Journal of Applied Sciences Research, 8(4): 2280-2285, 2012
ISSN 1819-544X
This is a refereed journal and all articles are professionally screened and reviewed

ORIGINAL ARTICLES

A study on occurrence of plasmid mediated AmpC ß-lactamases among gram negative clinical isolates and evaluation of different methods used for their detection

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ABSTRACT

AmpC enzyme is a ß-lactamase that can hydrolyze cephamycins as well as other extended spectrum cephalosporins and not inhibited by clavulanic acid. In gram negative bacteria, this enzyme is chromosomally mediated in most isolates and less commonly plasmid mediated in few isolates. There is no standard phenotypic method for detection of such enzyme. In this study, isolates were tested for the presence of AmpC ß-lactamase. In addition, the sensitivity and specificity, of different phenotypic methods used for its detection, have been evaluated. A number of 139 out of 240 gram negative clinical isolates were screen positive for AmpC ß-lactamases by standard disc diffusion method. These positive isolates were identified using Api 20E identification system and the presence of AmpC ß-lactamases were detected using multiplex PCR, AmpC disc test as well as cloxacillin and phenylboronic acid inhibitor based methods. Five plasmid mediated AmpC enzymes including three CIT and two DHA related types were identified in *Serratia marcescens, klebsiellae* spp. and *Escherichia coli* isolates. To our knowledge, this is the first time to be reported, at least locally, for the presence of plasmid mediated AmpC enzyme in *Serratia marcescens*. The best sensitivity and specificity (100 and 67%, respectively) for detection of such enzyme were recorded for AmpC disc test; it was efficient in detecting all plasmid and most chromosomally mediated AmpC enzymes. In General, AmpC disc test proved to be superior phenotypic method compared to other inhibitor based methods. We recommend routine testing for the presence of AmpC ß-lactamases in any gram negative isolates.

Key words: AmpC ß-lactamases, AmpC disc test, cloxacillin, inhibitor based method, phenylboronic acid.

Introduction

AmpC β-lactamases are cephalosporinases that can hydrolyze cephamycins as well as other extended-spectrum cephalosporins and are poorly inhibited by clavulanic acid (Singhal *et al*., 2005). Furthermore, in a strain with decreased outer membrane permeability, such enzymes can provide resistance to carbapenems (Philippon *et al*., 2002). Chromosomal AmpC enzymes are detectable in *Citrobacter* spp., *Enterobacter* spp., *Morganella morganii, Shigella* spp., *Hafnia alvei*, *Serratia marcescens* and *Yersinia* spp (Jacoby, 2009). On the other hand, Plasmid-mediated AmpC beta-lactamases were reported in *Escherichia coli* and *Klebsiella pneumoniae* nosocomial isolates (Thomson KS, 2001). According to Pérez-Pérez and Hanson (2002), six different groups of plasmid mediated AmpC were identified. These groups include ACC, DHA, CIT and EBC which originated from H*. alvei*, *M. morganii*, *C. freundii* and *E. cloacae*, respectively, as well as FOX and MOX (unknown origins).

There are currently no standardized phenotypic methods for screening and detection of AmpC enzymes (Tan *et al*., 2009). A recently developed multiplex PCR for the detection of plasmid-encoded AmpC genes has proved useful as a rapid screening tool for such genes (Pérez-Pérez and Hanson, 2002). However, since only expressed genes cause emergence of resistance, phenotypic tests seems to be more valuable for their detection (Manchanda and Singh, 2003)**.** Therefore, the present study aims to determine the spread of plasmid mediated AmpC enzymes, among local gram negative isolates, and to evaluate commonly known phenotypic tests used for their detection.

