

First Detection Of The *bla*OXA-23 Genes In A Clinical Isolates Of *Acinetobacter baumannii* IN Hillah Hospitals-IRAQ

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Abstract: Carbapenemase producing *Acinetobacter baumannii* is frequently associated with nosocomial infections. Increasing resistance to carbapenems, may significantly reduce the choice of effective antibiotics. This study was conducted to determine the occurrence of carbapenemase producing *A. baumannii* isolates obtained from Hillah hospitals. Isolates were identified according to biochemical tests and more confirmed using API 20E system. Carbapenem susceptibility was assayed by using disks diffusion test. Phenotypic detection of carbapenemase was performed using the imipenem-EDTA disk and modified Hodg tests. Then isolates were subjected to monoplex PCR targeting *bla*OXA-23 genes. 10 (0.76%) *A. baumannii* isolates were recovered from clinical samples. One (10%) of isolate was found to be imipenem and meropenem resistant (MIC > 512 µg/ml). 6(60%) gave positive result with the imipenem-EDTA disk and modified Hodg test. PCR experiments showed only four (40%) isolates were harbored *bla*OXA-23genes. The present findings suggest that emergence of *bla*OXA-23 carbapenemase producing *A. baumannii* clinical isolates in Hillah hospitals.

Keywords: *bla*OXA-23, *Acinetobacter*, Oxacillinase, Carbapenem, MDR

I. Introduction

Acinetobacter baumannii have been implicated in recent years as important nosocomial pathogen especially in intensive care unit (8). *A. baumannii* is responsible for 3-5% of nosocomial pneumonia, and one of the most common presentations in mechanically ventilated patients in intensive care units (14). Carbapenems are the drugs of choice for this pathogen and carbapenem-resistant *A. baumannii* has been frequently hospital encountered (13). Meropenem and imipenem are carbapenems that remain active against organisms carrying most Ambler classes of β -lactamases which include many Gram-negative bacilli, including *Acinetobacter* spp. However, carbapenem resistance is increasingly encountered in *Acinetobacter* isolates worldwide (1). One of the major mechanisms of carbapenem resistance in this pathogen is the production of carbapenem hydrolyzing β -lactamases. These specific groups of β -lactamases are categorized into class B metallo β -lactamases (MBLs) including IMP and VIM, NDM and class D (Oxacillinases) including OXA-23-like, OXA-24/40-like and OXA-58 (4).

In *A. baumannii*, the OXA-type class D β -lactamases are arranged into five families: the OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like and OXA-143-like groups, respectively. It is noteworthy that the genes encoding for the OXA-51 like enzymes are intrinsic to the species, while for other families the genes have been acquired from other sources (35).

The first oxacillinase gene was identified in *A. baumannii* on a plasmid, named ARI-1 but after its detailed characterization it was renamed OXA-23 (32).

Epidemics with *A. baumannii* strains harboring the *bla*OXA23 have been reported worldwide (9 ,17, 20, 45). In a recent survey, it was found that the isolates carrying the *bla*OXA23 are found globally and consistent with the clonal spread (21).

In Iraq, little attention has been paid to the carbapenemase producing isolates. However, in the Hillah city, no information are regarding the molecular studies of the occurrence of OXA-23-like *A. baumannii* producing clinical isolates. Hence, the proposed aim of this study is to identify and detect OXA-23-like producing *A. baumannii* isolates recovered from hospital settings in the Hillah city.

II. Material And Methods

Isolation and Identification of Isolates:

A total of 1300 clinical specimens (included 588 burn swabs, 136 wound swabs, 50 from throat, 204 urine, 110 stool, 20 sputum, 162 blood, 15 ears and 15 eyes) were collected from patients in Hillah hospitals over one year period starting from March, 2014 to March, 2015. Isolates were recovered from clinical samples after culturing on MacConkey agar and incubated for overnight at 37°C, non lactose fermenting bacteria were sub-cultured and incubated for additional overnights. Suspected bacterial isolates which their cells are Gram negative coccobacillary or diplobacillus and negative to oxidase which further identified using API20 E system.

