem. That was in accordance with other previous studies in USA ***(Pai et al., 2007)***, Canada ***(Baudry et al., 2008)*** and in Spain ***(Mata et al., 2009)***. However it should be used prudently as alteration in antibiotic access due to outer membrane porin loss can markedly change the susceptibility profile ***(Kaczmarek et al., 2006).*** Reduced imipenem susceptibility has been reported in porin-deficient clinical isolates of *K. pneumoniae* producing AmpC enzymes CMY-2 ***(Lee et al., 2007)***, CMY-4 ***(Cao et al., 2000)***, DHA-1 ***(Lee et al., 2007)***, or an uncharacterized AmpC-type enzyme ***(Palasulbramaniam et al., 2007).***

In conclusion, this study has revealed the occurrence of plasmid mediated AmpC beta-lactamase producing strains in clinically important bacterial isolates for the first time in our region. ESBL production may mask the phenotypic detection of pAmpC enzymes. Cloxacillin DPT is a simple and reliable method for detection of pAmpCs. MDDST and cloxacillin agar dilution tests may serve as reliable confirmatory tests for detection of ESBLs in AmpC-positive isolates. Occurrence of a large percentage of multidrug resistant strains has been observed. As AmpC beta-lactamase production is frequently accompanied by multidrug resistance, so conjugative dissemination of this AmpC beta -lactamase encoding plasmids may facilitate the spread of resistance

against a wide range of antibiotics among different members of *Enterobacteriaceae*. Imipenem is superior to other antibiotics for the treatment of serious infections due to AmpC beta-lactamase-producing enterobacteria.

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