

Molecular Detection of *AmpC* Gene Encoding Antibiotic Resistance among *Klebsiella* spp. Isolated from Different Infections

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Abstract :

One hundred and fifty five of Gram-negative-lactose fermented bacteria grown on MacConkey agar were collected from two main hospitals in Najaf province. The isolates were identified according to cultural characteristics and biochemical activities. The ability of *Klebsiella* spp. isolates to *AmpC* production were tested; the results have revealed that 9 (30%) isolates produce *AmpC* β -lactamase. PCR amplification results have shown that six *Klebsiella* spp. isolates possess *AmpC* β -lactamase gene.

Introduction

Members of the genus *Klebsiella* is straight gram-negative rods, about 0.3-1 μ m in width and 0.6-6 μ m in length, usually arranged singly, in pairs or in short chains. *Klebsiella* spp. are non-motile, facultative anaerobic bacteria which has both a respiratory and fermentative type of metabolism, optimal growth temperature is 37 C°, *Klebsiella* spp. exhibit a mucoid growth due to the large polysaccharide capsule. Recently, *Klebsiella* spp. considered as serious pathogen especially in hospital acquired infection such as burns, wound, respiratory tract and urinary tract infections because of their ability to resist a wide variety of antibiotics (Holt *et al.*, 1994).

One of the most important mechanisms of antibiotic resistance in *Klebsiella* spp. is the production of *AmpC* β -lactamases (Class C β -lactamases) which are an important group of enzymes that are broadly distributed in the world; it is the second most common β -lactamase group (Bush *et al.*, 1995). The inducible chromosomal *AmpC* genes were detected on plasmids of *Klebsiella* spp., *E. coli*, or *Salmonella* spp. In *E. coli* *AmpC* is poorly expressed, while in *Klebsiella* and *Salmonella* species the *AmpC* gene is missing from the chromosome and found on the plasmids (Philippon *et al.*, 2002). Recently, more than 100 different *AmpC* enzymes were commonly isolated from extended-spectrum cephalosporin-resistant Gram-negative bacteria (Jacoby and Bush, 2009).

Methods

Collection of specimens

One hundred and fifty five Gram-negative lactose fermenting isolates grown on MacConkey agar were collected from two hospital in Najaf province, Alsader Teaching Hospital and Alzahra Teaching Hospital. The isolates are composed of 64 burns infections, 22, 44 and 25 isolates from wounds, urinary tract and respiratory tract infections respectively. From those, 63 isolates from males and 92 from females.

The specimens were transferred immediately to the laboratory for culture and identification.

Identification of *Klebsiella* spp.

The bacterial isolates were identified according to the cultural and biochemical properties. *Klebsiellae* isolates were distinguished post growth on solid medium. It produces large, smooth, with pink color (lactose fermented), elevated and mucoid colony on MacConkey (Bharti *et al.*, 2008). Furthermore, the biochemical tests were performed for the identification of *Klebsiellae* isolates from other isolates. These tests included Indole test, Methyl red test, Voges-proskaur test, Simmon's Citrate test, Triple Sugar Iron test, Urease test, and Motility test.

Detection of *AmpC* Beta-lactamase

The modified three dimensional test was used to detect the production of *AmpC* β -lactamase enzyme; the method was described by Neelam *et al.*, (2008). The technique was standardized so as to obtain 10–15 mg of bacterial wet weight of fresh overnight growth for each sample. The growth was suspended in 1 ml peptone water and was pelleted by centrifugation at 3000 rpm for 15 min. Crude enzyme extract was prepared by repeated freezing and thawing. Five rounds of freezing and thawing gave satisfactory results. Lawn cultures of *E. coli* ATCC 25922 were prepared on Mueller–Hinton agar plates and cefoxitin (30 μ g) discs were placed on the plate. Linear slits (3 cm) were cut using a sterile surgical blade 3 mm away from the 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 wells could easily be loaded with the enzyme extract in 10 μ L increments until the well was filled to the top. The plates were kept upright for 5–10 min until the solution diffused, and then incubated at 37°C overnight. The isolates showing clear distortion of inhibition zone of cefoxitin were recorded as *AmpC* producers, whereas the isolates with no distortion were recorded as non-producers isolates.