Antimicrobial susceptibility testing:

Isolates were cultured on Mueller-Hinton agar and their susceptibilities to different antibiotics were tested by disk diffusion method according to the Clinical and Laboratory Standard Institute's guidelines (12).

MIC determination:

Depending on manufacturer's instructions the antibiotic stripes were applied to the agar surface, the antibiotic instantaneously diffuses into the surrounding medium in high to low concentration from one end of the strip to the other. The gradient remains stable after diffusion, and the zone of inhibition created takes the form of ellipse (Liofilchem manufacture).

Imipenem-EDTA double disks method:

Screening for metallo β -lactamases (MBL) was performed using disks containing 1900 μ g of EDTA plus 10 μ g of imipenem disk were placed on the inoculated plates containing Muller Hinton agar. An increase of ≥ 17 mm in zone diameter in the presence 1900 μ g of EDTA compared to imipenem alone indicated the presence of an MBL (25)

Modified Hodg test:

Imipenem was used for carbapenemase detection as described by Lee and his colleagues (24). Positive test has a clover leaf-like indentation of *E. coli* Top-10 growing along the test organism growth streak within the imipenem disk diffusion zone.

PCR amplification:

DNA was extracted from the isolates by using genomic extraction mini kit according to the manufacture instructions (Bioneer company, Korea). To amplify the genes encoding carbapenemases, a monoplex-PCR was run using the primers of *bla*_{OXA}-23 (501bp: F/5-GAT CGG ATT GGA GAA CCA GA-3' and R/5-ATT TCT GAC CGC ATT TCC AT-3') were described by Turton and his colleagues (43).

Amplification was performed in a 20 μ l volume as recommended by Promega Master mix instruction. PCR amplifications were carried out on a thermal cycler (Prime, England). The cycling conditions for amplification were as follows: initial denaturation of 94°C for 3 min and 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C, followed by 3 min at 72°C. Amplified products were detected by agarose gel electrophoresis in 1% Tris-borate-EDTA (TBE) agarose (Promega, USA) and staining with ethidium bromide. The electrophoresis result was detected by using gel documentation system (Claver, England).

III. Results

Based on the conventional biochemical tests and API20E system enabled to isolation and identification of 10 (0.76%) isolates as *A. baumannii* from the 1300 clinical samples (Table 1). As determined by disk-diffusion method, all *A. baumannii* isolates exhibited different pattern of resistance to different antibiotic agents (Fig.1), demonstrating highest resistance to penicillins (carbenicillin and ampicillin) with rate of resistance of 10(100%) isolates, whereas 3(30%) of isolates were resistance to piperacillin.

Resistance to other drug classes varied among the isolates. For cephalosporin antibiotics, a higher resistance was also detected with 6(60%) of isolates being resistant to ceftazidime and cefotaxime each other, and 7(70%) to cefepime. The results also revealed that were high resistant rates 8(80%) isolates for each amoxiclav and aztreonam of monobactam's antibiotics.

For the carbapenem antibiotics, imipenem, meropenem and ertapenem displayed the lowest resistant rate 1(10%) isolates. Aminoglycosides resistance was variable, 7(70%) to each of tobramycin, and gentamicin and 5 (50%) to amikacin.

The resistance to quinolones, (ciprofloxacin) was detected as 4(40%) isolates. Percentages of resistance of isolates to the remaining antibiotics were as follows: 2(20%) to each of tetracycline and doxycycline, trimethoprim-sulfamethoxazole 5(50%), chloramphenicol 8(80%), Polymyxin B 5(50%) and Colistin sulphate 7(70%). Results revealed that all tested isolates were resistant to at minimum of 3 classes of antibiotics, hence these isolates were considered to be multidrug resistant.

Table (1): Distribution of bacterial isolates recovered from clinical samples among different hospitals in Hillah city.

