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Prevalence of AmpC ß-lactamase producing carbapenem resistant clinical isolates of *Klebsiella pneumoniae* among different hospitals in Hilla City.

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Abstract

This study was performed to detect the presence of bla_{AmpC} among carbapenem resistant *Klebsiella pneumoniae* isolates.A total of 117 clinical isolates of *K. pneumoniae* were collected from 801 clinical sample from different hospitals in Hilla City,during the period from April to August 2011.High prevalence of *K. pneumoniae* isolates were detected in 38 (32.5%)of stool samples, followed by sputum 19(16,2%),All isolates were tested for antimicrobial susceptibility by Kirby-Bauer disk diffusion methods .High resistance rate was recorded for Carbencillin (98%),followed by Amox-clavulanic acid (95%).Carbapenem resistance was reported in 17 of *K. pneumoniae* isolates.These were screened for cefoxitin resistance.Results revealed that all these isolates were found to be cefoxitin resistant, among 17 cefoxitin resistant *K. pneumoniae* isolates,AmpC ß-lactamases production were phenotypically detected in 2(11.8%),3(17.6%) by AmpC disk and MTDT respectively.None of the isolates were positive for inducible AmpC ß-lactamase. AmpC ß-lactamases were dectectable in 13(76.4%) of isolates by PCR method.

Introduction

The predominant mechanism for resistance to ß -lactam antibiotics in Gram-negative bacteria is by the synthesis of ß- lactamases (Suranjana and Manjusiri., 2005). Among the ß -lactamases the production of extended spectrum beta Lactamases (ESBLs) and AmpC ß -lactamases are the most common (Coudron *et al*., 2000).

AmpC ß -lactamases are class C or group I cephalosporinases that confer resistance to a wide variety of ß- lactam antibiotics including 7 – α – methoxy cephalosporins (cefoxitin or cefotetan), oxyimino cephalosporins (cefotaxime, ceftazidimc, ceftriaxone), monobactam (aztreonam) and are not inhibited by ß -lactamses inhibitors such as clavulanic acid (Bush *et al*., 1995). Furthermore, in a strain with decreased outer membrane permeability, such enzymes can provide resistance to carbapenems (Philippon *et al*., 2002).

AmpC ß -lactamases are of two types, chromosomal inducible and plasmid mediated non-inducible, chromosome-mediated Ampc ß -Lactamases have been described in a wide variety of Gram-negative bacilli (Mohamudha *et al*., 2012). Overproduction of their chromosomal Ampc ß -lactamses by mutation is probably responsible for the resistance in these organisms (Yan *et al*., 2002). In most genera of the family *Entrobacteriaceae*, Ampc is inducible (Bush *et al*., 1995). Plasmid mediated AmpC ß -lactamases (PMABLs) have evolved by the movement of chromosomal genes on to plasmids and are found in *Escherichia coil*, *Klebsiella pneumoniae*, *Salmonella* spp., *Proteus mirabilis*, *Citrobacter freundii* and *Enterobacter aerogenes* which confer resistance similar to their chromosomal counterparts (Mohamudha *et al*., 2012). Unlike chromosome-mediated AmpC, most plasmid-mediated AmpC genes, such as MIR-1, are expressed constitutively even in the presence of a complete system for induction (Phillipon *et al*., 2002).

Organisms producing PMABLs are often associated with multidrug resistance, leaving a few therapeutic options and represent a new threat since they confer resistance to cephamycins and are not affected by ß -Lactamase inhibitors, this resistance mechanism has been found

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around the world, can cause nosocomial outbreak, appears to be increasing in prevalence. (Subha *et al*., 2003; Mohamudha *et al*., 2012).

Thus the present study was conducted to evaluate the prevalence of AmpC ß –lactamase producing *K. pneumoniae* in Hilla hospitals,the objectives of the study are: 1- Determine the antibiotic susceptibility pattern of clinical isolates of *K. pneumoniae* among different hospitals in Hilla City. 2- Detect the prevalence of AmpC ß -Lactamase gene by phenotypic and genotypic (PCR) methods.