Materials and methods:

A total of 240 gram negative clinical isolates were screened for AmpC β-lactamase production. The source of isolates was urine (114), endotracheal tube, sputum and bronchial lavage (54), stool (26), different body

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fluids (25) in addition to 21 isolates from wound secretions. The study was approved by the ethics committee of the faculty. The number of β-lactamase screen positive isolates was 139 and they were identified by API 20E identification system (biomérieux, Étoile, france) as *Escherichia coli* (39), *klebsiella* spp. (27), *Pseudomonas* spp. (33), *Acinetobacter* spp. (16), *Citrobacter* spp. and *Stenotrophomonas maltophilia* (five isolates of each), *Enterobacter cloacae* (four), two isolates of each of *Flavibacterium oryzihabitans*, *Proteus mirabilis*, *Serratia marcescens* and *Morganella morganii* in addition to two isolates identified as *Burkholderia cepacia* and *Vibrio fluvialis.* These isolates were subjected to multiplex PCR and phenotypic tests for AmpC β-lactamase detection.

Screening for AmpC ß-lactamase:

Screening was done by using the disc diffusion method of Lorian (2005). Isolates with inhibition zones of less than 18 mm, to cefoxitin (30 µg, oxoid**,** Cambridge, UK), were considered to be screen positive isolates (Coudron *et al*., 2003). However because group ACC of plasmid mediated AmpC enzymes may not confer cefoxitin resistance, further screening was carried out using the method of Mirelis *et al*. (2006), in which strains sensitive to cefoxitin and resistant to amoxicillin-clavulanic acid (30/10 µg, oxoid, Cambridge, UK), and either resistant or intermediate resistant to cefotaxime (30 µg, oxoid, Cambridge, UK) or ceftazidime (30 µg, oxoid, Cambridge, UK) and are negative for extended spectrum beta lactamase (ESBL) production, were also included. The phenotypic confirmation disc test, of the clinical laboratory standard institute (CLSI), for detection of ESBL was used. The CLSI test utilized both cefotaxime (30 µg) and ceftazidime (30 µg) each alone and in combination with clavulanic acid (10 µg). An increase of ≥5-mm in zone diameter of cefotaxime or ceftazidime or both in the presence of clavulanic acid was considered a positive result for an ESBL.

Multiplex PCR and sequencing:

Plasmid mediated AmpC ß-Lactamases were detected using polymerase chain reaction (PCR) method of Pérez-Pérez and Hanson (2002). Six primer pairs were used for detection of the six different families of plasmid mediated AmpC ß-Lactamases. Table (1) shows the sequence of the primer pair, used for the detection of each family and the corresponding amplicon size. All primer were synthesized and supplied by fermentas (Carlsbad, Canada). *Citrobacter freundii, Enterobacter cloacae* and *Morganella morganii* clinical isolates were used as positive control strains, for multiplex PCR reaction. For sequencing, PCR Amplification products were generated, purified using qiaquick PCR purification kit **(**Qiagen, Hilden, germany**)** and were sequenced. Sequence alignments and analyses were performed on-line using the BLAST program (www.ncbi.nlm.nih.gov).

Table 1: The sequence of primer pair used for detection of each family and the expected amplicon size.

Enzyme family	primers	Sequence (5´ to 3´, as synthesized)	Expected amplicon size (bp)
MOX	MOXMF MOXMR	GCT GCT CAA GGA GCA CAG GAT CAC ATT GAC ATA GGT GTG GTG C	520
CIT	CITMF CITMR	TGG CCA GAA CTG ACA GGC AAA TTT CTC CTG AAC GTG GCT GGC	462
DHA	DHAMF DHAMR	AAC TTT CAC AGG TGT GCT GGG T CCG TAC GCA TAC TGG CTT TGC	405
ACC	ACCMF ACCMR	AAC AGC CTC AGC AGC CGG TTA TTC GCC GCA ATC ATC CCT AGC	346
EBC	EBCMF EBCMR	TCG GTA AAG CCG ATG TTG CGG CTT CCA CTG CGG CTG CCA GTT	302
FOX	FOXMF FOXMR	AAC ATG GGG TAT CAG GGA GAT G CAA AGC GCG TAA CCG GAT TGG	190

Disc preparation:

In AmpC disc test, sterile filter paper discs were impregnated with 20µl saline-100X Tris-EDTA buffer (sigma-aldrich,St. Louis, USA) in 1:1 ratio, air dried and stored at 2-8°C for further use (Black *et al*., 2005). In inhibitor based method, discs were prepared by applying 20µl of inhibitor solution to each cefoxitin disc; air dried for 30 min and was used immediately.

