



Frequency of Metallo- β -Lactamases Producing *Klebsiella pneumoniae* in Burn Wound Isolates

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Abstract:

From the period 1 / 3 / 2016 to 30 / 8 / 2016, 210 swabs were collected from the burn patients hospitalized in different hospitals in Baghdad City: Al-Karama Teaching Hospital, Special Burn Hospital, Central Teaching Laboratories, Child protection Teaching Hospital, Imam Ali Hospital. Forty two isolates (37.5 %) showed a growth of *Klebsiella pneumoniae*, and the remaining isolates (62.5%) were belonged to other bacteria. Identification of all isolates were carried out depending on macroscopic, microscopic characterizations, conventional biochemical tests and Api 20E system. Metallo - β lactamase (MBL) enzymes were screen by three different phenotypic methods (DDST, CMDT and MHT) in which 10 μ g meropenem antibiotic disk has selected to all phenotypic methods.

Susceptibility test done with antibiotic disks of cefotaxime, ceftazidime, aztreonam, imipenem, meropenem, gentamicin, amikacin, ciprofloxacin, tetracycline and trimethoprim-sulfamethoxazole. The percentage of resistance isolates were presented as, Tetracycline (95.23%), Cefotaxime (85.71%), Trimethoprim- Sulfamethoxazol (83.33%), Aztreonam (71.42%), Ceftazidime (69.04%), Ciprofloxacin (59.52%), Gentamycin (26.19%), Imipenem (21.42%), Meropenem and Amikacin (19.04 %).

Key words: Metallo- β -Lactamases, *Klebsiella pneumoniae*, Burn Wound,

Introduction:

Metallo- β -lactamases (MBLs) are enzymes that confer and hydrolyze carbapenems and other β -lactam antibiotics, but are inhibited by chelating agents such as ethylene-diamine tetra-acetic acid (EDTA) (1). They are a type of carbapenemases that require zinc ion (Zn^{2+}) as a cofactor for enzyme activity (1,2) .

Risk factors for infections caused by carbapenemases-producing strains of *K. pneumoniae* include long hospital stay, and previous administration of antibiotics, especially broad-spectrum cephalosporins and β -lactamase inhibitor combinations (3). MBLs which hydrolyze and reduce inefficacious a wide variation of β -lactam antibiotics especially the Carbapenems; have become a serious public health problem for the treatment of bacterial related infections, (4) and their spread amongst clinically important bacteria (e.g. *E.coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) which inserted into the genetic mobile stages (plasmids, transposons, and integrons) (5). The carbapenems (e.g. imipenem and meropenem) are the drug of choice for the treatment of infections caused by multidrug resistant bacteria. The reduced susceptibility of bacterial pathogens to the carbapenems due to MBL production have been widely prevalence reported from different parts of the world. The remains infection with these MBL strains a challenge for treatment and can lead to morbidity and mortality (6). Early detection of MBL-producing bacteria like *K. pneumoniae* is critical due to the worldwide increase in the occurrence, types and spread of MBLs (including other drug-resistant pathogens) as well as developing new therapeutic rules and prophylactic strategies to control the bacterial infection in patients with burn wounds infection (7) further more in both the



community and hospital settings (8). Since the infection caused by Gram-negative bacilli producing MBL is difficult to treat, detection should be carried out. (9). Some of these tests like the double-disk synergy tests (DDST) using EDTA with meropenem disk (MEM), Combined EDTA disk test (CMDT) and the Hodge test (10).

This study aimed to detect MBL producing *K. pneumoniae* in burn wound patients, and to identify the antibiotic susceptibility pattern in such isolates in Baghdad City. In addition detect MBL in carbapenem resistant *K. pneumoniae* isolates by phenotypic methods, the Double disk synergy test (DDST), Combined disc test (CMDT) with EDTA and Modified Hodge test (MHT).

