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Research article

Prevalence of blaACC and blaMOX genes in *Klebsiella pneumonia* isolated from Al-Rumetha hospital in Al-Muthanna Province, Iraq

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ABSTRACT

Ampicillin hydrolyzing class C β-lactamase (AmpC) that confer resistance to extended spectrum cephalosporins and never inhibited by β- lactamase inhibitors. Results of this study confirmed that 37 (41.1%) isolates of *Klebsiella pneumonia* from 90 isolates tested for cefoxitin susceptibility by disk diffusion method. AmpC β-lactamase producers obtained from clinical isolates; 13 (35.1%) from urine, 10 (27%) from blood, 10 (27%) from wound, and 4 (10.8%) from vagina. AmpC β-lactamase producer isolates were confirmed in for cefoxitin resistance 37 *K. pneumoniae* isolates by modified three dimensional test and AmpC disk test. From 90 isolates, plasmid encoded AmpC genes were detected by multiplex PCR in 30 (33.3%) of the *K. pneumoniae* isolates. Of these, plasmid encoded AmpC genes belonging to the MOX family were detected in 14/30 (46.7 %) isolates. Gene of the family ACC type was present in 2/30 (6.7%) isolates. While, the other isolates 14/30 (46.7%): 7(23.3%), 4(13.3%), 2(6.7%) and 1(3.3%), isolates contained another families like DHA, FOX, CIT and EBC, respectively.

Keywords: **AmpC disk test, blaACC, blaMOX, *Klebsiella pneumoniae,* Multidrug resistance.**

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INTRODUCTION

Klebsiella is an opportunistic pathogen and has been associated with various infections such as urinary tract infection (UTI), septicemia, wound infection, respiratory tract infection and diarrhea [1]. β- lactams are the most widely used antibiotics in clinical medicine and resistance to β-lactams may become a severe threat because they have low toxicity and are used to treat abroad range of infections [2]. Cephalosporins, fluoroquinolones, aminoglycosides and carbapenems are effective for treating infections caused by *K. pneumoniae* [3]. The prevalence of multidrug resistant gram negative bacteria has increased continuously over

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the past few years, and bacterial strains producing ampicillin-hydrolyzing Class C β-lactamase (AmpC) and/or extended spectrum β- lactamases (ESBLs) are particular concern [4]. AmpC β-lactamases are clinically significant effect because they may confer resistance to penicillins, cephalosporins, oxyimino-cephalosporins (e.g. ceftriaxone, cefotaxime and ceftazidime), cephamycins (e.g. cefoxitin and cefotetan), and monobactams. AmpC β-lactamase activity is not affected by the ESBL inhibitor clavulanic acid [5]. Several studies were evaluate the antibacterial effect of antibiotics on Gram negative bacteria [6,7]

Genes for AmpC β -lactamases are commonly found on the chromosomes of several members of the family Enterobacteriaceae, including class C β-lactamase originating from *Hafnia alvei* (ACC-1),*Citrobacter freundii* origin for (CIT), EBC originating from *Enterobacter cloaca*, AmpC enzyme resist to moxalactam (MOX) and origin of this gene unknown. β-lactamase discovered at dhahran hospital in Saudi Arabia (DHA)and originating from *Morganella morganii*, another AmpC enzyme act on cefoxitin (FOX) and *Aeromonas caviae* is likely source of FOX derivative *ampC* gene [8]. The aims of the current study, Isolation and identification of *K. pneumoniae* isolates from clinical samples, detection of AmpC β- lactamase producing isolates by using polymerase chain reaction (PCR) and comparing the presence of AmpC β-lactamases by phenotypic methods. Moreover, the goal of present study is Studying the prevalence of β-lactam resistance in clinical isolates of *K. pneumoniae* and Evaluating the dissemination of blaACC and blaMOX genes among AmpC producing isolates by using PCR technique.

MATERIALS AND METHODS

Collection of specimens

Ninety clinical samples were collected from Al-Rumitha hospital in Al-Muthanna province from May to July 2015. The samples were included urine (30), wound swabs (30), vagina (7) and blood (23). The samples were cultured on MacConkey agar and incubated for overnight at 37 ^{o}C then identified by biochemical test according to MacFaddin [9] then reidentified by API-20E kit (Bio-Mereix, France) the manufacture's instructions of produced company was followed.