Detection of Plasmid Mediated *AmpC* Beta-Lactamase

The *AmpC* disk test described by Neelam *et al.* (2008) was performed to detect the plasmid mediated *AmpC* β -lactamase. *AmpC* disks were prepared by applying tris-EDTA to sterile filter paper disks; the surface of Mueller-Hinton agar plate was inoculated with a lawn of 0.5 McFarland suspension of cefoxitin susceptible *E.coli* ATCC 25922, then several colonies of each test organism were applied to a disk. Cefoxitin disk was placed on the inoculated surface of the Mueller-Hinton agar, the plate was then inverted and incubated overnight at 37 C. After incubation, plates were examined for either an indentation or flattening the inhibition zone, indicating that the isolate produce enzymatic inactivation of cefoxitin (positive result), or the absence of distortion, indicating no significant inactivation of cefoxitin (negative result).

Molecular Detection of *AmpC*

The method of Popiech and Neuman, (1995) was used for extraction and purification of bacterial DNA. The method depends on cell lyses using cell lysis buffer and SDS. Then the DNA was purified via phenol: chloroform: isoamylalcohol that precipitate the proteins. Finally, the DNA was washed via 70% ethanol. The purified bacterial DNA has been used as a template for PCR amplification according

to the method described by David *et al.* (2003) involving the use of 5 µl of bacterial DNA, 40 Pico moles of each primer (Alpha DNA, Canada) , 5 µl of 2X Go Taq Green master mix (Promega, USA) and 5 µl of nuclease-free water (Promega, U.S.A), giving a total PCR reaction volume of 25 µl. The oligonucleotide PCR primers specific for the *AmpC* gene were (F 5'-ATCAAAACTGGCAGCCG-3') and (R 5'-GAGCCCGTTTTATGCACCCA-3') and the length of PCR product was 510 bp.

The PCR conditions used were 35 cycles of amplification at a denaturation temperature of 94 C° for 30 seconds; the annealing primers temperature was 60 C° for 1 min, and an extension temperature was 72 C° for 1 min, followed by a final extension at 72 C° for 10 min. PCR products were resolved on 1% agarose gels stained with ethidium bromide, and photographed with UV illumination.

Results and Discussion

Specimens Collection and Identification

A total of one hundred and fifty five Gram-negative lactose fermented isolates grown on MacConkey agar were collected from two hospitals in Al Najaf province 129 isolates (83.2%) from Alsader teaching hospital and 25 (16.8%) isolates from ALzahra teaching hospital (table 1), 92 (59.3%) isolates from females and 63 (40.7%) from males, the isolates were represented by 64 (41.3%) from burns infections and 44 (28.4%), 25 (16.1%) and 22 (14.2%) from urinary tract, respiratory tract and wounds infections respectively table (3-1). The isolates have been collected from two groups of patients, 105 (67.7%) isolates from hospitalized patients and 50 (32.3%) from outpatients. The isolates were sub cultured on suitable media, including blood agar and MacConkey agar, and incubated at 37C° for 18-24 hour. However, 43 isolates have been distinguished as *Klebsiellae* among the 155 Gram-negative clinical bacterial isolates.

Table 3-1 Distribution of Bacterial Isolates According to Hospitals:

Hospital		Alsader		Alzahra		Total		Total
		Positive	Negative	Positive	Negative	Positive	Negative	
Burns infections	Hospitalized patients	17	47	–	–	17	47	64
	Out patients	–	–	–	–			
Urinary tract infections	Hospitalized patients	1	3	1	2	14	30	44
	Out patients	8	14	4	12			
Respiratory tract infections	Hospitalized patients	3	10	–	4	5	20	25
	Out patients	1	4	1	2			
Wounds infections	Hospitalized patients	7	10	–	–	7	14	22

	Out patients	–	4	–	–			
Total		37	92	6	19	43	112	155
		129		26		155		

Detection of *AmpC* β -lactamases-Producing Isolates

The results revealed that 9 (30%) of *Klebsiellae* isolates producing *AmpC* from the total number of 43 *Klebsiella* spp. isolates, which were represented by 4, 2 and 3 isolates from burns wounds and urinary tract infections respectively (Figure 1). The results indicated that all nine isolates have plasmid originated *AmpC* (Figure 2).