Material & Methods:

Isolation and Identification

During the period from 1 / 3 / 2016 to 30 / 8 / 2016, 42 *K. pneumoniae* isolates were isolated from 210 swabs of burn patients hospitalized in different hospitals in Baghdad City: Al-Karama Teaching Hospital, Special Burn Hospital, Central Teaching Laboratories, Child protection Teaching Hospital, Imam Ali Hospital. Specimens had been collected by sterile swabs after the removal of dressing and cleaning the wound surface by 70% alcohol. The isolation and identification of *K. pneumoniae* from wound specimens had been performed by conventional bacteriological methods.

Biochemical tests

- A. Specimens had been labeled and transported with aseptic technique to the laboratory within 1-2 hour then streaked on blood agar, MacConkey agar and EMB agar (Biomark Lab., Pune, India) and incubated at 37°C for 24hrs. Suspicious colony was sub cultured and purified. The isolates were identified as *K. pneumoniae* by manual biochemical tests that were used in accordance with the manufacturer's instructions; based on Gram staining, catalase test, oxidase test, triple sugar iron (TSI) fermentation, Indole test, Voges- Proskauer (VP) test, Methyl red (MR) test, Simmons Citrate test, Urease test, motility test, and string test (11). For final confirmation, biochemical tests embedded in the API-20E biochemical kit system (Bio-Merieux, France).
- B. Bacterial isolates were screened for metallo- β lactamase (MBL) enzymes by three different phenotypic methods in which 10 μ g meropenem antibiotic disk was selected to be used in all phenotypic methods.

Double disk synergy test (DDST):

This test has been performed by inoculating the tested organism onto Mueller-Hinton agar (MHA) plate. A 10 μ g meropenem disk and a blank Whatmann filter No.1 paper disk 6 mm in diameter were placed 10 mm apart from edge to edge, then 10 μ l of 0.5 molar EDTA solution was applied to the blank disk and dried immediately in an incubator before used, after 18 hours of incubation at 37°C. The presence of extension of zone towards the saturated EDTA disk has been interpreted as EDTA synergy test positive (12).

Combined EDTA disk test (CMDT):

This test has been performed by inoculating an overnight broth culture of the tested organism strain with an opacity adjusted to 0.5 McFarland standards onto plate of Mueller-Hinton agar. Four μ l of the sterilized EDTA solution was added to 10 μ g Meropenem disk, then the EDTA saturated antibiotic disks were dried immediately in an incubator and stored at -20 °C in



airtight vials without any desiccants until used (13). After drying of MHA plate, a 10 µg Meropenem disk and meropenem disk combined with EDTA had placed 20 mm apart (14), then incubated at 37 °C for 24 hours. Post incubation, an increase in the zone inhibition diameter of at least 7 mm around the Meropenem combined EDTA saturated disk compared to Meropenem disks alone was considered as MBL positive (15).

Modified Hodge test (MHT):

All tested isolates had exposed to MHT test as recommended by (16); Inoculating an overnight culture suspension of *E.coli* ATCC 25922 strain has prepared by adding two to three isolated colonies of *E. coli* strain to 5 ml of normal saline, and the suspension was further diluted by adding 1 ml of the suspension to 4 ml of 0.85% NaCl, the mixture was adjusted to 0.5 McFarland's standard and this suspension was streaked across the entire plate of Mueller-Hinton agar (MHA) plate. After drying 10 µg of Meropenem disk was placed at the center of the plate and up to 4 different isolates of tested organisms were streaked linearly from the periphery of the plate into the direction of Meropenem disk at the center then the test plate was incubated at 37°C for 18 hours. The presence of a clover leaf-like shaped zone of inhibition around each tested strain had interpreted as Carbapenemases producing strain or a positive result.