Antibiotic susceptibility testing

Antibiotic susceptibilities were determined by the standard disk diffusion test for the following antibiotics (Himedia, India): Ampicillin (10 µg), Amoxicillin (25 µg), Amoxiclav (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), Cefoxitin (30 µg), Ceftazidime (30 µg), Cefepime (30 µg), Cefixime (5 µg), Carbenicillin (100 µg), piperacillin-tazobactam (100/10 µg), Imipenem (10 µg), Aztreonam (30 µg), Amikacin (30 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Tetracycline (30 µg), co-trimoxazole (10 µg). *E. coli* ATCC 25922 was used as the control and the results were interpreted as per CLSI criteria [10]. Isolates showing resistance to cefoxitin (≤ 18mm inhibition zone diameter) were considered as initially AmpC β- lactamase producers [11].

Detection of AmpC β -lactamases

Modified three dimensional test (MTDT)

This test was carried out according to Manchanda and Singh [12] and Parveen *et al.* [13].

AmpC Disk Test

This test was carried out according to previous study [13, 14].

Molecular detection of AmpC -lactamase

Isolation of plasmid DNA by genomic DNA Mini kit

Plasmid DNA was purified by using the genomic DNA Mini kit, the manufacture's instruction of produced company was followed (Geneaid, korea).

Preparation of primers suspension

The DNA primers were resuspended by dissolving the lyophilized primers after spinning down with TE buffer depending on manufacturer instructions as stock suspension. Working primer tube was prepared by diluted with TE buffer (Promega, USA). The final picomoles depended on the procedure of each primer. All primers were synthesized and supplied by (Biocorp, Canada).

Detection of blaACC and blaMOXgenes by PCR

Multiplex PCR was carried out to detect two genes included *bla*ACC and *bla*MOX using specific primers (**table 1**). PCR mixture set up in 20 µl total volume consisting of 5 µl of lyophilized AccuPower® PCR PreMix (Bioneer, korea), 10 pico/ µl of each primer and 5µl of DNA template. The amplification of bla genes were run under the following conditions in (**table 2**).

Table 1. PCR Primers that used for detecting *blaACC* and *blaMOX* genes in *K. pneumonia*.

Primer's name	Gene	Oligo sequence (3'-5')	Product size (bp)	Reference
ACC-F ACC-R	*bla*ACC	F: AAC AGC CTC AGC AGC CGG TTA R: TTC GCC GCA ATC ATC CCT AGC	346	Perez-Perez and Hnson, [25]
MOX-F MOX-R	*bla*MOX	F: GCT GCT CAA GGA GCA CAG GAT R: CAC ATT GAC ATA GGT GTG GTG C	520	

Table 2. Duration of steps of PCR thermocycling.

Cycle number	Temperature (^{o}C)/ Time					Multiplex gene
	Final extension	Cycling condition			Initial denaturation	
		extension	annealing	denaturation		
25	72/5 min	72/1 min	61/30 sec	95/30 sec	95/2 min	***bla*ACC**
25	72/5 min	72/1 min	61/30 sec	95/30 sec	95/2 min	*la*MOX

RESULTS

The result of present study showed that 37 (41.1%) isolates of K. pneumoniae were isolated from 90 collected isolates. β-lactam resistant *K. pneumoniae* isolates were tested for cefoxitin susceptibility by disk diffusion method . AmpC β-lactamase producers obtained from clinical isolate: 13 (35.1%) from urine, 10 (27%) from blood, 10 (27%) from wound, and 4 (10.8%) from vagina (table 3). AmpC β-lactamase producer bacteria that attributed as cefoxitin resistance (37 *K. pneumoniae* isolates) was confirmed by the modified three dimensional test and AmpC disk test (fig 1). A clear distortion of the zone of inhibition of cefoxitin was observed in all 37 (100%) isolates that resistant to cefoxitin. Plasmid mediated AmpC β-lactamase genes were detected by multiplex PCR in 90 AmpC β lactamase producer isolates.

