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First Report of the blaOXA-23 Gene in a Clinical Isolates of *Acinetobacter baumannii* in Najaf Hospitals-Iraq

[1]Zuhair S. Alsehlawi, [1]Jamal M. Alshara, [2]Zainab J. Hadi and [2]Ali M. Almohana
[1]Department of Basic Science, College of Dentistry, Kufa University, Najaf, Iraq
[2]Department of Microbiology, College of Medicine, Kufa University, Najaf, Iraq

Corresponding Author: Zuhair S. Alsehlawi, Department of Basic Science, College of Dentistry, Kufa University, Najaf, Iraq Tel: 009647801294304

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 Najaf hospitals. Isolates were identified according to API 20NE system and more confirmed using 16Sr RNA gene. Carbapenem susceptibility was assayed by microbroth dilution and other antibiotics 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 blaOXA-23, blaIMP and blaVIM genes. Twelve (1.5.%) *A. baumannii* isolates were recovered from clinical infections. Five (41.6%) of isolates were found to be imipenem and meropenem resistant (MIC ranged 128-256 μg mL^{-1}), of which, 4 (66.6%) gave positive result with the imipenem-EDTA disk and modified Hodg test. PCR experiments showed only two (33.3%) isolates were harbored blaOXA-23 gene. The present findings suggest that emergence of OXA-23 carbapenemase-producing *A. baumannii* clinical isolates in Najaf hospitals.

Key words: *Acinetobacter baumannii*, carbapenem, OXA-23

INTRODUCTION

Acinetobacter baumannii have been implicated in recent years as important nosocomial pathogen especially in intensive care unit (Bergogne-Berezin and Towner, 1996). *Acinetobacter baumannii* is responsible for 3-5% of nosocomial pneumonia and one of the most common presentations in mechanically ventilated patients in intensive care units (Dijkshoorn *et al*., 1993). Carbapenems are the drugs of choice for this pathogen and carbapenem-resistant *A. baumannii* has been frequently hospital encountered (Dalla-Costa *et al*., 2003). 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 (Afzal-Shah and Livermore, 1998). 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 and class D (Oxacillinases) including OXA-23-like, OXA-24/40-like and OXA-58 (Ambler *et al*., 1991). Carbapenem-hydrolyzing OXA enzymes are the most important cause of carbapenem resistance in *A. baumannii* worldwide (Poirel and Nordmann, 2006). These began to be described over a decade ago, in 1993, first description of ARI-1, later renamed OXA-23, in an imipenem-resistant *A. baumannii* strain from a patient in the Edinburgh Royal Infirmary (Paton *et al*., 1993). Isolates

of *A. baumannii* producing oxacillinases, OXA-23, OXA-24 and OXA-58, are prevalent in Europe and Americas (Heritier *et al*., 2005; Villegas *et al*., 2007) and highly prevalent in Asia-Pacific region including Thailand (Mendes *et al*., 2009). In Iraq, little attention has been paid to the carbapenemase producing isolates. However, in the Najaf city, no information are regarding the molecular studies of the occurrence of OXA-23 carbapenemase *A. baumannii* producing clinical isolates. Hence, the proposed aim of this study is to identify and detect OXA-23 carbapenemase producing *A. baumannii* isolates recovered from hospital settings in the Najaf city.

MATERIALS AND METHODS

Isolation and identification of isolates: A total of 770 clinical specimens (included 450 sputum, 210 urine and 110 burns swabs) were collected from patients in Najaf hospitals over four months period starting from April to July, 2011. Isolates were recovered from clinical samples after culturing on MacConkey agar and incubated for overnight at 37°C, non lactose fermenting bacteria (colorless or slightly beige) were subcultured 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 NE system and confirmed by molecular detection using 16S ribosomal RNA gene according to (Misbah *et al*., 2005).

Antimicrobial susceptibility testing: Isolates were plated on Mueller-Hinton agar and their susceptibilities to different antibiotics were tested by disk diffusion method according to the NCCLS (2007).

Phenotypic detection of carbapenemase

Imipenem-EDTA disk method: Screening for Metallo β-lactamases (MBL) was performed using disks containing 1900 μg of EDTA plus 10 μg 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 μg of EDTA compared to imipenem alone indicated the presence of an MBL (Lee *et al*., 2003).

Modified Hodg test: Imipenem was used for carbapenemase detection as described by Lee *et al*. (2001). Positive test has a clover leaf-like indentation of *E. coli* ATCC 25922 growing along the test organism growth streak within the imipenem disk diffusion zone.