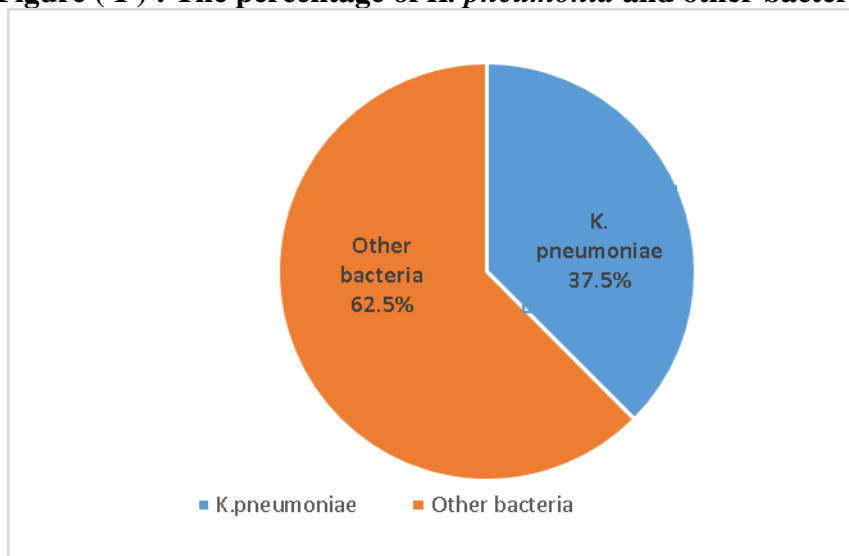
MHT negative result has no growth of *E. coli* ATCC 25922 along the test organism (17).

C. Antibiotics sensitivity was carried out as recommended by the Clinical and Laboratory Standards Institute rules (18), using Muller-Hinton medium (Biomark Lab., Pune, India), 5-10 colonies of each isolate had picked up with sterile loop and suspended into 5 ml of sterile distilled water and suspension has taken by a sterile cotton swab then streaked the surface of the plates in three different planes. Antibiotic disks were cefotaxime (CTX :30µg), ceftazidime (CAZ: 30 µg), aztreonam (ATM: 30 µg), imipenem (IMP:10 µg), meropenem (MEM:10 µg), gentamicin (GM: 10 µg), amikacin (AK:30 µg), ciprofloxacin (CIP:5µg), tetracycline (TET:30µg) and trimethoprim-sulfamethoxazole (TS:1.25/23.75 µg) (Mast group Ltd. England) were placed on the inoculated Mueller-Hinton Agar plates by using sterile forceps, and pressed firmly, gently into the surface of the agar, then incubated at 37°C for 18-24 hrs. After incubation the diameter of inhibition zones around the antibiotic disks were measured using reflected light and ruler. *K. pneumoniae* ATCC 700603, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used as control for antibiotic resistance. The antibiotic susceptibility results were interpreted as per the CLSI guidelines (18).

3. Results and Discussion:

The study population included of 31 % male and 69% female patients with an age range between 5 and 60 years. From (112 of 210) sample swabs isolates had been shown 42 (37.5 %) a growth of *Klebsiella pneumoniae*, and the remaining isolates (62.5%) (figure1) were belonged to other bacteria; 36 (32.14%) *Pseudomonas* spp., 20 (17.86%) *E. coli*, 10 (8.93%) *Staphylococcus aureus* and 4 (3.57 %) *Proteus* spp. (table 1). While mixed growth isolates (98 of 210) frequency as the following:

Klebsiella pneumoniae and *Pseudomonas* spp. 64 (65.31%), *Pseudomonas* spp. and *E. coli* 18 (18.37%), *Klebsiella pneumoniae* and *E. coli* 7 (7.14%), *Pseudomonas* spp. and *proteus* spp. 4 (4.08 %), *Klebsiella pneumoniae* and *Staphylococcus aureus* 3 (3.06 %) and *proteus* spp. and *E. coli* 2 (2.04 %).