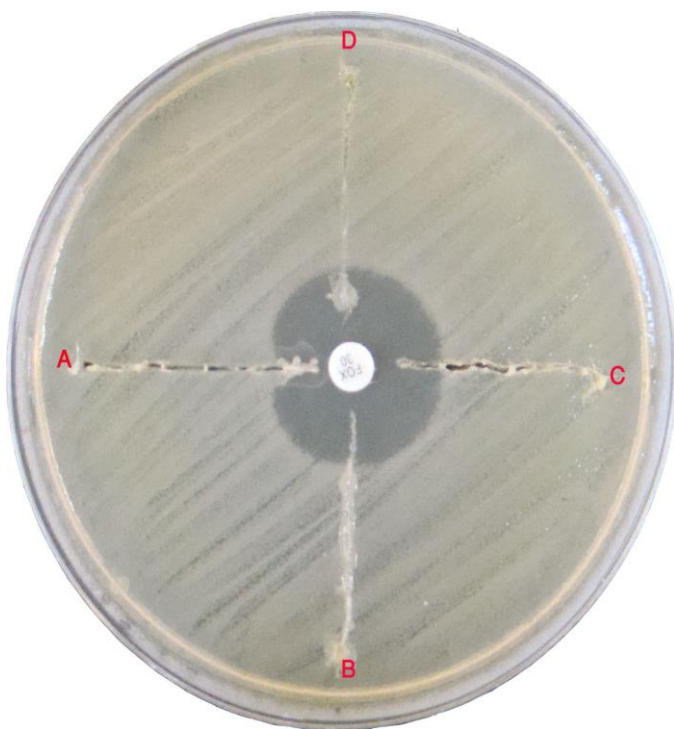


Figure 1, Three Dimensional Test, (A) *AmpC*-producing *Klebsiellae*, (B),(C) and (D) Negative Results.

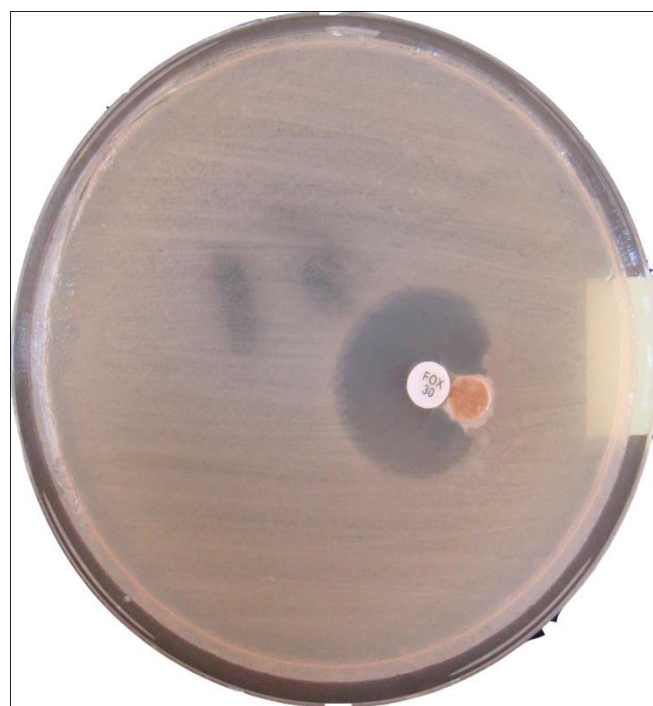


Figure 2, Positive Result of *AmpC* Disk Method

These findings are in agreement with those of Neelam *et al.* (2008) who found that the chromosomally encoded *AmpC* β -lactamases are very rare. Coudron (2005) reported that the susceptibility to third generation cephalosporins (e.g. ceftazidime and cefixime) serve as a poor marker for the identification of *AmpC*-producing bacteria. As mentioned by Black *et al.* (2005) that the plasmid mediated *AmpC*-producing isolates can some times appear falsely susceptible to the third generation cephalosporins.

Vikas *et al.* (2002) reported that the maximum incidence of *AmpC* producers was found among *Acinetobacter* spp. (42.8%) followed by *Klebsiella pneumoniae* isolates (33.3%) and (61%) of *AmpC* producers were found to be resistant to ceftiofur. The same study pointed out that among all *AmpC*-harbouring isolates those collected from the intensive care unit had the maximal number of *AmpC* positives (45%) followed by the burn unit (22%).

Molecular Detection of *AmpC* β -lactamase

Polymerase chain reaction technique has been used to amplify gene encoding the *AmpC* β -lactamase. The results clarify that 6 isolates carrying *AmpC* gene (Figure 3).