Materials and Methods

Bacterial isolates

A total of 801clinical samples were collected during the period of five months from April 2011 to August 2011 from different Hospitals in Hilla City included: Merjan Medical City, AL-Hilla Teaching Hospital, Babylon Teaching Hospital for Pediatric and Maternity and Chest Diseases Center. All samples were cultured on MacConkey agar (Himedia) and incubated at 37 C˚ for 24 hr.Bacterial isolates of *K. pneumoniae* were identified using the standarad biochemical tests according to Holt *et al* (1994), Baron and Finegold (1994) and MacFaddin (2000).

Antimicrobial Susceptibility

Antimicrobial susceptibility testing of *K. pneumoniae* isolates was performed on Mueller-Hinton agar (Oxoid) plates using Kirby-Bauer disk diffusion method (Bauer *et al*., 1966).The isolates were tested against the following antibiotics; Carbenicillin (100 µg), Piperacillin (100 µg), Amoxicillin-clavulanic acid (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg), Cefepime (30 µg), Cefoxitin (30 µg), Aztreonam (30 µg), Imipenem (10 µg), Meropenem (10 µg), Ertapenam (10µg), Gentamicin (10µg), Nalidixic acid (30µg) , Nitrofurantion (30µg), Chloramphenicol (30 µg) and Tetracycline (30µg). The cultures were incubated at 37 C˚ for 18 hr under aerobic condition and the diameter of the zones of inhibition of bacterial growth were measured and interpreted as recommended by the National Committee for Clinical Laboratory Standard guidelines. *E. coli* ATCC 25922(College of medicine,University of Kufa) was used as the reference strain for antimicrobial susceptibility testing (CISI, 2010).

Phenotypic Detection of AmpC ß –Lactamase

a. **Initial Screening of AmpC ß –Lactamase**

Bacterial isolates of *K. pneumoniae* were tested for cefoxitin susceptibility using standard disk diffusion method (CLSI, 2010). Isolates showing resistance to cefoxitin (inhibition zone diameter < 18mm) were considered as initially AmpC ß -Lactamase producers (Coudron *et al*., 2003).

b. **Confirmatory Tests of AmpC ß –Lactamase Modified Three-Dimensional Test (MTDT).**

Fresh overnight growth from Mueller-Hinton agar plate was transferred to a pre-weighed sterile eppendrof tube. The tube was weighed again to ascertain the weight of the bacterial mass. The technique was standardized so as to obtain 15 mg of bacterial wet weight for each sample. The growth was suspended in peptone water and pelleted by centrifugation at 3000 rpm for 15 minutes. Bacterial growth washed with normal saline 2 to 3 times, crude enzyme extract was prepared by repeated freeze-thawing (approximately 10 cycles). Lawn cultures of *E. coil* ATCC 25922 were prepared on Mueller-Hinton agar plates and cefoxitin (30 µg) disks

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were placed on the plate. Linear slits (3 cm) were cut using a sterile surgical blade 3 mm a way from the cefoxitin disk. 30 µl of extract was added in the wells made at outer edge of the slit. The plates were kept upright for 5-10 minutes until the solution dried, and then incubated at 37 C˚ overnight.Clear distortion of the zone of inhibition of cefoxitin were taken as AmpC producers (Coudron *et al*., 2003).

Ampc Disk Test

A lawn culture of *E. coil* ATCC 25922 was prepared on Mueller-Hinton agar plate. Sterile disks (6 mm) were moistened with sterile saline (20 µl) and inoculated with several colonies of each test organism. The inoculated disk was then placed beside a cefoxitin disk (almost touching) on the inoculated plate. The plates were incubated overnight at 37 C˚. A positive test appeared as flattening or indentation of the cefoxitin inhibition zone in the vicinity of the test disk. A negative test had an undistorted zone (Parveen *et al*., 2010).