**Figure (1) : The percentage of *K. pneumonia* and other bacteria****Table 1.Types of single pathogens isolated and percentage.**

Sequence	Type of bacteria	Frequency	Percentage %
1	<i>Klebsiella pneumonia</i>	42	37.5
2	<i>Pseudomonas spp.</i>	36	32.14
3	<i>E. coli</i>	20	17.86
4	<i>Staphylococcus aure</i>	10	8.93
5	<i>proteus spp.</i>	4	3.57
Total		112	100

The genus *Klebsiella* were opportunistic pathogens that were frequently isolated from various infections, in general *K. pneumonia* and other pathogens were involved in nosocomial infections and were considered the most medically important pathogens causing 75% to 86% of clinical *Klebsiella* infections (19,20). Table (1) showed that *Klebsiella pneumonia* 42 (37.5 %) the common single isolated pathogenic bacteria from burn wound infections. This percentage was considered as a lower than in another study Al- Charakh (21). This was agreement with Kehined *et.al* (22) who found that *Klebsiella spp.* (34.4%) was the most common isolate from infected burn wounds.

**Table 2. Types of mix pathogens isolated and percentage**

Sequence	Type of mix bacteria	Frequency	Percentage %
1	<i>Klebsiella pneumonia</i> and <i>Pseudomonas spp.</i>	64	65.31
2	<i>Pseudomonas spp.</i> and <i>E. coli</i>	18	18.37
3	<i>Klebsiella pneumonia</i> and <i>E. coli</i>	7	7.14
4	<i>Pseudomonas spp.</i> and <i>proteus spp.</i>	4	4.08
5	<i>Klebsiella pneumonia</i> and <i>Staphylococcus aureas</i>	3	3.06
6	<i>proteus spp.</i> and <i>E. coli</i>	2	2.04
Total		98	100

Also *Klebsiella spp.* with *Pseudomonas spp.* were the common mix growth isolates(65.31%)(table 2).The mix growth generated because the site of burns was suitable site for bacterial multiplication and infection, due to longer period, larger area complicated in the hospital (23), in addition to the contamination of hospital environment especially in the operating theatre, patient beds, medical instrument and hand carriers (24).

Phenotypic Detection of Metallo-Beta-Lactamases:

Metallo- β -lactamase enzymes (MBL) were identified worldwide from clinical strains with increasing frequency of these strains which producing MBL enzymes were responsible for prolonged nosocomial infections that lead to serious infections (24). So frequent isolates of *K. pneumoniae* have screened for metallo β - lactamase (MBL) enzymes by the following phenotypic methods:

Initially all bacterial isolates were screened for β -lactamase production by iodometric method which was described by (WHO) 1978 (25). The results showed that 30 isolates of the total 42 of *K. pneumonia* gave a positive result for the examination of production β -lactamase with percentage (71.4%), then the isolates subjected to detect the production of Metallo β -Lactamase (MBL) enzymes, the results showed that 20 isolates as positive results with percentage(66.6%). This percentage agreement with Charan *et al.*, (26) as the percentage of production *K .pneumoniae* for this enzyme from 71.9 - 50% and another study by Patel *et al.*, (27) pointed that the percentage of the production of bacteria for this enzyme was 40% which make bacteria resistant to a wide range of β -lactam by making antibiotics ineffective.

Double disk synergy test (DDST):

The results of Double disk synergy test showed a significant zone of enhancement between Meropenem disk and EDTA disk (Figure1) in 6 isolates (20%) out of 30 *Klebsiella pneumonia* which indicate a positive results. This ratio of the present study was close relative



with the study done by Saderi *et al.*, (28) which detected(26.5 %).In other studies held in India and Tunisia were detected a higher rates of DDST(45% , 33%) (24, 29).

