Egyptian Journal of Medical Microbiology Volume 28 / No.3 / July 2019 105-110 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Phenotypic and genotypic characterization of plasmid-mediated AmpC β-lactamases in *Klebsiella pneumoniae* isolates from Mansoura University Children Hospital

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Key words:
K. pneumoniae, AmpC β-lactamase, plasmid, pediatric patients.

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ABSTRACT

Background: *The plasmid mediated AmpC β-lactamases (pAmpCs) production is one of the most essential mechanism of drug resistance in Klebsiella pneumoniae (K. pneumoniae).* ***Objective:*** *Phenotypic and genotypic investigation of plasmid mediated AmpC β -lactamase production in K. pneumoniae and its antimicrobial profile among pediatric patients.* ***Methodology:*** *Ninety-two K. pneumoniae isolates were tested for pAmpC β-lactamase production by cefoxitin disk diffusion test. Multiplex polymerase chain reaction (multiplex PCR) was done to detect plasmid mediated AmpC β-lactamases genes in the cefoxitin resistant isolates. Antimicrobial susceptibility testing was done to pAmpC β- lactamase positive isolates by means of Kirby Bauer disc diffusion method.* ***Results:*** *Out of the 92 K. pneumoniae isolates, 37 strains (40.21%) were resistant to cefoxitin. Plasmid mediated AmpC genes were detected in 67.56% (25/37), ten isolates (40%) had CMY gene, 8 isolates (32%) had DHA gene and 7 isolates (28%) had FOX gene. All of the 25(100%) isolates were resistant to co-amoxiclav, ceftazidime, cefotaxime, cefuroxime, ceftriaxone and cefoxitin and 7 isolates (28%) showed resistance against imipenem with variable susceptibilities to the other antibiotics.* ***Conclusion:*** *Monitoring of Plasmid mediated AmpC β-lactamase producing K. pneumoniae is important because of its high prevalence among pediatric patients. Imipenem was the drug of choice for treatment of such infections.*

INTRODUCTION

The plasmid mediated AmpC β-lactamases production has become one of the most essential mechanism of drug resistance in *K. pneumonia* [1]. AmpC β lactamases are enzymes belonging to group I of Bush[2] or class C of Ambler[3]. A wide variety of β- lactam drugs including penicillins, cephalosporoins are hydrolysed by AmpC β-lactamases. Moreover, monobactams as aztreonam and oxyimino cephalosporins as ceftriaxone, ceftazidime, and cefotaxime can be hydrolyzed by AmpC β-lactamases. These enzymes are also resistant to cephamycins such as cefoxitin or cefotetan and β lactamase inhibitors like clavulanic acid, sulbactum and tazobactum to which class A enzyme; extended spectrum β lactamases (ESBLs) are sensitive. They are sensitive to cefepime, cefpirome and carbapenems[4].

AmpC β-lactamases were mediated by chromosomal genes and since the late 1980s they have spread through plasmid genes and nowadays have been considered a major clinical risk. The detection of plasmid-mediated AmpC β-lactamases (pAmpCs) has become crucial because of its emergence and spread in *Klebsiella spp.* with naturally deficient chromosomal cephalosporinase[5].

AmpC enzymes are found in periplasm, with molecular mass of 34 to 40 kDa and isoelectric points of >8.0[6]. Plasmids carrying these genes can spread among members of *Enterobacteriaceae* family which could be recognized in many countries. Plasmids carrying genes encoding AmpC β-lactamases frequently carry several resistant genes, such as genes for resistance to quinolones, aminoglycosides, chloramphenicol, tetracycline, sulfonamide, and trimethoprim and genes for other β-lactamases as *CTX-M-3*[7].

Plasmid mediated AmpC β-lactamase producing *K. pneumoniae* has been responsible for nosocomial outbreaks of infections and colonization. So, the recognition of pAmpC β-lactamases is essential for the improvement of the clinical use of antibiotics[8]. The present study aimed to investigate the genotype of pAmpC β-lactamases of *K. pneumoniae* isolated from clinical samples in Mansoura University Children Hospital, with recognition of their antibiotic resistance.

METHODOLOGY

Study design

Cross sectional descriptive study was done on 925 children within ten months from June 2017 to March

2018. All patients, in this study, were admitted in Mansoura University Children Hospital, Egypt.

Ethical approval

The study was accepted by Institutional Review Board of the Faculty of Medicine, Mansoura University; code number: R. 19.03.446.

Clinical samples and bacteriological examination

The collected specimens such as cerebrospinal fluid (CSF), blood, pus, ear swabs and different tips were cultured on blood agar, chocolate agar, and MacConkey's agar, while urine samples were cultured on CLED agar. *K. pneumoniae* isolates were recognized by film stained by Gram, colonial morphology and biochemical reactions using API20E (Biomerieux, France) [9].

