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Genotypic and phenotypic detection of carbapenemases in imipenem resistant clinical isolates of *Acinetobacter baumannii* from ICUs

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ABSTRACT

Acinetobacter baumannii is a significant nosocomial pathogen with multiple drug resistance and emerging resistance to carbapenems (the last resort drugs), so the treatment of *Acinetobacter baumannii* hospital-acquired infections is very complex. In this study the detection of the carbapenemase enzymes responsible for carbapenem resistance in imipenem resistant *A. baumannii* clinical isolates isolated from intensive care units (ICUs) was performed. This detection was carried out on a genotypic and phenotypic basis. Out of 32 isolates of *Acinetobacter baumannii* from ICUs of Zagazig University hospitals, Sharkia, Egypt, 24 imipenem resistant isolates were used to detect carbapenemases producers. The modified Hodge test, the AmpC Disk test, and Combined disk test using EDTA with CAZ and IPM were performed for the screening of carbapenemase, Amp C enzyme and metallo-β-lactamase production respectively. Polymerase chain reaction (PCR) assay were performed for the detection of genes encoding for OXA-23-like, OXA-24-like, OXA-51-like and OXA-58-like carbapenemase. All isolates showed 84.37% resistance to cefepime and cefuroxime, 81.25% resistance to cefotaxime, 78.1% resistance to ceftriaxone,75% resistance to ceftazidime, meropenem and imipenem,71.9% resistance to pipracillin, 65.6% resistance to amikacin trimethprim/sulfamethoxazole and ofloxacin, 68.75% resistance to ciprofloxacin, 59.37% resistance to and gentamicin, 46.88% resistance to levofloxacin. All isolates were sensitive to colistin. All of the 24 isolates (100%) showed positive results in the modified Hodge test and positive results in the Amp C disk test and Combined disk test. They all (100%) possessed the encoding gene for an intrinsic OXA-51-like carbapenemase and an acquired OXA-23-like carbapenemase in the PCR assay. The results revealed that all of the 24 Imipenem resistant *Acinetobacter baumannii* [IRAB] isolates acquired resistance to carbapenem by producing metallo-β-lactamase and OXA-23 carbapenemase.

Key Words: imipenem resistant *Acinetobacter baumannii* [IRAB], AmpC β- lactamases, metallo-β-lactamases, OXA-23-like carbapenamase.

INTRODUCTION

Acinetobacter baumannii has been emerging as one of the most important nosocomial pathogens in hospitals especially between Intensive Care Units (ICUs) patients worldwide (Bergogne-Bérézin and Towner, 1996; Villegas and Hartstein, 2003), particularly with increasing resistance to available therapy (Bergogne-Be´re´zin and Towner, 1996). The problem has been worsend by increasing resistance to broad-spectrum antibiotics including carbapenems, the drugs of choice for hospital acquired *A. baumannii* infections (Livermore, 2002; Abbo *et al.*, 2005). The efficacy of carbapenems is increasingly compromised by the emergence of carbapenem-hydrolysing β-lactamase enzymes of Ambler molecular class B and Ambler class D (oxacillinase) (Livermore, 2002; Poirel and Nordmann, 2002). The most widespread β-lactamases with carbapenemase activity in *A. baumannii* are carbapenem-hydrolysing class D β-lactamases (CHDLs) that are mostly specific for this species (Poirel and Nordmann,

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2006). Although metallo-β-lactamases (MBLs) are powerful carbapenemases (Walsh *et al*., 2005), oxacillinases possess the ability to hydrolyse imipenem (but not always meropenem) (Nordmann and Poirel, 2002). Poirel and Nordmann (2006) reported that MBLs are susceptible *in vitro* to EDTA inhibition, thus providing a means of their laboratory identification. Carbapenemases belonging to molecular class D (OXA enzymes) have emerged as the main mechanism responsible for this resistance (Woodford *et al*., 2006) The OXA carbapenemases of *Acinetobacter* spp. are divided into four phylogenetic subgroups: OXA-23-like; OXA-24-like; OXA-51-like; and OXA-58 (Brown and Amyes, 2006). Enzymes belonging to the OXA-51-like subgroup are intrinsic to *Acinetobacter baumannii* occurring in all strains, although they are very variably expressed (Heritier *et al*., 2005). A second intrinsic type of β-lactamase can be identified in all *A. baumannii* isolates is An AmpC-type cephalosporinase, expressed at a basal level and does not reduce the efficacy of expanded-spectrum cephalosporins (Pourel and Nordmann, 2006). However, introduction of the insertion sequence IS*Aba1* upstream of the bla_{AmpC} gene enhances β-lactamase expression considerably by providing promoter sequences, resulting in resistance to ceftazidime, but not to carbapenems (Corvec *et al*., 2003, Segal *et al*., 2004, Heritier *et al*., 2006). As observed for the natural bla_{OXA} gene of *A. baumannii*, IS*Aba1* might provide promoter sequences that enhance expression of associated genes. These promoter sequences are probably extremely efficient in *A. baumannii*, so that insertion of IS*Aba1* upstream of bla_{OXA-51}-like genes might represent a true mechanism of carbapenem resistance, or at least decreased susceptibility (Pourel and Nordmann, 2006). Recently the detection of the bla_{OXA-51}-like carbapenemase gene intrinsic to this species by polymerase chain reaction (PCR) is used for the identification of *A. baumannii* (Turton *et al*., 2006). The present study aimed to detect the carbapenemase enzymes responsible for carbapenem resistance in imipenem resistant *A. baumannii* clinical isolates from ICUs.