Ceftazidime-Imipenem Antagonism Test (CIAT)

This test consist of an imipenem disk (10 µg) placed 20 mm a part (edge to edge) from a ceftazidime disk (30 µg) on a Mueller-Hinton agar plate previously inoculated with a 0.5 McFarland bacterial suspension, and incubated for 24 hr at 35 C˚. For comparision a cefoxitin disk was also placed 20 mm a part from the ceftazidime disk. Antagonism indicated by a visible reduction in the inhibition zone around the ceflazidime disk adjacent to the imipenem or cefoxitin disks, was regarded as positive for the inducible AmpC ß -lactamase production. (Cantarelli *et al*., 2007).

Genotypic detection of *bla*$_{\text{AmpC}}$ gene

- DNA preparation

DNA preparation from bacterial cells was performed by salting out method as described by Pospiech and Neuman (1995) with some modification and used as a template for PCR reaction.

-PCR amplification of *bla*$_{\text{AmpC}}$ gene. -

Polymerase chain reaction was used to amplify the entire sequence of *bla*$_{\text{AmpC}}$ gene. The primer (Bioneer) used for the amplification of this gene was : *bla*$_{\text{AmpC}}$ /F (5˙-ATCAAAACATGGCACCCA ˙3˙) and AmpC /R (5˙-GAGCCCTTTTATGCACCCA-3˙).Amplification reaction mixture was carried out in a 25 µl reaction volume using 12.5 µl Go Taq Green Master Mix 2X (Promega), 5 µl DNA template, 2.5 µl of 10 pmol/ µl of specific up stream primers and, 2.5 µl of 10 pmol/ µl of specific down stream primers, 2.5 µl nuclease-free water. PCR conditions as follows : an initial denaturation at 94 C˚ for 30 sec, followed by 35 cycles of dematuration at 94 C˚ for 30 sec, anneling at 60 C˚ for 1 min, extension at 72 C˚ for 1 min, and a find extension step of 72 at 10 min. The resulting PCR product was run in 1.5 % agarose gels and electric current was allowed at 70 volts for 2 hr. DNA bands were observed using UV- Transilluminator and photographed with Gel documentation system. 100 bp DNA Ladder (Bioneer) was used to assess PCR product size.

Print ISSN: 2073-8854 & Online ISSN: 2311-6544

Results

Bacterial isolates

A total of 117 *K.pneumoniae* isolates were obtained from 801 clinical samples over a period of five months. The distribution of *K. pneumoniae* isolated from various clinical specimens was 38 (32.5 %) were obtained from stool, 19 (16.2%) from sputum, 18 (15.4%) from vagina and burn, 10 (8.5%) from urine, 8 (6.8%) from wound, 3 (2.5%) from blood, 2 (1.7%) from ear, 1(0.9%) from eye and 0 (0%) from throat. Table -1).

Antibiotic susceptibility test

All 117 *K. pneumoniae* isolates were screened for their antibiotic resistance against selected antibiotic agents of different classes (Fig.1).

In the present study higher resistance rate were recorded for carbenicillin (98%), the next most resistance antibiotic was Amox-clav (95%), followed by cefoxtin(91.4%),ceftazidime (86%),cefotaxime (84%), (82%) for piperacillin and cefepime,ceftriaxone (81,2%), aztreonam (79%),tetracycline (63%) nitrofurantin (60%),nialidix acid (43%), gentanicin ,(41%),chloramphenicol (39,3%),ertapenem (15%), meropenem (14%)and imipenem (8%).

Phenotypic Detection of AmpC ß –Lactamase

-Cefoxitin Susceptibility

Cefoxitin susceptibility of 17 carbapenem resistant *K. pneumoniae* isolates was tested by standard Kirby-Bauer disk diffusion method. Results revealed that all carbapenem resistant *K. pneumoniae* isolates yielded cefoxitin zone diameter less than 18 mm, these isolates may be AmpC ß -lactamase producers.