Screening for AmpC ß-lactamase-producing isolates

K. pneumoniae isolates were screened using cefoxitin 30 µg (Oxoid, England) by Kirby-Bauer disk diffusion test. Isolates showing a diameter zone of inhibition measuring ≤18 mm could be considered AmpC ß-lactamase producers, in reference to the CLSI Antimicrobial Susceptibility Testing (AST) Standards [10].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was done by means of Kirby Bauer disc diffusion method[11]. Bacterial suspensions were adjusted in reference to the 0.5 McFarland's turbidity standard. The suspension was streaked on the Mueller Hinton agar plate (Bio-Rad, USA). The antibiotic discs of amikacin, gentamicin, co-amoxiclav, cefotaxime, ceftriaxone, ceftazidime, cefepime, cefuroxime, cefoxitin, ciprofloxacin, levofloxacin, and imipenem (Oxoid, England) were used for the susceptibility testing. The diameter of each zone of inhibition was measured after overnight incubation at 35-37°C. Result of each isolate were recognized as resistant, intermediate or sensitive to the antibacterial disc according to the Clinical and Laboratory Standard Institute (CLSI) manual 2013[11].

Detection of pAmpC β-lactamase genes using multiplex PCR

Different genes of pAmpC β-lactamases were distinguished. The primer sequences and the size of each[12,13] are listed in table 1. The colony PCR method was used [14]. In brief, a colony was taken and put in LB broth medium (Sigma, USA) at 37°C overnight. From 50 to 100 µl were cultured on selective LB agar. The cultures were incubated at 37°C for 16 to 18 hours. Then, 2 to 4 colonies from the culture were taken at random to be heated in 50 µl H2O at 95°C for 5 min, then centrifuged for 1 minute at 13000 rpm. Next, Dream Taq™ Green PCR Master Mix (Fermentas, USA) and the specific-group primers were added to the supernatant. PCR steps were: initial denaturation of 5 min at 95°C; 40 cycles for 20 seconds at 94°C, 30 seconds at 60°C and 45 seconds at 72°C and final extension of 7 minutes at 72°C. From each reaction, an aliquot was evaluated on a 1.2% agarose gel stained with ethidium bromide and compared with 100 bp DNA ladder (Thermo Fisher Scientific, USA).

Table 1: Primer sequences of pAmpC β-lactamases target genes

Target gene	Primer sequence (5'→3')	Fragment length (bp)
DHA	F: GGAATTCCACGGAAGGTTAATTCTGATG R: GCAAGCTTTTATTCCAGTGCACTCA	1140
EBC	F: TCGGTAAAGCCGATGTTGCGG R: CTTCCACTGCGGCTGCCAGTT	302
MOX	F: GCTGCTCAAGGAGCACAGGAT R: CACATTGACATAGGTGTGGTGC	520
FOX	F: AACATGGGGTATCAGGGAGATG R: CAAAGCGCGTAACCGGATTGG	190
CIT	F: TGGCCAGAACTGACAGGCAAA R: TTTCTCCTGAACGTGGCTGGC	462
CMY	F: TGG CCG TTG CCG TTA TCT AC R: CCC GTT TTA TGC ACC CAT GA	870

RESULTS

Of the 925 clinical samples processed, 92(9.66%) isolates were *K. pneumoniae*. Out of the 92 *K. pneumoniae* isolates, 37 strains were resistant to cefoxitin, with a rate of 40.21% (37/92). Plasmid mediated AmpC genes was detected in 67.56% (25/37); table 2. *CMY* gene was detected in 10 of 25 pAmpC-positive isoalates (40%), the *DHA* gene was found in 8 isolates (32%) and *FOX* gene was detected in 7 isolates (28%). The *EBC*, *MOX* or *CIT* genes were not detected; table 3 and figure 1. AmpC β- lactamase producing *K. pneumoniae* were isolated from 15 (60%) male and 10(40%) female patients and there were 11(44%) neonates,8(32%) infants and 6(24%) children; table 4.

Out of the 25pAmpC β-lactamase producing *K. pneumoniae* isolates, 10(40%) were obtained from blood, 8(32%) from urine and 7(28%) from pus; table 5.

All of the 25(100%) isolates were resistant to co-amoxiclav, ceftazidime, cefotaxime, cefuroxime, ceftriaxone and cefoxitin. There were7(28%) isolates which showed resistance against imipenem. The 25 pAmpC β- lactamase positive isolates showed variable susceptibilities to the other antibiotics; table 6.