MATERIAL and METHODS

A total of two hundreds and nineteen clinical and eleven environmental samples were collected over the period from September 2011 to January 2012. All specimens were obtained from different surgical intensive care units (ICUs) of Zagazig University Hospitals, Sharkia, Egypt. *Acinetobacter baumannii* isolates were presumptively identified according to Peleg *et al*. (2008) as Gram negative catalase-positive, oxidase-negative, non-motile and non-fermenting coccobacilli with pale punk non lactose fermenting colonies on MacConkey agar and able to grow at 44°C and 37°C, non-hemolytic on blood agar, glucose oxidation in OF test (Hugh and Leifson's medium) containing 1% glucose and negative gelatin hydrolysis. Species-level identification was performed according to Turton *et al*. (2006) by using molecular method (PCR amplification of bla_{OXA-51}-like gene fragment). *Acinetobacter baumannii* isolates were stored in Mueller Hinton broth and 20% glycerol at -20° C.

Antibiotic susceptibility tests

Antibiotic sensitivity of the isolated *A. baumannii* strains was carried out by disc diffusion breakpoint assay as described by Clinical and Laboratory Standard Institute (CLSI) (2013). The antibiotic disks (Oxoid) used were amikacin (AK,30µg) cefepime (FEP, 30µg), cefotaxime (CTX, 30µg), ceftazidime (CAZ, 30µg), ceftriaxone (CRO,30µg), cefuroxime (CXM, 30µg), ciprofloxacin (CIP, 5µg), colistin (CT, 25µg), gentamicin (CN,10µg), imipenem (IPM,10µg), levofloxacin (LEV, 5µg),

meropenem (MEM, 10µg), ofloxacin (OFX, 5µg), pipracillin (PRL, 100µg), and sulfamethoxazole/trimethoprim (SXT, 1.25/23.27µg).

Phenotypic detection of carbapenemases

The imipenem resistant strains were subjected to the following tests:

Modified Hodge test [MHT]

According to Lee *et al.* (2001), an overnight culture suspension of *Escherichia coli* ATCC 25922 adjusted to 0.5 McFarland standard according to CLSI guidelines (2012) was inoculated using a steril ecotton swab on the surface of a Mueller-Hinton agar (MHA) (Oxoid Limited, Hampshire, United Kingdom). After drying, 10 µg imipenem disk was placed in the center of the plate and the test strain was streaked straight from the edge of the disk to the periphery of the plate. The plate was incubated overnight at 37°C. The presence of a distorted inhibition zone 'cloverleaf shaped' zone of inhibition indicates carbapenemase production by the test strain.

AmpC Disk Test

According to Singhal *et al.* (2005), MHA plates were surface inoculated with overnight broth culture of *E.coli* ATCC 25922. Saline moistened disks (6mm) were inoculated with several colonies of test organism and placed beside a cefoxitin disk (Oxoid) (almost touching) on the inoculated plate. The plates were incubated overnight at 35°C. The test was interpreted as positive if a flattening or indentation of the cefoxitin inhibition zone in the vicinity of the test disk is appeared.

Detection of metallo-beta-lactamases Combined disk test using EDTA with CAZ and IPM

According to Yong *et al.* (2002), MHA plates were surface inoculated with overnight broth culture of the IRAB isolates. Two imipenem discs (10µg) and two ceftazidime (30µg) discs were placed on the surface of the agar plate and 5 µl of 0.5 M EDTA solution was added to either one of them to obtain a desired concentration of 750 µg of EDTA. The inhibition zones of the imipenem and ceftazidime and their EDTA-impregnated discs were compared after incubation at 37°C. A zone size difference of ≥ 7 mm was taken as indicative of metallo-beta-lactamase production.