- Plasmid Mediated AmpC ß -Lactamase Production Out of 17 carbapenem resistant *K.pneumoniae* isolates, AmpC ß -lactamase production was phenotypically confirmed by MTDT and AmpC disk test in 3 (17.6 %) and 2 (11.8 %) of cefoxitin resistant isolates, respectively.(Table -2).

Inducible AmpC ß -Lactamase Production -

Results of the present study revealed no blunting of the ceftazidime zone adjacent to imipenem disk among the 17 cefoxitin resistant isolates. (Table- 2).

Genotypic Detection of AmpC ß –Lactamase

Among 17 carbapenem resistant *K. pneumoniae* tested, a 550 bp fragment corresponding to AmpC gene was detected by PCR in 13 (76.4 %) of isolates. (Fig -2).

Print ISSN: 2073-8854 & Online ISSN: 2311-6544

Table (1): *Klebsiella pneumoniae* isolates among different clinical specimens.

Clinical specimen	No. of specimens	No. (%) of *K. pneumoniae* isolates
Stool	141	38 (32.9 %)
Sputum	128	19 (16.2 %)
Vagina	116	18 (15.4 %)
Burn	153	18 (15.4 %)
Urine	97	10 (8.5 %)
wound	60	8 (6.8 %)
Blood	58	3 (2.5%)
Ear	30	2 (1.7 %)
Eye	8	1 (0.9 %)
Throat	10	0 (0 %)
Total	801	117 (100%)

Table(2): AmpC-ß-Lactamase production in carbapenem resistant *K. pneumoniae* isolates by three phenotypic confimaty methods.

No. (%) of cefoxitin resistant isolates	No. (%) of AmpC- ß -lactamase detected with		
	MTDT	AmpC disk test	Ceftazidime-imipenem test
17	3 (17.6 %)	2 (11.8 %)	0 (0 %)

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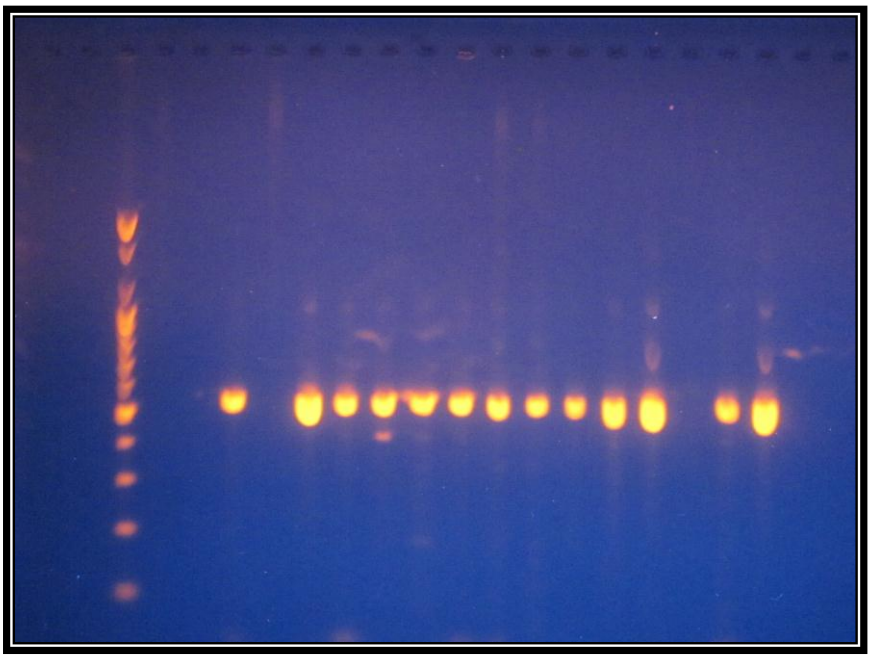

Figure(3): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA amplified with primers *bla*$_{AmpC}$ F and *bla*$_{AmpC}$ R. Lane L:DNA molecular size marker (100- bp ladder),K: *K. pneumoniae* isolate,K3,5,6,7,8,9,10,11,12,13,14,16,17 show positive result with *bla*$_{AmpC}$ primer ; K 1,2,4,15 show negative result with *bla*$_{AmpC}$ primer.