PCR amplification: DNA was extracted from the isolates by boiling one to three colonies in 100 μL of sterile ultrapure water for 10 min followed by centrifugation for 1 min at 14,000 rpm (Brown *et al*., 2005). To amplify the genes encoding carbapenemases, a monoplex-PCR was run using the primers of blaOXA-23 (1065 bp: F/5'-GATGTGTCATAGTATTCGTCG-3' and R/5'-TCACAACAACTAAAAGCACTG-3'), blaIMP (587bp:F/5' CGGCCKCAGGAGMGKCTTT-3' and R/5'-AACCAGTTTTGCYTTACYA-3') and blaVIM (633bp: F/5'-ATTCCGGTCGGRGAGGTCCG-3' and R/5'-GAGCAAGTCTAGACCGCCCG-3') were described by Yin *et al*. (2008) and Yu *et al*. (2006). Amplification was performed in a 25 μL volume as recommended by Promega Go Tag Green Master mix instruction. PCR amplifications were carried out on a thermal cycler (Gene Amp, Singapore). The cycling conditions for amplification were as follows: For blaOXA-23 gene, initial denaturation of 5 min at 95°C and 30 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C, followed by 7 min at 72°C, for blaIMP and blaVIM initial denaturation at 93°C for 3 min and 40 cycles of 1 min at 93°C, 1 min at 55°C and 1 min at 72°C, followed by 7 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 (Biometra).

RESULTS

Based on the conventional tests and amplification of 16S ribosomal RNA gene enabled to isolation and identification of 12 (1.5%) isolates as *A. baumannii* from the 770 clinical samples, excluding scant growth (Table 1).

According to the results of the antimicrobial susceptibility testing by disk diffusion for 12 *A. baumannii* isolates, all isolates were resistant to most of the antimicrobials tested. The highest rates of susceptibility were observed with piperacillin-tazobactam (41.6%; n = 5), ampicillin-sulbactam (50%; n = 6) and amikacin (58.3%, n = 7) (Fig. 1).

Microbroth dilution assay showed that 5 (41.6%) isolates were found to be carbapenem resistant, MICs ranged from 128 to ≥256 μg mL^{-1}, of which, 4 (33.3%) demonstrated enhancement of inhibition zone with the imipenem-EDTA test and modified Hodg test, while no remarkable distinct change was noticed in the others isolates (Table 2).

Two isolates (2/5; 40%) of carbapenem resistant *A. baumannii* gave blaOXA-23 PCR products using specific primers gene. Moreover, the amplification of DNA of 5 isolates of carbapenem resistant with blaIMP and blaVIM primers, observed presence only one (1/5; 20%) of blaIMP positive isolate. The blaVIM was not detected in this study (Fig. 2).

Consequently, Table 3 shows the isolates that harbored OXA-23 gene appeared as Extensive Drug Resistant (XDR) which exhibited resistance to at least 5 classes of antibiotics were used in this study.

Table 1: Distribution of *Acinetobacter baumannii* isolates among clinical samples recovered from Najaf hospitals

		Acinetobacter baumannii isolate	
Clinical sample	No.	No.	%
Sputum	450	4	0.88
Urine	210	6	2.80
Burn swab	110	2	1.80
Total	770	12	1.50

Table 2: Frequency of carbapenem susceptibility and carbapenemase producing *A. baumannii* isolates

		Phenotype of carbapenemase production	
Acinetobacter baumannii isolate (n = 12)*	Resistance to carbapenem (MIC μg mL^{-1})	Imipenem-EDTA test	Modified Hodge test
AU1	8	-	-
AU2	128	-	-
AU3	≥256	+	+
AU4	≥256	+	+
AS5	8	-	-
AS6	4	-	-
AS7	8	-	-
AB8	4	-	-
AB9	≥256	+	+
AB10	128	+	+
AS11	8	-	-
AS12	4	-	-
Total	5 (41.6%)	4 (33.3%)	4 (33.3%)

*AU: *Acinetobacter baumannii* isolated from urine, AS: From sputum and AB: From burn wound