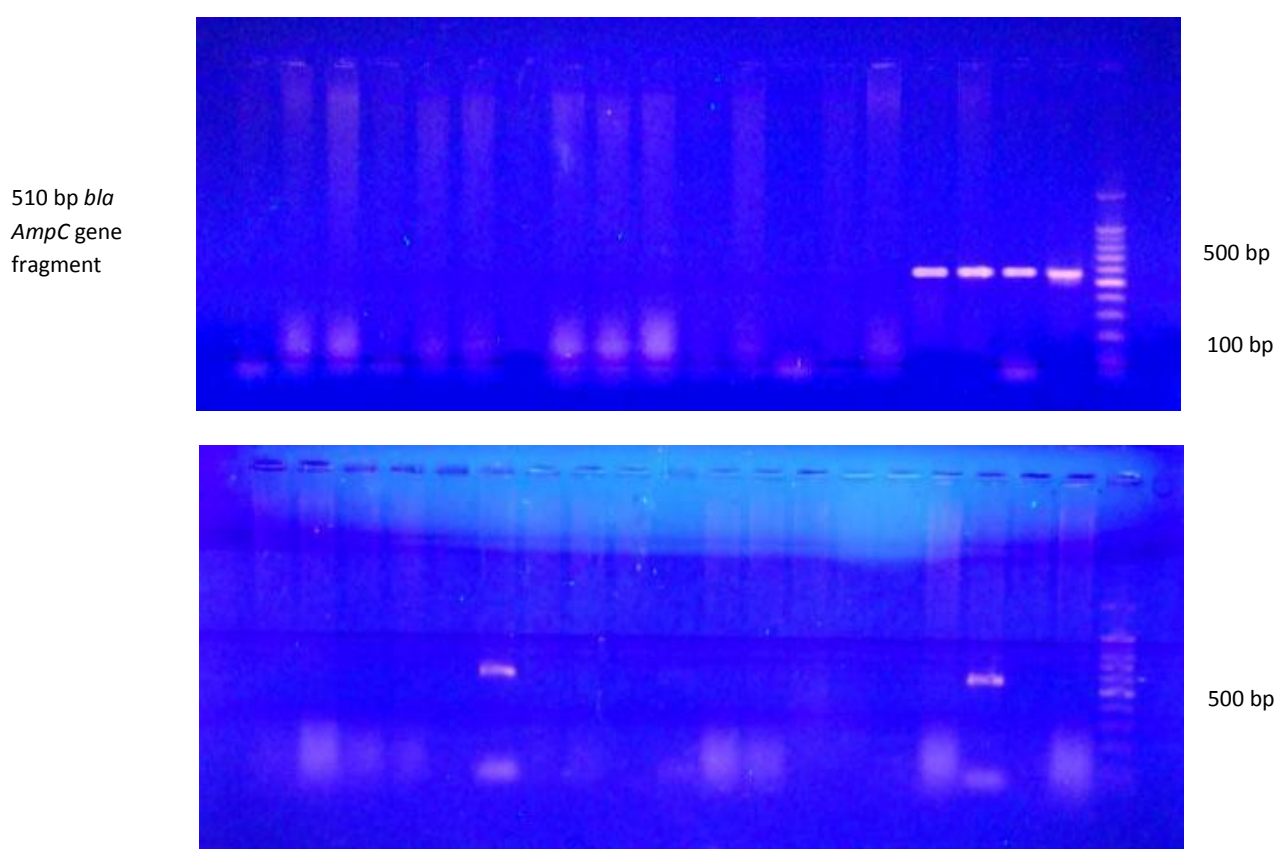


Figure 3, PCR Amplification of *AmpC* β -lactamase Gene.

AmpC enzyme was first reported in 1988 as plasmid mediated enzyme (Bauernfeind *et al.*, 1989). Plasmid mediated *AmpC* have arisen through the transfer of chromosomal genes for the inducible *AmpC* β -lactamase into plasmids; this transfer has resulted in plasmid-mediated *AmpC* β -lactamases in isolates of *K.pneumoniae*, *E.coli* and *Enterobacter aerogenes* as reported by Bauernfeind *et al.* (1989). The same study also pointed out that plasmid-mediated *AmpCs* differ from chromosomal *AmpCs* in being uninducible. *AmpC*-encoded β -lactamases are now predominant in many strains of *Klebsiella* spp., *E.coli*, *Proteus mirabilis* and *Salmonella* spp. (Koh *et al.*, 2007). Plasmid-mediated *AmpC* have been described from diverse geographic areas including the United States, United Kingdom and Asia (Hernandez *et al.*, 2000). Lee *et*

al. (2005) pointed out that the reduced susceptibility to cefoxitin in *Enterobacteriaceae* may be an indicator to *AmpC* activity, but cefoxitin resistance may also be mediated by alterations to the outer membrane permeability. Organisms overexpressing *AmpC* β -lactamases a major clinical concern because these are usually resistant to all β -lactam drugs except for cefepime, ceftazidime and carbapenems, but in contrast to ESBLs *AmpC* β -lactamases hydrolyze cephamycins and are not inhibited by β -lactamase inhibitors (Girlich *et al.*, 2000). Robberts *et al.* (2008) reported that *K.pneumoniae* harbours only plasmid mediated *AmpC*, the same study revealed that 29 (70.7%) of *K.pneumoniae* isolates were positive for *AmpC* β -lactamase, from those only 14 isolates were detected to be carrying *AmpC* gene using PCR technique.