Genotypic identification and detection of carbapenem resistance genes by PCR method

The PCR was performed according to the method described by Woodford *et al.* (2006). PCR was done for the detection of the four families of OXA-type carbapenamases found in *Acinetobacter baumannii*. Sequences of primers used for the detection of genes encoding bla_{OXA-23} like, bla_{OXA-24} like, bla_{OXA-51} like and bla_{OXA-58} like genes are given in table1 (Woodford *et al.*, 2006).

The amplification cycles were as follow: Initial denaturation at 94°C for 5 min, 33 cycles of 94°C for 25 s, 53°C for 40 s and 72°C for 50 s, followed by an elongation step at 72°C for 6 min. The PCR products were visualized by agarose gel electrophoresis.

Table (1). Primers used in PCR method for detection of OXA enzymes.

primer	Primer sequence (5'-3')	Target gene	Amplicon size (b
OXA-51-like F	5'-TAA TGC TTT GAT CGG CCT TG-3'	bla_{OXA-51} like	353
OXA-51-likeR	5'-TGG ATT GCA CTT CAT CTT GG-3'		
OXA-23-like F	5'-GAT CGG ATT GGA GAA CCA GA-3'	bla_{OXA-23} like	501
OXA-23-likeR	5'-ATT TCT GAC CGC ATT TCC AT-3'		
OXA-24-like F	5'-GGT TAG TTG GCC CCC TTA AA 3'	bla_{OXA-24} like	246
OXA-24-like R	5'-AGT TGA GCG AAA AGG GGA TT 3'		
OXA-58-like F	5'-AAG TAT TGG GGC TTG TGC TG 3'	bla_{OXA-58} like	599
OXA-58-like R	5'-CCC CTC TGC GCT CTA CAT AC 3'		

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RESULTS

Strain identification

Thirty-two A. baumannii strains were the total collected isolates. Twenty-five clinical isolates were isolated from tracheal aspirate, surgical wounds, blood, urine specimens and 6 isolates from the patient surrondings like ventilators, cupboards, bed sides and floor. One environmental isolate outside the hospital was isolated from a plant.

The collected *A. baumannii* strains were 24 isolates (75%) in the surgical ICU and 7 isolates (21.88%) in trauma ICU and one sample (3.12%) isolated from outside the hospital. Ten isolates (16. 7%) were obtained from 60 tracheal aspirate specimens, 7 isolates (25.9%) from 27 swabbing the environment around patients, 7 isolates (25%) from 28 wounds (pus), 3 isolates (5.1%) from 59 urine samples, 2 isolates(16.7%) from 12 blood samples , 2 isolates (50%) from 4 tracheoctomy swabbing and one isolate(9%) from a plant from eleven environmental samples(table 2). One hundred and ninty eight specimens of varied sources were negative for *Acinetobacter baumannii*.

Table (2) Percentage of *Acinetobacter baumannii* isolated from different sources

Specimens type	Number of specimens	Number of isolates	Percentage%
Tracheal aspirate	60	10	16.7
Blood culture and central venous line	12	2	16.7
Wound swab	28	7	25
Urine	59	3	5.1
Tracheoctomy swab	4	2	50
hospital environmental	27	7	25.9
Environmental	11	1	9

Antimicrobial susceptibility tests

In the present study, the highest resistance to antimicrobial agents was recorded with cefepime and cefuroxime 84.37%, cefotaxime 81.25% and ceftriaxone 78.1% while the same resistance was detected with ceftazidime, imipenem and meropenem 75%. Resistance to piperacillin was 71.9%. A lower resistance to ciprofloxacin was exhibited as 68.75%. Resistance to amikacin, trimethoprim/sulfamethoxazole and ofloxacin was the same 65.6%, while resistance to gentamicin was 59.37%. The lowest resistance was recorded with levofloxacin 46.88%. Only to colistin, all isolates were susceptible (Table 3).

Table 3 Susceptibility pattern of *A. baumannii* isolates to different antibiotics in percentage.

Antibiotic Disk	*Acinetobacter baumannii* (n=32)					
	R		I		S	
	N	%	N	%	N	%
AK	21	65.63	0	0	11	34.37
PIP	27	84.37	2	6.25	3	9.38
CTX	26	81.25	3	9.38	3	9.38
CAZ	24	75	2	6.25	6	18.75
CIP	25	78.125	3	9.38	4	12.5
LEV	27	84.37	2	6.25	3	9.38
OFX	22	68.75	0	0	10	31.25
CN	0	0	0	0	32	100
IPM	19	59.37	0	0	13	40.63
MEM	24	75	0	0	8	25
SXT	15	46.88	6	18.8	11	34.37
TIC	24	75	1	3.12	7	21.88
PRL	21	65.63	1	3.12	10	31.25
CZ	23	71.9	4	12.5	5	15.6
	21	65.6	2	3.1	9	28.1

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The modified Hodge, AmpC disk and the Combined disk test

Among the 24 imipenem-resistant *A. baumannii* isolates, all 24 isolates (100%) showed positive results in the modified Hodge test (fig. 1a, 1b, 1c) AmpC disk test (fig.2a, 2b) and Combined disk test (table 4 and figure 3).