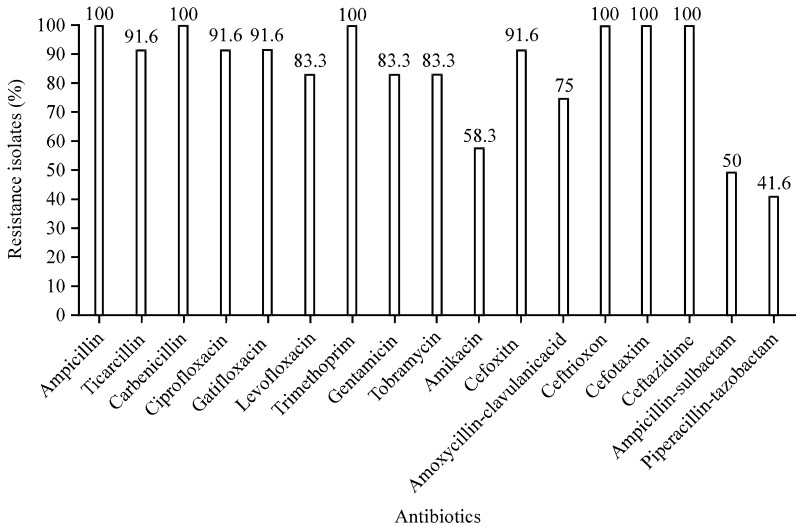

Fig. 1: Antibiotic susceptibility pattern of *Acinetobacter baumannii* isolates (n = 12) according to NCCLS (2007)

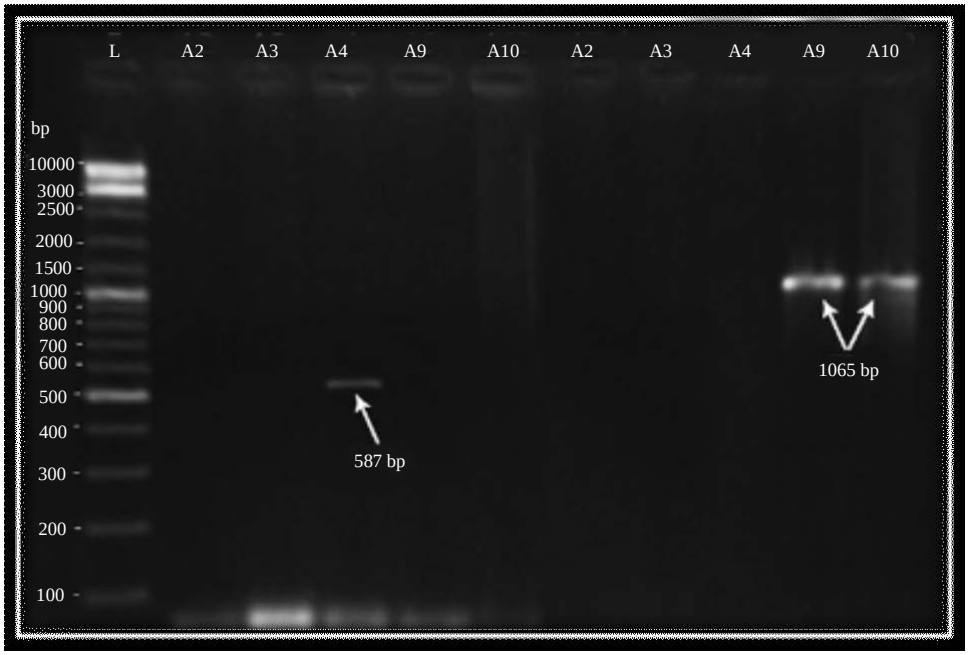

Fig. 2: Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of carbapenem resistant *Acinetobacter baumannii* isolates and amplified with blaIMP and blaOXA-23 gene primers. The electrophoresis was performed at 70 V for 1.5 h. Lane (L): DNA molecular size marker (l0000 bp ladder), Lane A4: *Acinetobacter baumannii* isolates show positive results with blaIMP (587 bp), Lanes (A2, 3, 9 and 10): Show negative results with blaIMP gene. Lanes A9 and A10: Show positive results with blaOXA-23 gene (1065 bp), lanes A2, A3 and A4: Show negative results

Table 3: Antibiotic susceptibility profiles of blaOXA-23 gene harbored *Acinetobacter baumannii* clinical isolates

Isolate code no.	Resistant antibiotics
AB9	AMP, AC, CTX, CI, CAZ, ATM, FOX, TOB, PTP, CN, TEP, CIP, GT, TIC, PY and A/S
AB10	AMP, AC, CTX, CI, CAZ, ATM, FOX, TOB, CN, CIP, GT, LEV, TIC, PY and A/S

AMP: Ampicillin, AC: Amoxiclav, CTX: Cefotaxime, CI: Ceftriaxone, CAZ: Ceftazidime, ATM: Aztreonam, FOX: Cefoxitin, TOB: Tobramycin, PTP: Piperacillin-tazobactam, CN: Gantamycin, TEP: Trimethoprime, CIP: Ciprofloxacin, LEV: Levofloxacin, GT: Gatifloxacin, TIC: Ticarcillin, PY: Carbenicillin and A/S: Ampicillin-sulbactam

DISCUSSION

The current report provided an evidence that the *A. baumannii* may be silently spread with low proportion 1.5% (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 (Kollef and Schuster, 1994).