Hospital's name	No. of samples	No. (%) of <i>Acinetobacter baumannii</i> isolates	No. (%) of other bacterial spp. isolates	No. (%) of no growth cultures
Al- Hillah Teaching Hospital	885	7 (0.8%)	710 (80%)	168 (18%)
Babylon Teaching Hospital for Maternity and Pediatric	415	3 (0.7%)	235 (56.6%)	177 (42.6%)
Total	1300	10 (0.76%)	945 (72.69%)	345 (26.5%)

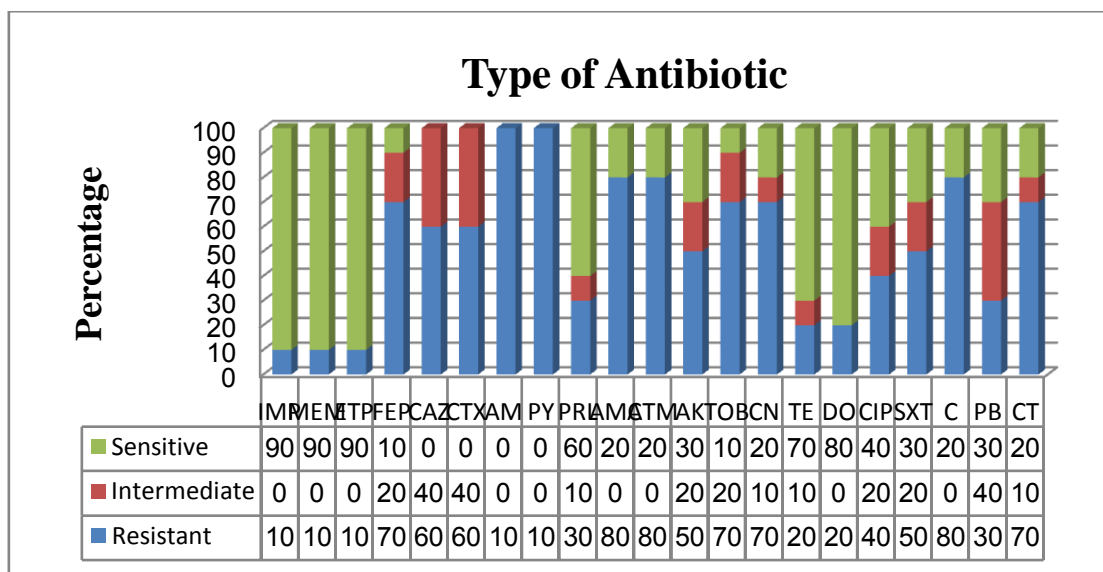


Figure (1) : Antibiotics susceptibility profile of *A. baumannii* isolates by disk diffusion method (n=10) (IMP, Imipenem ; MEM, Meropenem ; ETP, Ertapenem ; FEP, Cefepime ; CAZ, Ceftazidime ; CTX, Cefotaxime ; AM, Ampicillin ; PY, Carbenicillin ; PRL, Piperacillin ; AMC, Amoxi-clav ; ATM, Aztreonam ; AK, Amikacin ; TOB, Tobramycine ; CN, Gantamicin ; TE, Tetracycline ; DO, Doxycycline ; CIP, Ciprofloxacin ; SXT, Trimethoprim-Sulfamethoxazole ; C, Chloramphenicol ; PB, Polymyxin B ; CT, Colistin sulphate).

MIC determination:

Minimum inhibitory concentrations of *A. baumannii* isolates against carbapeneme antibiotics (imipenem, meropenem, ertapenem) were determined by using E-test and microbroth dilution methods according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Table (2) revealed that *A. baumannii* isolates were resistant to imipenem, meropenem, and ertapenem with concentrations beyond values: 0.032 µg /ml - >512 µg /ml.

Table (2) : MIC of carbapenem antibiotics

Isolates No.	MIC (µg/ml)		
	IMP	MER	ERT
1	0.75	0.032	0.032
2	0.25	0.38	2
3	1	0.047	0.023
4	0.19	0.38	1.5
5	0.38	1.5	6
6	> 512	> 512	> 512
7	0.19	0.75	3
8	1	0.5	2
9	1.9	0.047	0.032
10	1	0.064	0.047

Phenotypic detection of carbapenem production:

1(10%) isolate demonstrated enhancement of inhibition zone, with the imipenem-EDTA test whereas six isolates showed positive results with modified Hodg test.

