

Full Length Research Paper

Occurrence and detection of AmpC β -lactamases among some clinical isolates of Enterobacteriaceae obtained from Mansoura University Hospitals, Egypt

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The increasing incidence of β -lactam resistance due to AmpC β -lactamases in Egypt necessitated this study which aimed to evaluate four different phenotypic methods for detection of AmpC β -lactamases among some clinical isolates of Enterobacteriaceae and compare these results with those obtained using polymerase chain reaction. The distribution of five AmpC β -lactamases genes (*AmpC*, *CIT-M*, *Fox-1*, *ACC-1*, *ACT-1*) were determined among the clinical isolates. Among 180 clinical isolates of Enterobacteriaceae, only 57 isolates were AmpC producers by phenotypic methods and 108 were AmpC producers by polymerase chain reaction. Phenotypic methods adapted in this study gave variable results with the most discriminatory results given by direct inoculation of both the enzyme extract and the bacterial culture in the wells. Of these, the best results were given by enzyme inoculation methods where 43 isolates exhibited positive result by this method. The distribution of AmpC β -lactamases gene among the clinical isolates showed that *AmpC* gene predominated in *Escherichia coli*. *Fox* gene was predominantly present in *Enterobacter cloacae* and *E. coli* isolates. *ACT-1* predominated in *E. cloacae*. In contrast, enzymes from *CIT-M* and *ACC-1* group were rarely present in Enterobacteriaceae.

Key words: Betalactam resistance, Enterobacteriaceae, AmpC betalactamases, polymerase chain reaction.

INTRODUCTION

AmpC β -lactamases are clinically important enzymes because they may be associated with the resistance to a wide variety of β -lactam drugs except carbapenems and cefepime (Odeh et al., 2002). AmpC enzymes class β -lactamases hydrolyze penicillins, cephalosporins and cephamycins such as cefoxitin or cefotetan. They can also hydrolyze oxyimino cephalosporins such as ceftazidime, cefotaxime and ceftriaxone and monobactams such as aztreonam but the hydrolysis rates for cefepime, ceftazidime and carbapenems are very low (Coudron et al., 2000). They differ from extended-spectrum β -lactamases (ESBLs) by their ability to hydrolyze cephamycins and they are not affected by available β -lactamase inhibitors (Manchanda and Singh, 2003). Genes of AmpC β -lactamases were encoded on the chromosomes of several

members of the family Enterobacteriaceae (Barlow and Hall, 2002). The bacteria lack or poorly express a chromosomal *bla AmpC* gene that may contain transmissible plasmids that carry acquired genes for AmpC enzymes (Jacoby, 2009). Plasmids with these genes can spread among members of the family Enterobacteriaceae and have been documented in many countries (Coudron, 2003). Plasmids carrying genes for AmpC β -lactamases often carry multiple resistances including genes for resistance to aminoglycosides, quinolones, chloramphenicol, sulfonamide, tetracycline, and trimethoprim as well as genes for other β -lactamases such as CTX-M-3 (Chen et al. 2007).

The ability to detect AmpC enzymes is important to improve the clinical management of infections, but at present, there are no standardized phenotypic screening methods that are readily available for detection of these enzymes. Increased resistance to cefoxitin in the Enterobacteriaceae may be an indicator of AmpC activity, but cefoxitin resistance may also be mediated through altera-

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tions of outer membrane permeability (Hernandez-Alles et al., 2000). Various workers used double disc-synergy and three dimensional extract test for detection of AmpC harboring Gram-negative organisms. Since it was found that the disc diffusion test was non specific and three dimensional extract test is a labour-intensive procedure, there is always a search for newer methods and the aim is to make existing methods more user-friendly to incorporate them in routine diagnostic laboratories (Manchanda and Singh, 2003).

The objective of the present study is to establish a user friendly and effective protocol for the detection of AmpC enzymes. This was attained through a comparative study between the reported phenotypic screening methods and genotypic detection methods using PCR.

MATERIALS AND METHODS

Clinical isolates

A total of 180 clinical isolates of Enterobacteriaceae were collected from different patients distributed among university hospitals in Mansoura, Dakahlia governorate, Egypt. The isolates were collected from urine and sputum samples. These isolates were identified as *Escherichia coli* (87 isolates), *Klebsiella pneumoniae* (61 isolates) and *Enterobacter cloacae* (32 isolates) using standard biotyping methods (Crichton, 1996). All isolates were tested for AmpC β -lactamase production using phenotypic and genotypic methods.