References

- Bauernfeind** A, Chong Y, and Schweighart S. (1989). Extended broadspectrum β -lactamase in *Klebsiella pneumoniae* including resistance to cephamycins. *Infection*; 17:316-21.
- Bharti** Arora & D.R. Arora (2008). Practical Microbiology, Maharaja Agrasen Medical Collage, Agroha, Hisar, Haryana (India). ISBN: 978-81-239-1595-1.
- Black** JA, Moland ES and Thomson KS. (2005). *AmpC* disk test for detection of plasmid-mediated *AmpC*- β lactamases in *Enterobacteriaceae* lacking chromosomal *AmpC*- β -lactamases. *J Clin Microbiol*; 43 : 3110-3.
- Coudron** PE. (2005). Inhibitor-based methods for detection of plasmid-mediated *AmpC* beta-lactamases in *Klebsiella spp.*, *Escherichia coli* and *Proteus mirabilis*. *J Clin Microbiol*; 43 : 4163-7.
- Girlich** D, Naas T, Bellais S, Poirel L, Karim A. and Nordmann P. (2000). Heterogeneity of *AmpC* cephalosporinases of *Hafnia alvei* clinical isolates expressing inducible or constitutive ceftazidime resistance phenotypes *Antimicrob. Agents Chemother.*; 44:3220–3223.
- Hernandez-Alles**, S., M. Conejo, A. Pascual, J. M. Tomas, V. J. Benedi, and L. Martinez-Martinez. (2000). Relationship between outer membrane alterations and susceptibility to antimicrobial agents in isogenic strains of *Klebsiella pneumoniae*. *J. Antimicrob. Chemother.* 46:273–277.
- Jacoby**, J. A. and K. Bush (2009). Amino Acid Sequences for TEM, SHV and OXA Extended-Spectrum and Inhibitor Resistant β -Lactamases. from
- Koh**, T. H., L. H. Sng, G. Wang, L. Y. Hsu, R. T. Lin, and N. W. Tee. (2007). Emerging problems with plasmid-mediated DHA and CMY *AmpC* betalactamases in *Enterobacteriaceae* in Singapore. *Int. J. Antimicrob. Agents* 30:278–280.
- Lee**, K., S. G. Hong, Y. J. Park, H. S. Lee, W. Song, J. Jeong, D. Yong, and Y. Chong. (2005). Evaluation of phenotypic screening methods for detecting plasmid-mediated *AmpC* beta-lactamases-producing isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *Diagn. Microbiol. Infect. Dis.* 53:319–323.

- Neelam** Taneja, Pooja Rao, Jitender Arora & Ashok Dogra (2008). Occurrence of ESBL & AmpC β -lactamases & susceptibility to newer antimicrobial agents in complicated UTI. Indian J. Med. Res. 127, pp 85-88.
- Robberts**, P. C. Kohner, and R. Patel. (2009). Unreliable Extended-Spectrum Lactamase Detection in the Presence of Plasmid-Mediated AmpC in *Escherichia coli* Clinical Isolates. Journal of Clinical Microbiology; p.358–361.
- Vikas** Manchanda and Narendra P. Singh. (2002). Occurrence and detection of AmpC β -lactamases among Gram-negative clinical isolates using a modified three-dimensional test at Guru Tegh Bahadur Hospital, Delhi, India. Journal of Antimicrobial Chemotherapy; 51, 415–418.

التشخيص الجزيئي للجين المشفر Ampc لمقاومة المضادات الحيوية بين عزلات *klebsiella spp.* والمعزولة من اصبات مختلفة .

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الخلاصة

تم جمع 155 عزلة من البكتريا السالبة لصبغة كرام المخمرة لسكر اللاكتوز والنامية على وسط الماكونكي الصلب من مختبرات مستشفى الصدر التعليمي ومستشفى الزهراء التعليمي في محافظة النجف الاشرف، شخضت العينات اعتمادا على الصفات الزرعوية والصفات الكيميائية الحيوية. تحرت الدراسة عن قابلية عزلات بكتريا *Klebsiellae* على إنتاج إنزيمات البيتا لاکتاميز نوع *AmpC* مظهريا، حيث أظهرت النتائج ان 9 عزلات (30%) كانت منتجة لهذا الإنزيم. تم التحري عن الجين المشفر لإنزيم *AmpC* باستخدام تقنية الـ PCR حيث أظهرت النتائج ان 6 عزلات حاوية لهذا الجين.