Discussion

Results of the present study revealed the presence of 117 (14.6%) of *K. pneumoniae* isolates obtained from 801 clinical samples. This result is in agreement with a previous study in Hilla by Al-Saedi (2000) who found that *K. pneumoniae* isolates comprised (15.3%) from 711 clinical samples. In another study, Al-Sehlawi (2012) reported that the detection rate of *K. pneumoniae* was (14%) of all pathogen isolated from clinical samples in Najaf hospitals.

However, most *K. pneumoniae* isolates were from stool samples 38 (32.5%), followed by 19 (16.2%) from sputum, vagina and burn 18 (15.4%) each alone, 10(8.5%) from urine, 8(6.8%) from wound, 3 (2.6 %) from blood, 2 (1.7%) from ear, 1 (0.9%) from eye and 0 (0%) from throat (Table – 1).

K. pneumoniae predominantly isolated from stool samples, since it's a common member of the human intestinal flora. High prevalence of *K. pneumoniae* in stool samples was demonstrated by other researchers,Al-Gharakh (2005) in Hilla,Ali *et al* (2010) in Jorden. In sputum, prevalence rate of *K. pneumoniae* was (16.2 %), this in accordance with the result of AL-Sehlawi (2012) who found that *K. pneumoniae* comprised (16%) in sputa of 450 patients in Najaf hospitals. Results of the present study revealed that *K. pneumoniae* isolates confer high level of resistance against antibiotics tested (Fig.1). This may be due to in appropriate and incorrect administration of antimicrobial agents and lack of appropriate infection control strategies (Zakaria, 2005).

The results revealed that higher resistance rate was found for carbenicillin (98 %), piperacillin (82%), this result is in agreement with a pervious study in Hilla by AL-Hilli (2010) who stated that all *K. pneumoniae* isolates were resistance to carbenicillin (100%) and (81%) to piperacillin,high resistance to carbenicillin and piperacillin may be due to widespread use of these antibiotics in Hilla hospitals.

Print ISSN: 2073-8854 & Online ISSN: 2311-6544

Higher resistance was noted for Amox-clav (95 %). Similar finding also was recorded by Lim *et al*.,(2009) who found that out of 51 *K. pneumoniae* isolates, 49 (96%) were resistance for Amox-clav in Malaysia.There is a higher resistance to cefoxitin (91.4%) in clinical isolates of *K. pneumoniae*. Resistance to cefoxitin may be due to high level expression of plasmid mediated AmpC ß-Lactamase and / or development of porine – deficient mutants(Tan *et al*.,2009). However, levels of resistance were (86 %) for cefotazidime and (84 %) for cefotaxime .In a study conducted by Aminizadeh and Kashi (2011) in Iran (87%) of *K. pneumoniae* isolates were found to be resistant to ceftazidime. For cefotaxime our result was higher than that reported in Jorden by Al-Shara (2011) who showed that resistance rate for cefotaxime was (42.9%).

High level of resistance could be attributed to the presence of ESBLs, since it mediate resistance to broad – spectrum cephalosporins (e.g., ceftazidime, ceftriaxone, cefotuxime) and aztreonam (Umadevi *et al*., 2011).

Regarding resistance to carbapenem, resistance rate of ertapenem was(15%) ,other study showed that out of (98%)ertapenem resistant *Enterobacteriacae*, *K. pneumoniae* comprised (54.9%) of isolates (Patel *et al*.,2011).