Two inhibitors were tested: cloxacillin sodium (sigma-aldrich, St. Louis, USA), a ß-lactam inhibitor, as a solution in sterile distilled water (10 µg/ µl) and phenylboronic acid (PBA), a non ß-lactam inhibitor, as 120 mg of PBA (sigma-aldrich, St. Louis, USA) in three ml of dimethyl sulfoxide (sigma-aldrich, St. Louis, USA) to which equal volume of sterile distilled water was then added.

J. Appl. Sci. Res., 8(4): 2280-2285, 2012

AmpC disc test:

The test was done according to the method of Black *et al*. (2005). Briefly, Cefoxitin-susceptible *E. coli* ATCC 25922 was surface inoculated, on Mueller-Hinton agar plate, following the instructions of the standard disc diffusion method; immediately prior to use, the dried AmpC discs were rehydrated with 20 µl of saline and several colonies, of each test organism, were then applied to each disc. The discs were placed on the inoculated surface, with the colonies in contact with the medium, close to 30µg cefoxitin disc. The plates were incubated overnight, in inverted position, at 35°C and were examined for the presence of an indentation or a flattening of the inhibition zone. This indicates enzymatic inactivation of cefoxitin which means positive result.

Inhibitor-Based Methods for Detection of AmpC ß-Lactamases:

The standard disc diffusion method was applied using inoculated Mueller-Hinton agar plates as described earlier. A 30 µg cefoxitin disc, alone or in combination with the test inhibitor, were placed on the surface of the medium and the plates were incubated overnight at 35°C. An increase of 4 or 5 mm, in presence of cloxacillin or PBA, respectively, in the inhibition zone diameter, was interpreted as an AmpC producer organism (Manchanda and Singh, 2003; Tan *et al*., 2009).

Data analysis:

The performances of various phenotypic test methods, for the detection of plasmid mediated AmpC ß-lactamases, were evaluated by comparing their results to that of multiplex PCR method. For each test, sensitivity was defined as the probability that a positive test result is true for the presence of plasmid mediated AmpC ß-lactamases and was calculated as follow: sensitivity= number of true positive isolates by phenotypic test / number of positive isolates by multiplex PCR . On the other hand, specificity was defined as the probability that a negative test result is true for the absence of this enzyme and was calculated as follow: specificity= number of true negative isolates by phenotypic test/ number of negative isolates by multiplex PCR (Lee *et al*., 2009).

Results:

Among the screen positive isolates (139), only 34 isolates (22%) were sensitive to cefoxitin but were either resistant to cefotaxime or ceftazidime or were resistant to both antibiotics. Of these isolates, 17 and 12 were identified as *Escherichia coli* and *klebsiella* spp., respectively.

In addition, applying the multiplex PCR method, only five out of 139 isolates (3.5 %) were recorded to be plasmid mediated AmpC ß-lactamases. These isolates were resistant to cefoxitin and were identified as *Serratia marcescens* (one isolate), *klebsiella* spp. (two isolates) and *Escherichia coli* (two isolates). The results of multiplex PCR and sequencing showed that the origin of AmpC ß-lactamase genes of these isolates was of CIT group genes (CMY-4 genes) in case of *Serratia marcescens* and one *klebsiella* isolate, CIT (CMY-6 gene) in one *Escherichia coli* isolate. However, the genetic origin was of DHA group genes (DHA-1 genes) in one *klebsiella* and one *Escherichia coli* isolates.