Genotypic detection of *bla*OXA-23 genes :

*bla*OXA-23 genes were appeared in (40%) of *A. baumannii* isolates PCR products using specific primers gene. (Fig. 2). Consequently, table (3) shows the isolates that harbored *bla*OXA-23 genes appeared as extensive drug resistant (XDR), which exhibited resistance to at least 5 classes of antibiotics were used in this study.

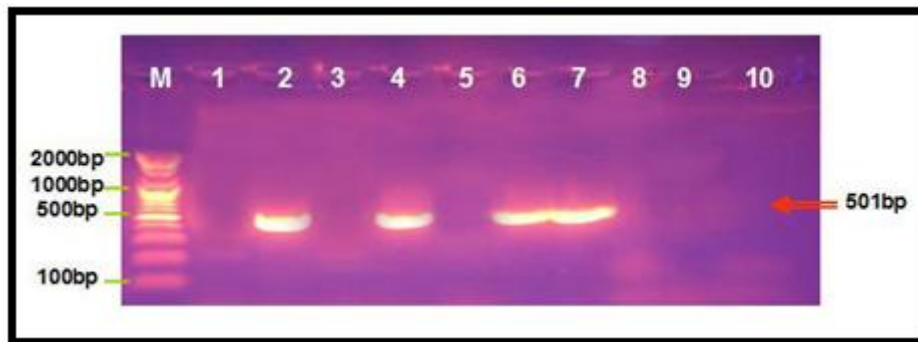


Figure (2): Agarose gel electrophoresis (1.5% agarose,70 volt for 1-2 hrs) for *bla*OXA-23 gene product (amplified size 501bp) using DNA template of *Acinetobacter baumannii* isolates extracted by using genomic extracting Mini Kit. Lane (M), DNA molecular size marker (100- bp Ladder). Lanes (2 , 4, 6, and 7) of *A. baumannii* isolates show positive results with *bla*OXA-23 gene . lanes (1, 3, 5, 8, 9 and 10) show negative results with *bla*OXA-23 gene.

Table (3) : Antibiotics resistant pattern of carbapenemase genes positive *A. baumannii* isolates.

Type of resistance	No. (%) of <i>A. baumannii</i> isolates (N=10)	No. of antibiotics classes resisted
MDR	10 (100%)	3 or more
XDR	2 (20%)	9
	3 (30%)	7
	4 (40%)	6
	5 (50%)	5
PDR	1 (10%)	11

IV. Discussion

The current report provided an evidence that the *A. baumannii* may be silently spread with low proportion 0.76% (Table 1) in a hospital settings and highlighted the threat of undetected reservoirs. However, The source of infection may include health care device or the environment can involve with transfer of microorganisms between staff and patients (22).

Results from Figure (1) revealed that higher resistant rate was found for ampicillin and carbenicillin were (100%) for each other, while for piperacillin was (30 %). This result in agreement with a pervious study in Hillah by Al-Warid (3), who found that all 11 (100%) β -lactam resistant *A. baumannii* isolates were resistant to ampicillin , carbenicillin and piperacillin . Alsehlawi and his colleagues (2) stated that all *A. baumannii* isolates were resistant to ampicillin , carbenicillin (100%) for each other and (91.6%) to ticarcillin . High resistance to this class of antibiotics may be due to widespread use of these antibiotics in Hilla hospitals. The present study showed a high level of resistance to cephalosporins : ceftazidime and cefotaxime (60%) each other, and (70%) for cefepime. There is also a wide range of resistance to aztreonam (80%). Alsehlawi, and his colleagues (2) reported that resistance rate of *A. baumannii* isolates to ceftazidime , cefotaxime and cefepime were (100%). A report by Chaiwarith and his colleagues (10) documented that susceptibility to ceftazidim and cefpirome were equal , (70%) in *A. baumannii* isolates collected from Thailand hospitals. High level of resistance to third generation cephalosporins could be attributed to the production of ESBLs, since it mediates resistance to broad spectrum cephalosporins (e.g. ,ceftazidime ,ceftriaxone and cefotaxime) and aztreonam (37). In the present study there was a high level of resistance (80%) to β -lactam / β -lactamase inhibitor combination (amoxicillin / clavulanic acid). This is likely due to the heavy selection pressure from overuse of this antibiotic and seem to be losing the battle. A similar result was recorded by Alsehlawi and his colleagues (2) who noted that (75%) of *A. baumannii* were resistant to amoxi-clav. In spite of the restricted use of cefotaxime in treatment of bacterial infection in Iraq , results of this study revealed a higher resistance (70%) to cefotaxime among clinical isolates