Table 2: Frequency of pAmpC β-lactamase producing *K. pneumoniae*

	AmpC Screening by cefoxitin disc		Plasmid AmpC detection by multiplex PCR	
	No	%	No	%
Positive	37/92	40.2	25/37	67.56
Negative	55/92	59.79	12/37	32.44

Table 3: Distribution of *PAMPC* genes among the pAmpC β-lactamase producing *K. pneumoniae* (No=25)

PAMPC gene type	Number of isolates	%
CMY	10	40
DHA	8	32
FOX	7	28

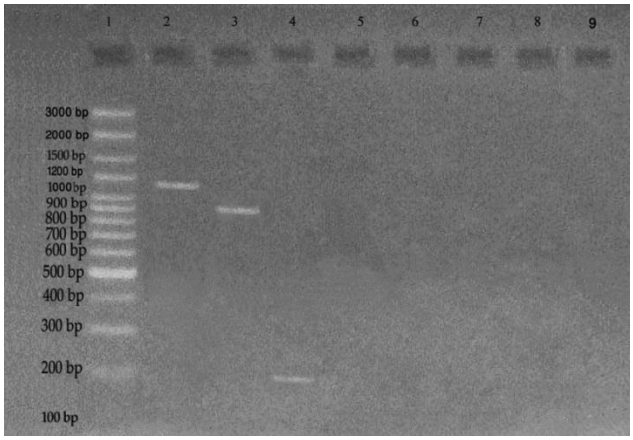

Fig. 1: Lanes 1: 100 bp DNA size marker, lane 2: *DHA* gene (1140 bp), lanes 3: *CMY* gene (870bp) lane 4: *FOX* gene (190bp).

Table 4: Demographic distribution of patients infected with pAmpC β-lactamase producing *K. pneumoniae* (No=25)

	No	%
Gender		
Male	15	60
Female	10	40
Age groups		
Neonates	11	44
Infants	8	32
Children	6	24

Table 5: Source distribution of pAmpC β-lactamase producing *K. pneumoniae* (No=25)

Clinical Specimens	No	%
Blood	10	40
Urine	8	32
Pus	7	28

Table 6: Antimicrobial resistance profile of PAmpC β –lactamase producing *K. pneumoniae* (No=25)

Antibiotics	Resistant No (%)	Intermediate No (%)	Susceptible No (%)
Co-amoxiclav	25(100)	0(0)	0(0)
Ceftazidime	25(100)	0(0)	0(0)
Ceftriaxone	25(100)	0(0)	0(0)
Cefotaxime	25(100)	0(0)	0(0)
Cefuroxime	25(100)	0(0)	0(0)
Cefoxitin	25(100)	0(0)	0(0)
Cefepime	20(80)	0(0)	4(20)
Ciprofloxacin	20(80)	2(8)	3(12)
Levofloxacin	18(72)	3(12)	4(16)
Gentamicin	19(74)	0(0)	6(24)
Amikacin	17(68)	4(16)	4(16)
Imipenem	7(28)	3(12)	15(60)

DISCUSSION

Serious infections caused by *K. pneumonia*e in pediatric wards have been reported[15]. Antibiotic resistance is a worldwide alarm, and wide-spectrum β-lactam resistance caused by AmpC β-lactamases is a rising international problem[16]. *K. pneumoniae* pAmpC β-lactamases were first identified in 1989 in South Korea[17]. From that time, 1 or 2 new pAmpC β-lactamase enzymes have been recognized yearly, which are highly expressed and can be transmitted to other bacteria throughout either plasmid conjugation or chromosomal transformation, to trigger antimicrobial resistance[18]. Serious infections caused by pAmpC β-lactamase-producing *K. pneumoniae* makes anti-bacterial therapy more difficult[19].

Regarding to cefoxitin disk, it is helpful in AmpC screening although it is not specific. In our research, out of the 92 *K. pneumoniae* isolates, 37 strains were resistant to cefoxitin, with a positive rate of 40.21% (37/92) but plasmid mediated AmpC genes was detected in 67.56% (25/37). Several reasons could elucidate cefoxitin resistance among AmpC-negative *K. pneumoniae*: First, it may be due to porin channel alterations and mutations. Second, cefoxitin-resistance can be caused by the chromosomal *AMPC* gene over expression as a result of the promoter or attenuator regions mutations leads to change of bacterial cell permeability toward cefoxitin. Third, cefoxitin may activate efflux pump in the isolates[20].

In the present work, the prevalence of plasmid mediated *AMPC* genes among the 37 cefoxitin resistant isolates was 67.56% (25/37). Similarly, Barwa et al[14] reported 60% frequency of pAmpC β-lactamase producing *K. pneumoniae* by multiplex PCR at Mansoura University Hospital, Egypt. Plasmid-mediated AmpC prevalence in our study was higher than those reported by Fam et al[21] and El-Hefnawy et al[22] from Egypt (28.3% and 34%, respectively), and that reported by Tan et al[23] from Singapore (26%). In the first study, multiplex PCR was used, while in the second one, three dimensional test was used, and in the third work three dimensional test and the multiplex PCR together were applied. Other findings reported in other parts of the world; in Spain (0.43%)[24], in China (2.79%)[25] and in USA (3.3%)[26] were much lower than our results. The study population variations and the epidemiological variations of different geographic areas may make these different results.