In general, comparing the results of different used phenotypic tests to that of multiplex PCR reaction, and according to table 2**,** plasmid mediated AmpC ß-lactamase negative *Stenotrophomonas maltophilia*, by multiplex PCR method, were also negative by the other phenotypic methods. However, in case of *klebsiella* spp. and *Proteus mirabilis*, except few isolates, the majority of the isolates were also undetectable, by phenotypic methods, for AmpC ß-lactamase as in multiplex PCR method. The AmpC ß-lactamases were detectable by PBA, AmpC disc test and cloxacillin method only in 43%, 11% and 5.4%, respectively of *Escherichia coli* PCR negative isolates. None of *Pseudomonas* isolates (33) was identified as AmpC ß-lactamase positive by multiplex PCR or cloxacillin method and only one of these isolates was positive by PBA technique. Dissimilar to that, about 50% of these isolates recorded as AmpC ß-lactamase positive by AmpC disc test. Similar results were observed for *Acinetobacter spp*., where all isolates (16 isolates) were negative for plasmid mediated genes by multiplex PCR as well as by cloxacillin method. However, the AmpC ß-lactamase enzymes in about 63% (10 out of 16) and 56% (9 out of 16), of the isolates, were detectable by PBA and AmpC disc test, respectively. Regarding *Serratia marcescens* isolates (only two), one of them showed positive multiplex PCR genotypic test, for the plasmid mediated AmpC ß-lactamase, but both were negative for all phenotypic methods except AmpC disc test. In almost all *Citrobacter* spp. (five isolates), *Enterobacter cloacae* (four isolates), *Morganella morganii* (two isolates) and *Burkholderia cepacia* (one isolate), the AmpC ß-lactamase enzyme was undetectable by multiplex PCR method but was positive by all other phenotypic methods applied.

As shown in table 3, the overall sensitivity, for detection of AmpC ß-lactamase, was the highest for AmpC disc test (100%) followed by PBA (22%) and cloxacillin (20%); AmpC disc test was capable of detecting all isolates that showed positive result for AmpC enzyme, by multiplex PCR method, while in case of cloxacillin

and PBA only one *klebsiella* out of five different ß-lactamase positive isolates was detected. On the other hand, the specificity of these methods was proved to be the highest with cloxacillin (88%) followed by AmpC disc test (67%) or PBA (61%).

Table 2: Comparison of phenotypic and genotypic methods for detection of plasmid-mediated AmpC β-lactamase.

Tested isolate	*Multiplex PCR*		*AmpC disc test*			*Inhibitor-Based Methods using*			
						cloxacillin		*phenylboronic acid*	
			+	W	-	+	-	+	-
Escherichia coli	+	2	1	1	0	0	2	0	2
	-	37	4	0	33	2	35	16	21
klebsiellae spp.	+	2	2	0	0	1	1	1	1
	-	25	1	0	24	1	24	7	18
Pseudomonas spp.	+	0	0	0	0	0	0	0	0
	-	33	5	12	16	0	33	1	32
Acinetobacter spp.	+	0	0	0	0	0	0	0	0
	-	16	9	0	7	1	15	10	6
citrobacter spp.	+	0	0	0	0	0	0	0	0
	-	5	4	1	0	5	0	5	0
stenotrophomonas maltophilia	+	0	0	0	0	0	0	0	0
	-	5	0	0	5	0	5	0	5
Enterobacter cloacae	+	0	0	0	0	0	0	0	0
	-	4	4	0	0	4	0	4	0
Proteus mirabilis	+	0	0	0	0	0	0	0	0
	-	2	0	0	2	1	1	0	2
Serratia marcescens	+	1	0	1	0	0	1	0	1
	-	1	1	0	0	0	1	0	1
Morganella morganii	+	0	0	0	0	0	0	0	0
	-	2	2	0	0	1	1	0	2
Burkholderia cepacia	+	0	0	0	0	0	0	0	0
	-	1	0	0	1	1	0	1	0
Flavibacterium oryzihabitans	+	0	0	0	0	0	0	0	0
	-	2	1	0	1	0	2	0	2
Vibrio fluvialis	+	0	0	0	0	0	0	0	0
	-	1	0	0	1	0	1	0	1

Table 3: Calculated specificity and sensitivity of different phenotypic methods for detection of AmpC β-lactamase.

calculated sensitivity and specificity	*AmpC disc test*	*Inhibitor-Based Methods using*	
		cloxacillin	*phenylboronic acid*
Total specificity	67	88	61
Total sensitivity	100	20	22
Specificity(no chromosomal AmpC ß-lactamase)	83	94	61
Sensitivity(no chromosomal AmpC ß-lactamase)	100	25	25

Discussion:

To our knowledge, no one reported locally the true rate of existence of AmpC ß-lactamase positive strains among gram negative isolates. Generally, Very few reports are available about the rate of existence of these enzymes and similar to Empel *et al*. (2008), the percentage of AmpC ß-lactamase positive strains recorded in the present study, among the collected gram negative isolates, was only 3.5%.