of *A. baumannii*. This result is in accordance with the findings recorded by other researchers, Al-Warid,(100%) in Hillah(3) and AlSehlawi and his colleagues,(100%) in Najaf(2). The resistance to cephalosporins may be as a result of the development of porin-deficient mutants (28). In addition, increasing numbers of bacterial strains express different types of β -lactamases including inducible and / or plasmid mediated AmpC type of enzyme may also increase the chance for resistance to cephalosporins (38).

Results from (Fig.1) showed that resistance rates to imipenem, meropenem and ertapenem were (10%). This result is lower than that reported by other studies conducted in Turkey which reported that the resistance rate of *A. baumannii* isolates collected from clinical samples to imipenem was (46.7%) and to meropenem was (53.3%) (31). While in a local study in Najaf , AlSehlawi and his colleagues (2) found that only four isolates (41.6 %) of *A. baumannii* were resistant to imipenem and meropenem antibiotics . Leepethacharat and Oberdorfer demonstrated (65%) and (50%) resistance to imipenem and meropenem , respectively by *A. baumannii* in a surveillance study at Chiang Mai University Hospital in Thailand (27). While in USA Trotter and his colleagues who found that (87%) of *A. baumannii* resist to imipenem(43).

Meropenem is well tolerated and offers several potential advantages, including greater *in vitro* activity against Gram negative pathogens and the option of bolus administration (46) . Beside these , problem of renal metabolism of imipenem , and risk of seizures (36), and availability of meropenem only in Hillah hospitals might be the reasons behind possible greater use of meropenem over imipenem and hence the high prevalence of resistance.

Ertapenem is the least active carbapenem against most strains producing carbapenemase and therefore the first marker that indicates the likelihood of carbapenemase occurrence (30,41). Specificity is questioned because bacteria with ESBL and porin mutations are also resistant to ertapenem (19)

Reasons behind resistance may be due to inappropriate duration of antibiotic therapy and sub-therapeutic concentrations of the drug (6,34) .

Results of the present study revealed that amikacin was more effective (80%) than other aminoglycosides, gentamicin and Tobramycin (60%) for each other. This result was parallel with other studies worldwide , as with Leepethacharat and Oberdorfer in Thailand (27) and Özdemir and his colleagues In Turkey(31). In another study in Najaf Alsehlawi and his colleagues who found that resistance against aminoglycosides were (58.3%) to amikacin , whereas gentamicin and Tobramycin (83.3%),(2).

High efficiency of amikacin may be due to its less vulnerability to bacterial enzymes than other aminoglycosides . In both *A. baumannii*, gentamicin and Tobramycin resistance is often due to the expression of a variety of modifying enzymes including aminoglycoside modifying enzymes (AME), acetylases , phosphorlyases and adenylases which can impair the effectiveness of antibiotics. Other resistance mechanisms include changes in bacterial membrane permeability and altered ribosomal proteins (7) .

As shown in figure (1) resistance to quinolones antibiotics , (ciprofloxacin) was (40%). Quinolone resistance is typically encoded chromosomally .In this study, resistance against fluoroquinolones may reflect antibiotic pressure in Hilla hospitals. In China , Zhou and his colleagues reported high resistance to quinolones (> 95%) among clinical isolates of *A. baumannii* (48). In another study in Najaf Alsehlawi and his colleagues who found that resistance against ciprofloxacin was (91.6 %),(2).

Quinolone resistance results from mutations in the chromosomally encoded type II topoisomerases, and via the up regulation of efflux pumps, or point- related genes (15,42). The plasmid *qnr* genes play an emerging role in the dissemination of fluoroquinolone resistance (18).

Results of present study show highly resistant level to colistin sulphate (80%) with moderate resistance to Polymyxin B (50%). This result had been convergent with study at Chiang Mai University Hospital in Thailand (27).