In the present work, multiplex PCR was used to detect plasmid mediated *AmpC* genes among cefoxitin resistant *Klebsiella pneumoniae* isolates. One of the most significant diagnostic means is multiplex PCR which can recognize the most prevalent pAmpC genes. PCR is a rapid, accurate, low-cost. Moreover, it facilitates epidemiological screening the appearance and spread of these genes[27]. The PCR can be used also for phenotypic methods verification and differentiation of various *AMPC* genes amongst clinical isolates[14].

In this work, *CMY* gene was detected in 10 among 25 pAmpC-positive strains (40%), *DHA* gene was found in 8 isolates (32%) and *FOX* gene was detected in 7 isolates (28%). Our result was supported by Peng and colleagues[28] who reported that the most common gene type of AmpC ß-lactamase in the world is *CMY* one. Also, Fam et al[21] in Egypt reported that 76.5% of the AmpC-positive isoalates had *CMY* genes and 23.5% of them had *DHA* genes . However, other results done in China[12] and Egypt[27] reported that the most commonly detected plasmid mediated AmpC β-lactamase genes were *DHA* .

In our study, AmpC β-lactamase producing *K. pneumoniae* were isolated from 15(60%) male and 10(40%) female patients. Similar results were obtained from other studies in Nigeria[29], Pakistan[30] and Korea[31] (60%, 66.7% and 66.3% prevalence of AmpC b-lactamases producers in males, respectively). In our study, AmpC β- lactamase producing *K. pneumoniae* were isolated from 11(44%) neonates,8(32%) infants and 6(24%) children. Other studies reported highest occurrence rate of AmpC ß-lactamase-producing *K. pneumoniae* in neonates[30].

Out of the 25pAmpC β-lactamase producing *K. pneumoniae* isolates in this research, 10(40%) were obtained from blood, 8(32%) from urine and 7(28%) from pus. Similar finding was reported by Younas et al[30] who found that 46% of AmpC β-lactamase-producing *K. pneumoniae* were isolated from blood. Another study done in Turkey by Yilmaz et al[32] found that 86.8%, 7.7%, and 5.5% of AmpC β-lactamase-producing *K. pneumoniae* isolates were from urine, blood cultures and other body sites respectively.

In this research, all of the AmpC β-lactamase-producing *K. pneumoniae* isolates were resistant to co-amoxiclav, ceftazidime, cefotaxime, cefuroxime, ceftriaxone and cefoxitin. Similar results were obtained in another study in Pakistan[30]. conversely, another research reported that among AmpC β-lactamase producing isolates, 18.2%, 67.5%, 81.0% and 63.0% were resistance to coamoxiclav, cefuroxime, cefotaxime and ceftazidime respectively[33]. As regard the fourth generation cephalosporin (cefipeme), 80% of AmpC β-lactamase-producing isolates were resistant to it. Lui et al[12] reported 78.6% cefepime resistance and due to this high resistance, they did not recommend the fourth generation cephalosporins for treatment of AmpC-producing *K. pneumoniae* . AmpC-producing *K. pneumonia* isolates, in our study, were highly resistant to ciprofloxacin, levofloxacin, Gentamicin and amikacin (80%, 72%, 74% and 68% resistance, respectively). Similar results were reported in two studies done in tertiary hospitals of China[12] and in a children hospital of Pakistan[30]. However, results reported from a study in Spain[24] were lower than our result. The different sensitivity patterns from one study to another may be due to variable utilization of these antibiotics for common infections[34].

In our study, 28% of the AmpC β-lactamase-producing *K. pneumoniae* were imipenem resistant. Also, other studies reported imipenem resistance of 14.3%[12] and 25.4%[30]. On the contrary, All of the organisms were susceptible to imipenem in other studies[21,24]. Carbapenems are recommended for treating the infection caused by AmpC-producing *K. pneumoniae* because of their stability against ß-lactamases and high affinity to penicillin-binding proteins[35]. Nevertheless, imipenem must be prescribed cautiously to avoid loss of outer membrane porin that could change the antibiotic access with marked change of the sensitivity pattern[36-37]. Resistance to imipenem in AmpC ß-lactamase *K. pneumoniae* producers were reported because of outer membrane porin deficiency[38].

CONCLUSION

It is recommended to monitor *K. pneumoniae* producing AmpC β-lactamases. Imipenem was the drug of choice to treat infections caused by AmpC-producing *K. pneumoniae*. However, imipenem must be prescribed cautiously to avoid loss of outer membrane porin that could change the antibiotic access with marked change of the sensitivity pattern.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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