On the other hand and in agreement with Alvarez *et al*. (2004), *Escherichia coli*, *Klebsiella pneumoniae* and *P. mirabilis* enteric isolates that lack chromosomal AmpC ß-lactamase (Livermore, 1995; Bonomo *et al.*, 2006; Jacoby, 2009), showed plasmid mediated AmpC ß-lactamases in only 6% of them. However, the results are dissimilar to that of tan *et al*. (2008), Patel *et al*. (2010) and Ingram *et al.* (2011) who reported higher rates of about 26, 20 and 30%, respectively. It has to be noted, and to our knowledge, that it is the first time to detect plasmid mediated AmpC ß-lactamases in *Serratia marcescens* clinical isolates. Being a nosocomial microorganism (Yatsuyanagi *et al.*, 2006), and dissimilar to Thomson (2010) opinion, detection of such plasmid mediated enzyme resistance is highly recommended in *Serratia marcescens* clinical isolates.

Because of the lack of standard CLSI methods available for AmpC enzyme detection, different phenotypic methods; including AmpC disc test and inhibitor based methods using cloxacillin or PBA, have been evaluated. In agreement with Singhal *et al*. (2005), the best overall results were observed with AmpC disc method. However, the low specificity recorded may be referred to the interference of chromosomal encoded AmpC ß-lactamases in *Escherichia coli*, *Pseudomonas* spp., *Acinetobacter* spp., *citrobacter* spp. *Enterobacter cloacae, Proteus mirabilis*, *Serratia marcescens* and *Morganella morganii* (Jacoby, 2009). However, with all phenotypic methods tested, the detection of chromosomal AmpC b lactamases was not fulfilled in all of the tested isolates. The same was reported by Manchanda and Singh (2003).

For more accurate determination of specificity and sensitivity, of the different tested phenotypic methods, only test microorganisms lacking chromosomal resistance were selected. The results indicated increase in sensitivity and specificity of all methods used. But, the highest values still recorded for AmpC disc method. The same was recorded recently by Ingram *et al.* (2011).

On the contrary to Mirelis *et al.* (2006), who reported 100% sensitivity for cloxacillin method, the sensitivity recorded, for cloxacillin and PBA methods, didn't exceed 25%. This may be explained to be due to emergence of cloxacillin resistance during 5 years ago as a result to the use of such ß-lactam as an inhibitor.

In PBA method, using a cut-off value of 4mm increase in the inhibition zone diameter instead of 5mm, increased the sensitivity of the test but decreased the specificity. Similar observation was reported by Lee *et al.* (2009) and Tan *et al*. (2009). In addition, by this method 26% false positive results, in case of *klebsiella* isolates, were recorded. This could be due to the presence of another type of enzymes namely KPC-type β-lactamase, an enzyme also sensitive to PBA (Thomson, 2010). The PBA method failed to detect DHA type β-lactamase enzyme, in one *Escherichia coli* and one *klebsiella* isolate. Similar results have been reported by Lee *et al.* (2009), an observation that deserves further investigation.

Conclusion:

In conclusion, as the universal existence of AmpC ß-lactamase enzyme was reported, the presence of such enzyme was also detectable in local clinical isolates. The co-existence of both chromosomal and extra chromosomal ß-lactamases should be tested for. The most reliable phenotypic method, for detection of such enzyme, was the AmpC disc technique, which proved to be suitable method for detection of both plasmid as well as chromosomal AmpC ß-lactamases. The method is characterized by simplicity, convenience, and accuracy (Black *et al.*, 2005).

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