Resistance to meropenem was (14%), this result is higher than that reported by AL-Sehlawi (2012) in Najaf who found that only four isolates (3.9%) of *K. pneumoniae* were resistance to meropenem. Resistance to impienem was (8%), but this resulti s higher than that reported by Aminizadeh and Kashi (2011) in Iran who found that resistance rate of *K. pneumoniae* to imipenem was (2%). Other study showed that the susceptibility of 150 clinical isolates of *K. pneumoniae* to imipenem was (100%) in Nigeria (Iroha *et al*., 2011).

Result also revealed that all 17 (100 %) carbapenem resistant *K. pneumoniae* isolates yielded cefoxitin zone diameter less than 18 mm, these isolates may be AmpC ß -Lactamase producers. In a study from India, Mohamudha *et al* (2012) showed that out of 109 clinical isolates of *K. pneumoniae* collected from hospitalized patients 83 (76.1 %)of isolates were found to be resistant to cefoxitin. Other study characterized that 84 (72 %) of *Klebsiella* isolates were found to be resistant to cefoxitin in Chennai (Subha *et al*., 2003). Of the 17 cefoxitin resistant *K. pneumoniae* isolates, 2 (11.8 %) isolate revealed positive result by AmpC disk test (Table- 2). Other study by Mohamudha *et al* (2012) reported that AmpC ß -Lactamase was confirmed in 137 (73.2 %) of cefoxitin resistant *E. coli* and *K. pneumoniae* isolates.In this study there is low prevalence in AmpC ß-lactamase in comparison with cefoxitin susceptibility result ,this may be due to a lack of permeation of porin or that some isolates may have *AmpC* genes ,but not expressed in all the isolates.They might have silent genes or there might be low level expression of *AmpC* genes that was not detected (Jacoby,2009).

Results also showed that of 17 cefoxitin resistant *K. pneumoniae*, AmpC production was confimed by MTDT in 3 (17.6 %)of isolates (Table -2). In a related study in Hilla by Al-Hilli (2010), AmpC ß -lactamase was produced by MTDT and AmpC disk test in 1 (4 %) of 7 cefoxitin resistant *K. pneumoniae* isolates collected from Merjan teaching hospital. In another study, Al-Sehlawi (2012) estimated that 31 (42.5 %) of *K. pneumoniae* isolates were AmpC ß -Lactamase producer using MTDT in Najaf.

Many Gram -negative bacteria harbor chromosomal AmpC beta -lactamase genes,which are constitutively expressed at low level, these bacteria can acquire plasmid-encoded AmpC genes resulting in a stably derepressed resistance phenotype (Thomas,2007;Polsfuss *et al* .,2011). In this study, screening for inducible AmpC ß -Lactamase was done by the disk antagonism test, results showed that none of the isolates were positive for inducible AmpC ß -

lactamase (Table- 2). This result in agreement with previous study in Hilla by Al-Hilli (2010) who found that among 7 (28 %) cefoxitin resistant isolates, no *K. pneumoniae* isolates was positive for inducible AmpC ß -Lactamase production.

Using PCR method, result demoustrated that 13 (76.4 %) isolates were amplified with bla_{AmpC} primers (Fig. -2). These isolates showed cefoxitin resistant in cefoxitin susceptibility test. Cefoxitin resistant in AmpC non-producers could be due to some other resistance mechanism. Hernandez-Alles *et al* (2000) demonstrated that the interruption of a porin gene by insertion sequences is a common type of mutation that causes loss or decrease of outer membranes porin expression and increased cefoxitin resistance in *E. coli* and *Klebsiella* spp.

It is pertinent to note that in this study only 2 (11.8 %) of cefoxitin resistant isolates were detected by AmpC disk test and 3 (17.6 %) by. MTDT .Phenotypic tests alone may not reflect the true number of AmpC ß -Lactamase producers, hence molecular studies, although not possible routinely in clinical laboratories, need to be employed in surveillance studies.

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