Table 3. Distribution of cefoxitin resistance *K. pneumoniae* isolates in different clinical samples. a, variable is constant. Chi-Square test cannot be performed. b, 0 cells (0.0%) have expected frequencies less than 5 isolates; The minimum expected cell frequency is 9.3.

Sample	No. of observed cells	No. of expected cells	Residual	Test statistics		
				Chi-Square	df	Asym.Sig
Urine	13	9.3	3.8	4.622[a]	3	.202
Blood	10	9.3	.8			
Wounds	10	9.3	.8			
Other	4	9.3	-5.3			
Total	37					
Positive	37	37.0	0.0			
Total	37[b]					

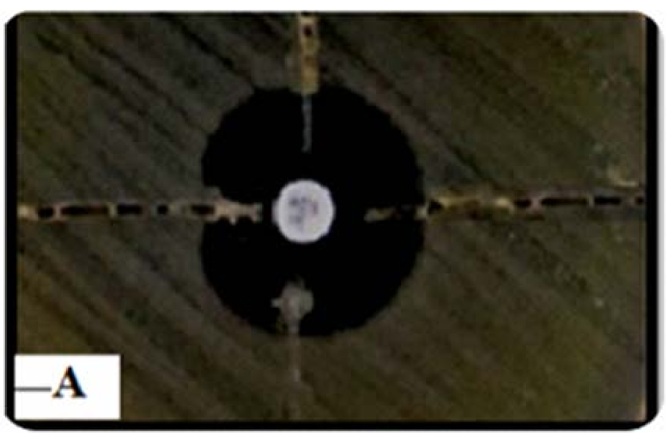

Fig 1. AmpC β-lactamase production in *K. pneumonia* by modified three dimensional tests. A, *AmpC*-producing *Klebsiella.*

Among the 90 isolates, plasmid encoded AmpC genes were detected in 30 (33.3%) of the *K. pneumoniae*. Plasmid encoded AmpC genes belonging to the MOX family were detected in 14/30 (46.7 %) isolates. Gene of the family ACC type was present in 2/30 (6.7%) isolates (fig 2 and 3). While, the remaining 14/30 (46.7%) contained other families that include: DHA, FOX, CIT and EBC, which had different percentage 7 (23.3%), 4 (13.3%), 2 (6.7%) and 1 (3.3%) respectively. The 90 (100%) PMABLs producers were resistant to Ampicillin, Amoxicillin, Carbenicillin, piperacillin-tazobactam and Amoxi-clav as well as 80 (88.9%) were resistant to gentamicin, amikacin and co-trimoxazole, while 78 (86.7%) were resistant to tetracycline and 4 (4.4%) isolates were resistant to imipenem.

DISCUSSION

The AmpC resistance phenotypes occur due to over expression of the chromosomal AmpC gene, acquisition of a plasmid AmpC gene, alteration in permeability of the cell to cefoxitin or a combination of the above factors. Lack of a standardized phenotypic method for screening and detection of this type of resistance makes the surveillance and characterization of such strains difficult [4,5]. Distinguishing between cefoxitin resistant AmpC producers from cefoxitin resistant non-AmpC producers could guide treatment options, *i.e*. extended spectrum cephalosporins for cefoxitin resistant non-AmpC, non-ESBL producers and carbapenems for the cefoxitin-resistant AmpC producers. Differentiation between these

Fig 2. Ethidium bromide-stained agarose gel of multiplex PCR amplified products from extracted DNA of *bla*AmpC positive *K.pneumoniae* isolates and amplified with bla_{ACC} and bla_{MOX} genes primers (forward and reverse). Lane (L), DNA molecular size marker (2000-bp ladder), Lanes (1 and 3) of *K.pneumoniae* isolates showed positive results with *bla*MOX (520 bp) genes and Lanes (6 and 9) showed positive results with *bla*ACC (346bp) gene.

types of organisms would prevent the unnecessary usage of cephalosporins and carbapenems resulting in the selective pressure driving the AmpC or plasmid mediated class A carbapenem resistance gene propagation [15].