Combined EDTA disk test (CMDT):

CMDT were used which showed positive results with zone inhibition size of at least 7 mm around the Meropenem combined EDTA saturated disk compared to Meropenem disks alone in 20 isolates (66.6%) out of 30 *Klebsiella pneumonia*. The results has close relative with the study which was done by Eser *et al.*, (30) that found 76% positive CMDT among Gram negative bacteria. While disagree with Gupta *et al.*, (31) who pointed that the sensitivity of CMDT was equal to that of E-test.

3. Modified Hodge test (MHT):

The results showed the presence of a clover leaf-like shaped zone of inhibition around each tested strain towards the Meropenem disk that was understood as Carbapenemases producing strain or a positive result (figure3).The present study showed that 21of 30 isolates (70 %) MHT positive. This results was close relative with the study which was done by Metwally *et al.*, (32) that the percentage of the MHT test as a positive results (85%).But this study disagree with the studies done by Noyal *et al.*, (33) and Hashemi *et al.*, (34) in India and Iran respectively which showed small percentages (12% and 16%).

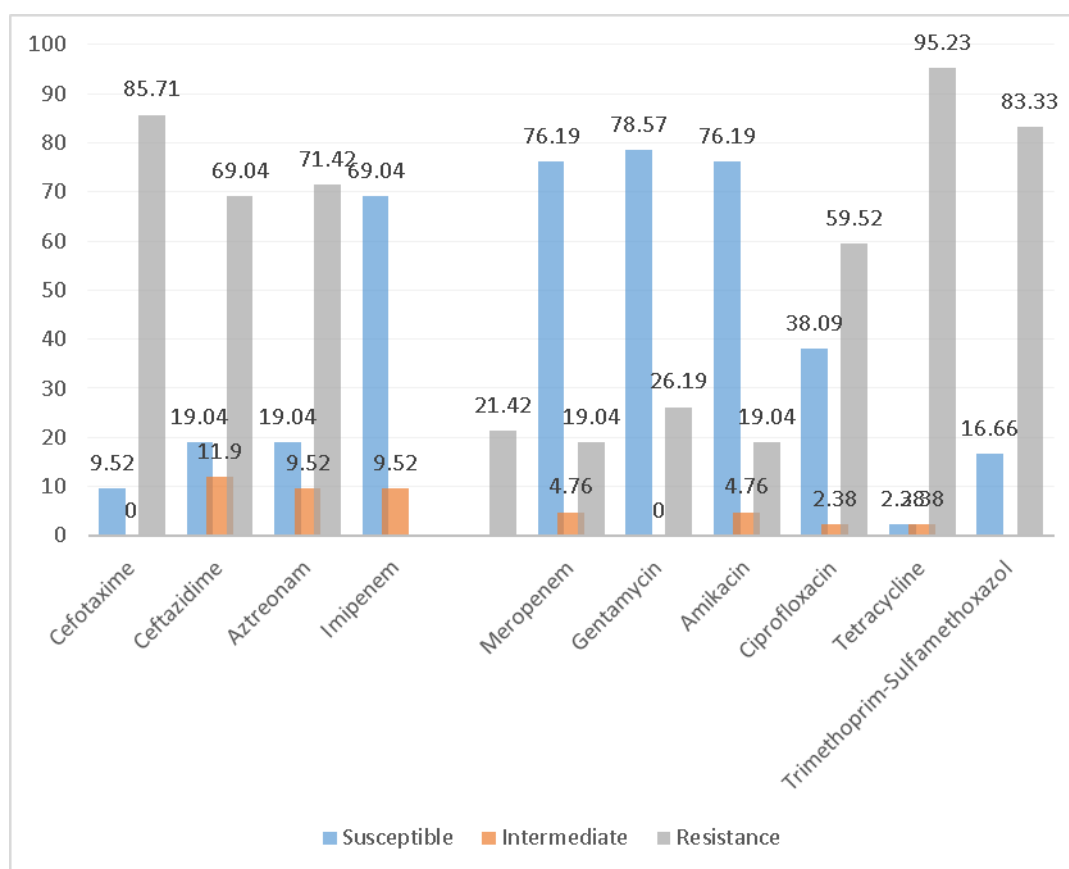
Antimicrobial sensitivity tests:

All the 42 *Klebsiella pneumonia* isolates have tested for their antibiotic susceptibility against the selected 10 antibiotics. Table (3) and showed that the resistance frequency of isolates to: Tetracycline were (95.23%), Cefotaxime(85.71%), Trimethoprim- Sulfamethoxazol(83.33%), Aztreonam(71.42%), Ceftazidime (69.04%), Ciprofloxacin(59.52%), Gentamycin(26.19%), Imipenem (21.42%), Meropenem and Amikacin(19.04 %).The most active antibiotic against all isolates of *Klebsiella pneumonia* were Gentamycin(78.57%) followed by Meropenem and Amikacin (76.19%), Imipenem(69.04%)and Ciprofloxacin (38.09%), while the minimum active antibiotic was Tetracycline(2.38%).In local study at Baghdad city; Al-Qafaji (35) pointed that 100% and 94.5% of *K. pneumoniae* isolates were resistance to Cefotaxime and Ceftazidime, respectively. Another study; Al-Obadi (36) referred that 90.5%, and 97.5% of isolates were resistant to Cefotaxime, and Imipenem, respectively. The present study gave percentage resistance (85.71%) and (21.42%) to Cefotaxime and Imipenem respectively, in compared to local studies but not agree with Imipenem result in local study. While Kevin *et al.*, (37) pointed that the resistance of *K. pneumoniae* isolates to Cefotaxime (69.5%), Ceftazidime (68.5%) and these results relatively in agreement to the present study .



Table1. antibiotic susceptibility tests of isolated strains of *K. pneumonia*

Antibiotics	Susceptible	Intermediate	Resistance
Cefotaxime	4 (9.52%)	-	36 (85.71%)
Ceftazidime	8 (19.04 %)	5 (11.90%)	29 (69.04%)
Aztreonam	8 (19.04%)	4 (9.52%)	30 (71.42%)
Imipenem	29 (69.04%)	4 (9.52%)	9 (21.42%)
Meropenem	32 (76.19%)	2 (4.76%)	8 (19.04 %)
Gentamycin	33 (78.57%)	-	11 (26.19%)
Amikacin	32 (76.19%)	2 (4.76%)	8 (19.04 %)
Ciprofloxacin	16 (38.09%)	1 (2.38%)	25 (59.52%)
Tetracycline	1 (2.38%)	1 (2.38%)	40 (95.23%)
Trimethoprim- Sulfamethoxazol	7 (16.66%)	-	35 (83.33%)

Figure 2 : antibiotic susceptibility tests and their percentage of isolated strains of *K. pneumonia*

The isolates in present study showed susceptible to Carbapenems (meropenem, imipenem) which consider the drugs of choice for many infections caused by G+ve and G-ve bacteria (Nicolau,(38). The results of this study agreement with Abdolaziz *et al.*,(39) who reported among burns patients that; the resistant rate to various antibiotics were cefotaxime (91%), aztreonam (89%), trimethoprim- sulfamethoxazole (83%), tetracycline (80%), gentamicin (72%), amikacin (71%) and imipenem (54%),while related to gentamicin and the lowest rate was associated to imipenem, meropenem, and ceftazidime. In this study it had been found that the isolated showed multi-drug resistant (MDR) which lead to high prevalence of *K. pneumonia* and have become a serious public health problem for the treatment of bacterial related infections (40).

Conclusion:

MBL enzyme have been increasing wide world which hydrolyzes all classes of β -lactam antibiotics and their quickly spread among other bacteria and that it could cause an increase in both mortality , morbidity and because of the high rate of multi-drug resistant (MDR) strains in such isolates; the early diagnosis, appropriate infection control measures and guidelines are needed to prevent the spread of the infections among patients.

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