Phenotypic methods for detection of AmpC β -lactamase activity

AmpC enzyme production was estimated by modified three-dimensional test (M3D) with several modifications in previous works (Coudron et al., 2000; Manchanda and Singh, 2003). Cefoxitin (30 μ g) discs were used for AmpC enzyme detection. The methods were divided into two groups:

Methods directly using the bacterial isolates

Lawn cultures of sensitive *E. coli* were prepared on Mueller–Hinton agar and the plates were incubated for 15 min at 37°C, for drying. Then 30 μ g cefoxitin disc was placed on the surface of the inoculated MH agar plate using a sterile forceps. The adopted methods were:

Method 1: Linear slits (3 cm) were cut using a sterile surgical blade 3 mm away from the edge cefoxitin disc. Small circular wells were made on the slits at 5 mm distance, inside the outer edge of the slit, by stabbing with a sterile pasture pipette on the agar surface. The method was modified by making the wells within, outside and approximately at the margin of the zone of inhibition with and without linear slit. The well was loaded approximately with 30 to 40 μ l of 0.5 McFarland bacterial suspension prepared from an overnight broth suspension of the tested isolates in 10 μ l increments until the well was filled to the top. The plate was kept upright for 5 to 10 min until the solution dried, and was then incubated at 37°C overnight (Manchanda and Singh, 2003).

Method 2: A heavy inoculum of the tested isolate was streaked over the agar surface in a linear fashion, beginning 5 mm from cefoxitin disc and moving outwards. The plate was then incubated at 37°C overnight (Shahid et al., 2004).

Method 3: Five to six colonies of the tested isolate were spot inoculated at a distance of 9 to 10 mm from the edge of cefoxitin disc approximately at the zone of inhibition. The plate was then incubated at 37°C overnight (Shahid et al., 2004).

Methods using the enzyme extract (three dimensional extract method)

Lawn cultures of *E. coli* were prepared as described previously and cefoxitin (30 μ g) discs were placed on the inoculated Mueller–Hinton agar plates. Crude enzyme was extracted from nutrient agar slope culture of the tested isolate as described previously (Livermore et al., 1984). A linear slit and a circular well were cut in the agar as described in conventional method 1. The well was loaded with 30 to 40 μ l of the enzyme extract. The plate was kept upright for 5 to 10 min until the solution dried, and was then incubated at 37°C overnight (Manchanda and Singh, 2003).

Two different kinds of result were recorded in phenotypic methods. The isolates showing clear distortion of zone of inhibition of cefoxitin were taken as AmpC β -lactamase producers. The isolates with no distortion were taken as AmpC β -lactamase non-producers.

Detection of ampC genes using PCR

Different ampC β -lactamase genes namely: *AmpC*, *CIT-M*, *Fox-1*, *ACC-1* and *ACT-1*, were detected. Each was detected with gene specific primer, the sequences and the amplicon size of which are listed in Table 1. The method adapted was colony PCR. Briefly, single colony was picked up from our collected isolates and grown on LB broth medium overnight at 37°C. Different amounts (50 to 100 μ l) were plated on selective LB agar plates. The plates were incubated for 16 to 18 h at 37°C. Subsequently, 2 to 4 colonies of each plate were randomly picked with sterile toothpicks and heated in 50 μ l H₂O for 5 min at 95°C. Following centrifugation at 13000 rpm for 1 min, direct colony PCR of the supernatant was performed with Dream Taq™ Green PCR Master Mix (Fermentas). PCR cycling parameters were as follows: initial denaturation at 95°C for 5 min; 40 cycles at 94°C for 20 s, 60°C for 30 s and 72°C for 45 s with a final extension at 72°C for 7 min. An aliquot of each reaction was analyzed on a 1.2% agarose gel (Agarose MP) stained with ethidium bromide and compared with 100 bp plus DNA Molecular Weight Marker to verify the success of the PCR reaction.

Statistical analysis

The phenotypic methods were compared using Chi square test. Statistical calculations were carried out using the InStat-2 computer program (GraphPad Software Inc. V2.04, San Diego, Calif., USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Detection of AmpC activity using different phenotypic methods and PCR indicates that 122 (67.7%) of the tested isolates exhibited AmpC activity. Although 108 (60%) isolates were positive by PCR, only 57 (31.6 %) were positive by different phenotypic methods.

Table 1. Specific amplification primer sets for the tested AmpC β -lactamase genes.