Antibiotic resistance is a major clinical problem in treating infections that caused by this bacteria. Using the disk diffusion method, results showed high resistant rates (83.3-100)% of *A. baumannii* to carboxypenicillin (carbenicillin and ticarcillin), fluoroquinolones (ciprofloxacin, gatifloxacin and levofloxacin), extended spectrum cephalosporins (cefotaxime, ceftriaxone, ceftazidime and cefoxitin) and aminoglycosides (gentamycin and tobramycin). The highest resistant were also found against amoxiclav and trimethoprim (Fig. 1). This result is in accordant with the results being reported in Carvalho *et al*. (2011). Reports hypothesized that the versatility of plasmids together with the abuse of antimicrobials in human medicine may have largely contributed to the spread of antimicrobial resistance worldwide (Carattoli, 2009). In the same manner, piperacillin-tazobactam, ampicillin-sulbactam and amikacin were the most active drug after carbapenem, study results similar to report from Iran, Feizabadi *et al*. (2008) found that 42.1 and 38.2% of carbapenem resistant *A. baumannii* were susceptible to piperacillin-tazobactam and amikacin, respectively. These findings suggest the presence of another resistance mechanism that has not been previously investigated, such as extended spectrum β-lactamases, the modification of penicillin-binding proteins and the up-regulation of the efflux system (Zarrilli *et al*., 2009).

Microbroth dilution assay (Table 2) revealed high degree of resistant to carbapenem (MIC range 128-≥256 μg mL^{-1}). Resistance to this antibiotic has emerged due to the production carbapenem-hydrolyzing enzyme (carbapenemases) among *A. baumannii* (Kohlenberg *et al*., 2009). Moreover, the resistance to carbapenems may involve several combined mechanisms other than carbapenemases, include modifications to outer membrane permeability and hyperproduction of AmpC β-lactamases (Nordmann *et al*., 2009).

Recently, modified Hodge test and imipenem-EDTA disc test became the most acceptable confirmatory test to detect all type of carbapenemases for infection control and epidemiological purpose (Lee *et al*., 2003). Phenotypic screening for carbapenemase production confirmed that 4 (33.3%) *A. baumannii* isolates were carbapenemase producer by both methods (Table 2). The main enzymes may involve in carbapenem resistance are OXA-carbapenemases (Kohlenberg *et al*., 2009).

Interestingly, nosocomial outbreaks with *Acinetobacter* strains producing these enzymes have been reported from various region in the world (Stoeva *et al*., 2008). Present study recorded the first two blaOXA-23 harbored carbapenem resistant *A. baumannii* clinical isolates in Najaf hospital (Fig. 2). This result is correspond with same study in Irish Republic was the first report of two OXA-23 carbapenemases in *Acinetobacter* spp. isolated from a biliary drain fluid specimen and the second isolate was cultured from a sputum specimen (Boo *et al*., 2006). However, many strains of OXA-23 producing *A. baumannii* from the same clone were responsible for an epidemic of nosocomial infection from 2005-2007 in Tunisia (Mansour *et al*., 2008).

As in the present study, such isolates (blaOXA-23 positive *A. baumannii*) exhibit resistance to most antimicrobials that recommended by NCCLS (2007) and appeared to be extensive antibiotic resistance (XDR) (Table 3), this may creating a serious problem for choice of therapy, this result 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 (Kim *et al*., 2006). Hence, the occurrence of isolates contain blaOXA in Najaf hospital may resulted from transfer of plasmid among resistant isolates rather than, several isolate may produce identical restriction pattern suggest the dissemination of OXA-23 due to a clonal spread of resistant *A. baumannii* isolates (Chang *et al*., 2012). In present study amplification for blaIMP metallo β-lactamases (class B) were negative but study detected the blaVIM type metallo β-lactamases allele in one (20%) isolate of carbapenem-resistant *A. baumannii* (Fig. 2). Plasmid mediated VIM enzymes which are located within a variety of integron structures which they have been incorporated as gene cassettes in *A. baumannii*. In addition, carbapenem-resistant *A. baumannii* have been reported which possess metallo β-lactamase instead of plasmid mediated class D (OXA type enzyme) (Walther-Rasmussen and Hoiby, 2006).

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

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