Percentages of resistance of isolates to the remaining antibiotics were as follows : tetracycline (20%), doxycycline (20 %) , trimethoprim-sulfamethoxazole (50%) and chloramphenicol (80%). These results were parallel with previous studies in Brasil (41) and with Patwardhan, and his colleagues at India (33).This may be due to multiresistance plasmid harboring *A. baumannii* (33).

The high levels of resistance to antibiotics in the present study may be as a result of both intrinsic and acquired mechanisms .The resistance is widespread and constitutes serious clinical threat (29). In addition, the selection pressure of antibiotics in hospital environment lead to multiple resistance to these drugs. El-Astal, mentioned that inappropriate and incorrect administration of antimicrobial agents and lack of appropriate infection control strategies may be the possible reasons behind increasing resistant rate of *A. baumannii* to common used antimicrobial drugs (16).

All *A. baumannii* isolates were screened by two phenotypic tests for carbapenemase production. The present study showed that 6 (60%) of the isolates gave positive results by imipenem- EDTA disk test . Different studies which have used the IMP-EDTA to detect MBLs production in *A. baumannii* reported that (33%) of isolates have enhancement of inhibition zone, with the IMP-EDTA test (2) . However, there are four isolates which gave negative results with EDTA disk synergy test.

The most easily performed test for Carbapenemase detection is the modified Hodge's test, which has been found to be 100% sensitive for the detection of the carbapenemase (24). Out of the 10 *A. baumannii* isolates which were enrolled in this study, 6 (60%) isolates were found to produce the carbapenemase enzyme by MHT and all the remaining isolates were found to be carbapenemase negative. In a previous local study, Alsehlawi and his colleagues reported that 4 (33.3%) of *A. baumannii* isolates recovered from Najaf hospitals were confirmed as carbapenemase producer using modified Hodge's test, whereas the same isolate gave negative result with imipenem-EDTA synergy test (2). Another study from Croatia the Hodge test showed that 74% (72/97) of the *A. baumannii* isolates were positive for carbapenemase production. (18), whereas in a study from Pakistan has shown that 17% of *A. baumannii* were positive for carbapenemase production by MHT (5).

Oxacillinases are only weakly active against carbapenems and are largely confined to *Pseudomonas* and *Acinetobacter* species and only rarely in Enterobacteriaceae (47). *bla*OXA-23 represented a new subset of the OXA family. It has been identified in outbreaks of carbapenem-resistant *Acinetobacter* in Brazil, Korea and United Kingdom (13, 22, 39, 44). Result from present study show that 4(40%) *A. baumannii* isolates had *bla*OXA-23 genes (Fig. 2). In another study in Iraq, Alsehlawi, and his colleagues who found 2(40%) *A. baumannii* isolates had *bla*OXA-23 genes positive in Najaf hospitals (2). In contrast in Taiwan study, Lee, and his colleagues documented only one isolate (4%) of *A. baumannii* was harbored *bla*OXA-23 gene (26).

As in the present study, such isolates (*bla*OXA-23 positive *A. baumannii*) exhibit resistance to most antimicrobials that recommended by CLSI (2012) and appeared to be extensive antibiotic resistance (XDR) (Table 3), this may creating a serious problem for choice of therapy, this results was more identical with the report of emergence XDR in *A. baumannii* isolates from patients in ICUs of Samsung Medical Center in Seoul, South Korea (23). Hence, The occurrence of isolates contain *bla*OXA-23 in Hillah hospitals may resulted from transfer of plasmid among resistant isolates rather than, several isolate may produce identical restriction pattern suggest the dissemination of *bla*OXA-23 due to a clonal spread of resistant *A. baumannii* isolates (11).

V. Conclusion

Our study has shown the spreading of multidrug resistant and *bla*OXA-23 harbored *A. baumannii* isolates among patients with different infections. Hence, it is suggested that, such isolates, which consequently poses an increased threat to hospitalized patients in Hillah hospitals and more importantly, avoiding misuse and overuse of antibiotics may reverse the undesired effects of multidrug resistant and OXA-23producing bacteria.

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