Gene name	Type	Sequence	Amplicon size (bp)
<i>AmpC</i>	Fw	ACACGAGTTTGCATCGCCTG	254
	Rv	CTGAACTTACCGCTAAACAGTGGA AT	
<i>CIT-M</i>	Fw	TGGCCAGAACTGACAGGCAAA	462
	Rv	TTTCTCCTGAACGTGGCTGGC	
<i>Fox-1</i>	Fw	GCAAACCAGCAATACCATCCA	642
	Rv	GCTCACCTTGTTCATCCAGCTC	
<i>ACC-1</i>	Fw	AGCTGTTATCCGTGATTACCTGTCT	248
	Rv	AGCGAACCCACTTCAAATAACG	
<i>ACT-1</i>	Fw	CATGCTGGATCTGGCAACCT	343
	Rv	CTTCAGCGTCCAGCATTCT	

Fw: Forward, Rv: Reverse



Figure 1. Detection of AmpC enzymes production using enzyme extract method. The enzyme extracts of *E. coli* isolate No. 71 and 72 were loaded in wells made at a distance of 10 to 12 mm from cefoxitin disc with linear slits (both enzymes exhibited distortion of inhibition zone "positive result"). The enzyme extract of the *E. coli* isolate No. 69 was loaded in a well made at a distance of 10 to 12 mm from cefoxitin disc without linear slits "it shows positive result".

Regarding the phenotypic methods for detection of AmpC production, enzyme extract method showed the best results where 43 isolates exhibited clear distortion in the inhibition zone of cefoxitin and were considered posi-

tive AmpC producers (Figure 1).

Similar results were shown with the method using broth suspension of the tested isolates where 39 isolates were positive AmpC producers by this method. But, only 25 of

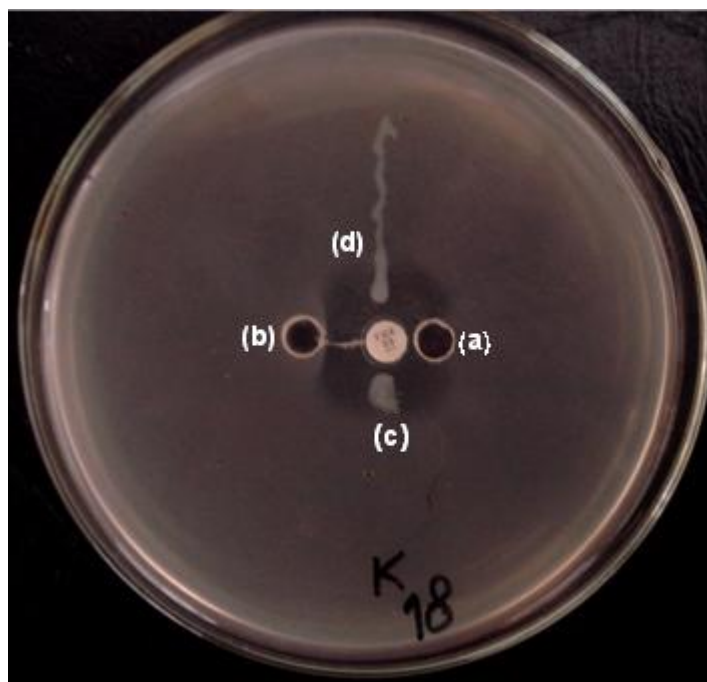


Figure 2. Detection of AmpC enzymes production directly using the *Klebsiella* isolates (no. 18): (a) Bacterial suspension loaded in a well made within the inhibition zone without linear slit (it shows clear distortion of inhibition zone indicating positive results); (b) bacterial suspension loaded in a well made approximately at the margin of the inhibition zone with linear slit (it shows clear distortion of inhibition zone); (c) spot inoculation of the tested isolate at a distance of 6 mm from the edge of the centrally placed cefoxitin disc (it shows no distortion of inhibition zone); (d) streaking of the test organism over the surface of the agar (it shows no distortion of inhibition zone).

Table 2. Statistical analysis of phenotypic methods for detection of AmpC β -lactamase.

Methods		Significance
M3D using the bacterial suspension	enzyme extract	Non statistically significant
Streaking culture	spot inoculation	Non statistically significant
Enzyme extract	streaking culture	Moderate statistically significant (P value = 0.0064)
Enzyme extract	spot inoculation	High statistically significant (P value = 0.0004)
M3D using the bacterial suspension	streaking culture	Low statistically significant (P value = 0.0255)
M3D using the bacterial suspension	spot inoculation	Moderate statistically significant (P value = 0.0024)
All phenotypic methods used for detection of AmpC.		High statistically significant (P value = 0.0006)

M3D: modified three dimensional

these isolates were considered positive by enzyme method (Figure 2).