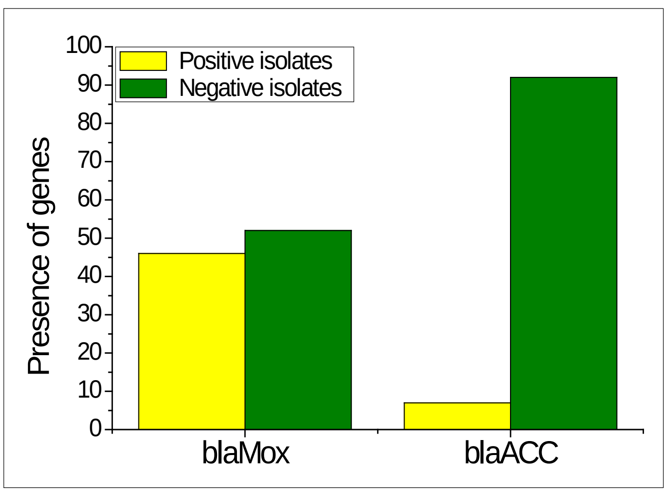

Fig 3. Occurrence of *bla*MOX and *bla*ACC genes among β-lactam resistant *K. pneumonia* isolates (n, 30).

Detection of AmpC is essential to improve the clinical management of patients suffering from infections. However, there are no clinical and laboratory standard institute guidelines for detection of AmpC mediated resistance in Gram negative clinical isolates and hence, it usually poses a problem due to misleading results, especially so in phenotypic tests [16]. In this study, the occurrence of AmpC producing strains with multiplex PCR from the 90 isolates tested, 30 (33.3%) carried plasmid encoded AmpC genes. In a nationwide study from China, the prevalence of plasmid mediated AmpC β-lactamases was (10.1%) in *K. pneumoniae* strains [17]. Similarly, the previous study showed that from 241 isolates 92 (38.1%) were carried *bla*AmpC gene in *K. pneumoniae* and (45.5 %) in *Escherichia coli* [18]. On the contrary, compared to present results, the highest prevalence of AmpC genes were reported in a Korean, surveillance showing 73% of *E. coli* and 77% of K*. pneumoniae* carrying plasmid mediated AmpC genes [19]. Cefoxitin resistance in non-AmpC β-lactamase producers may be due to some other resistant mechanisms such as lack of permea-bility of porins [20]. Another study has demons-trated that the interruption of a porin gene by insertion sequences is a common type of mutation that causes loss or decrease of outer membrane porin expression and increase cefoxitin resistance in *Klebsiella* spp. [21]. In current study, AmpC β-lactamase production was observed in 37 isolates by using modified three dimensional test and AmpC disk test. The occurrence of AmpC β-lactamase in *K. pneumoniae* isolates tested may reflect two modes of production: hyper production of chromosome mediated and plasmid-mediated AmpC β-lactamase [22]. The result of present study is similar to another study established by Soha and Lamia [1]. They reported that 50 (33.8%) of cefoxitin resistant *K. pneumoniae* and *E. Coli* isolates were attributed as AmpC producer by modified three dimension test and AmpC disk test. Additionally, this result disagreement with Al-Shamarti [23] who estimated that 9 (20.9%) of *Klebsiellae* isolates producing *AmpC* β- lactamase by the modified three dimensional test and AmpC disk test from the total number of 43 *Klebsiella* spp. In the present study, the data were obtained by multiplex PCR, out of 30 isolates yielded amplification products with AmpC-PCR specific primers, 14 (46.7%) had a bands compatible with *bla*MOX gene, this family was the most prevalent AmpC-type enzyme. In addition, 2 (6.7%) had a bands compatible with *bla*ACC gene. Compared to the result of Parveen *et al.* [18] who reported that DHA and CIT type genes were predominantly present in nosocomial isolates of *K. pneumonia* and *E. coli* followed by MOX and ACC types in *E. coli*. On the other hand the result of Montgomery *et al.* [24] who reported that 22 AmpC genes were detected in 25.8% of the positive cefoxitin screened isolates of which 40.9% belonged to each of the MOX and FOX families, 13.6% belonged to the EBC family and 4.5% belonged to the CIT family. Fam *et al*. [25] reported that CMY-2 and DHA-1 were the most common gene clusters of AmpC, while other studies reported that DHA-type enzymes have been previously identified in Taiwan [26, 27].

Conflict of interest

The author declares that she has no conflict of interests.

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