In contrast, detection of AmpC activity using streaking culture (method 2) and spot-inoculation (method 3) showed poor results with only 23 and 18 isolates showing clear distortion of zone of inhibition of cefoxitin by the two methods, respectively (Figure 2).

The four phenotypic methods were statistically significant from each other as $P = 0.0006$. However, upon comparing the M3D using the bacterial suspension and

the enzyme extract only, it was found that they showed no significant difference. In addition, the method that used streak culture and the one that used spot inoculation were also statistically non significant from each other. Table 2 illustrates the statistical analysis of all phenotypic method.

From the distribution of different AmpC β -lactamase genes among the clinical isolates (Table 3), it was observed that enzymes from the AmpC group were predominantly present in *E. coli* isolates (62%). In contrast, it was

Table 3. Distribution of different *AmpC* genes within the study isolates.

Organism	Number of isolate	AmpC type
<i>E. coli</i>	39	<i>AmpC</i>
	1	<i>AmpC, CIT-M</i>
	3	<i>Fox-1</i>
	4	<i>AmpC, CIT-M, Fox-1</i>
	10	<i>AmpC, Fox-1</i>
<i>Klebsiella pneumoniae</i>	9	<i>Fox-1</i>
	3	<i>Fox-1, ACT-1</i>
	4	<i>ACC-1</i>
	9	<i>ACT-1</i>
<i>Enterobacter cloacae</i>	1	<i>Fox-1, AmpC</i>
	7	<i>Fox-1</i>
	1	<i>ACT-1, AmpC</i>
	2	<i>Fox-1, ACC-1</i>
	11	<i>Fox-1, ACT-1</i>
	1	<i>Fox-1, ACT-1, AmpC</i>
	3	<i>Fox-1, ACC-1, ACT-1</i>

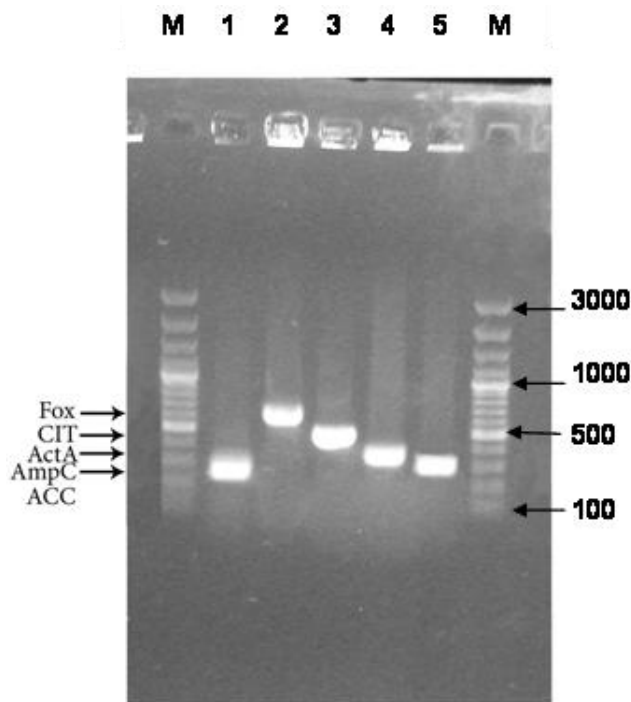


Figure 3. Agarose gel electrophoresis of five *AmpC* β -lactamase genes amplified from different isolates. Lane M is 100 bp DNA marker. Lanes from 1 to 5 are the amplified product of *AmpC* (254 bp), *Fox-1* (642 bp), *CIT-M* (462 bp), *ACT-1* (343 bp) and *Acc-1* (248 bp) respectively. The amplified product from each PCR is indicated on the left, and the size of the marker in base pairs is shown on the right.

present in a minority of *Enterobacter cloacae* isolates (9.3%). *Fox-1* like enzymes was predominantly present in

E. cloacae (89.2%) but was also found in *E. coli* isolates (19.5%) and *Klebsiella pneumoniae* isolates (19.6 %). Enzymes from *ACT-1* predominantly present in *Enterobacter cloacae* (50%) but were also found in *K. pneumoniae* (19.6%). On the other hand, it was found that enzymes from *CIT-M* and *ACC-1* group were rarely present in Enterobacteriaceae as they were found in only 5 and 9 isolates, respectively. The amplification of products for each family member of a particular set resulted in a single amplicon of the predicted size. For example, the amplified product of the *Fox-1* family resulted in large amplicon of 642 bp. In addition, single amplicons of 462 and 343 bp were generated for the family members *CIT-M* and *ACT-1*, respectively. The small amplicon of 254 and 248 were generated for the family members *AmpC* and *ACC-1* respectively (Figure 3).

DISCUSSION

With the spread of *AmpC* producing strains all over the world, it was necessary to estimate the prevalence of these strains in hospitals. Detection of resistant isolates would allow doctors to formulate a policy of empirical therapy in high-risk units where infections due to resistant organisms are much higher. Therapeutic and infection control considerations argue that recognition of plasmid-mediated *AmpC* enzymes is necessary for the average laboratory. *AmpC*-producing isolates may appear to be susceptible *in vitro* to some cephalosporins and aztreonam yet fail to respond if those agents are used, so that a specific test for their presence is necessary (Thomson, 2001).

In this study, we evaluated the accuracy of various phenotypic techniques used to screen for AmpC activity among Enterobacteriaceae.

Thomson and Sanders (1992) first reported three-dimensional test, since then, many modifications have been made. The original method was intricate as it needed customized low-speed rotator and careful loading of slit without spilling the extract. Coudron et al. (2000) modified the original method; they used linear slits instead of circular slits. The method could overcome the need for rotator, but filling these slits without spillage was not solved. Subsequently, Manchand and Singh (2003) modified the procedure; they created a well at the outer edge of the slit and the enzyme extract was put into the well. The method overcame all the problems of three-dimensional test. But the M3D test is laborious, technically demanding requiring careful cutting of slit and well, time consuming and needs experience. Streaking culture and spot inoculation test (Shahid et al., 2004) with direct bacterial colonies were introduced to overcome these problems. But these tests were less sensitive than M3D test.

To determine the placement of wells, more experiments were designed. The zone of inhibition of cefoxitin discs for the inoculated *E. coli* was noted and the wells were punched within the zone of inhibition (5 to 6 mm from the edge of discs), at the zone of inhibition (9 to 10 mm) and outside the margin of the zone of inhibition. More positive results were obtained with wells punched within, and at the zone of inhibition.

Phenotypic methods adapted in this study gave variable results. The most discriminatory results were given by both direct inoculation of the enzyme extract in the wells made in agar and by direct inoculation of the organism in the wells. Of these, the best results were given by enzyme inoculation methods where 43 isolates exhibited positive result by this method. A major draw back of using direct inoculation of bacterial suspension is that it is not specific for AmpC activity where cefoxitin resistance may also be mediated by alterations to outer membrane permeability (Hernandez-Alle et al., 2000). This can explain the presence of 14 isolates exhibited AmpC activity by suspension method, and is considered negative by enzyme inoculation method and PCR.

All phenotypic methods used in this study detect 57 isolates as positive AmpC producer (31.6%) but they are unable to differentiate the different types or families of AmpC β -lactamases (Thomson et al., 1991). Therefore, it is necessary to use other techniques for confirmation and detection of different AmpC gene.

We reported here the use of PCR as simple, rapid and accurate technique for confirmation of phenotypic methods and discrimination of different AmpC gene among different isolates. Although 57 isolates were reported as positive AmpC producer by phenotypic methods, detection by PCR indicated that 108 isolates were positive AmpC producer. This great variation in the results ob-

tained by phenotypic detection and by PCR may be due to the fact that chromosomal AmpC genes present in *E. coli*, are not usually expressed because of the presence of a transcriptional attenuator coupled with a weak promoter (Tracz et al., 2007). Therefore, not all isolates detected as AmpC producers by PCR can be detected by different phenotypic methods.

In conclusion, this report evaluated several phenotypic methods for screening of AmpC enzymes and demonstrated that using enzyme inoculation method accurately detects the AmpC-positive isolates. For confirmation of AmpC-positive isolates and discrimination of different AmpC genes among different isolates, PCR can be used as simple, rapid and accurate technique.

Further studies will be needed to establish the reliability of these methods in detecting AmpC enzymes among other pathogens, such as *Pseudomonas aeruginosa*, as well as among pathogens possibly coproducing extended-spectrum β -lactamases.

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