Fig.1a

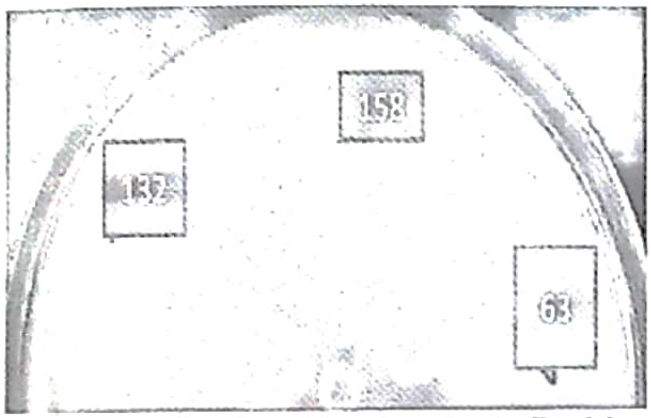

Fig.1b

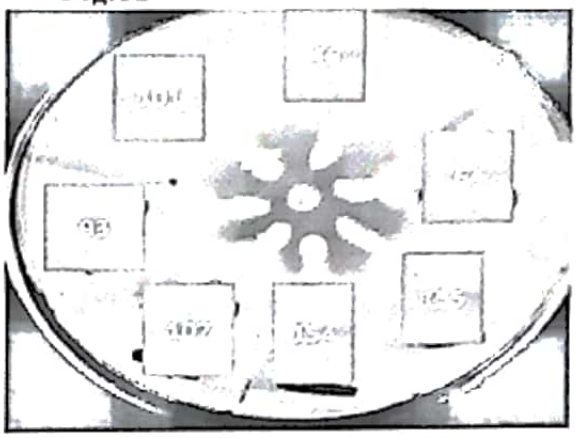

Figure1. Modified Hodge test. Positive strain shows a "cloverleaf shaped" zone of inhibition. Isolates 46, 132, 63, 158, 101, 64, 107, 93, 154 and155 are positive.

Fig.2a

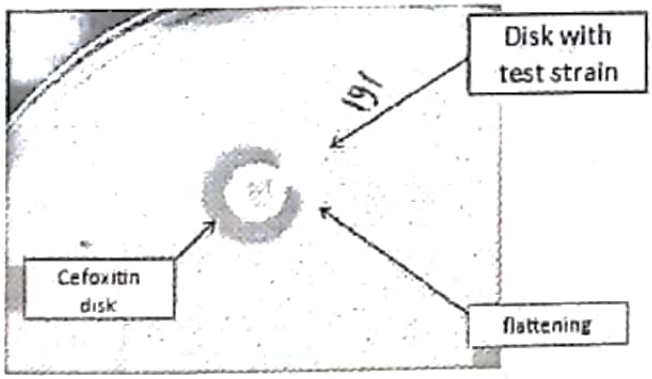

Fig.2b

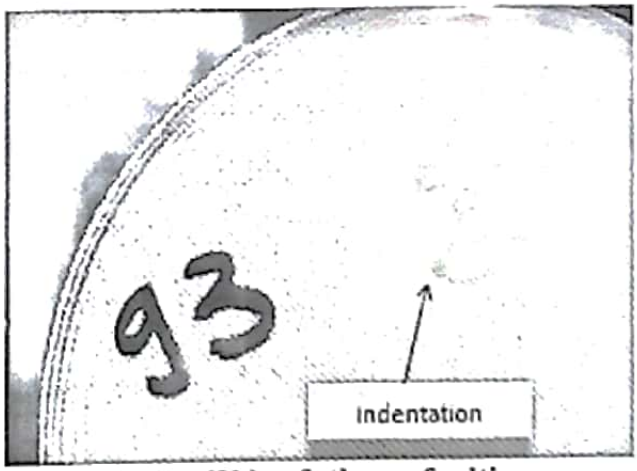

Figure 2. AmpC disk test. Flattening (2a) or indentation (2b) of the cefoxitin zone of inhibition is seen in the vicinity of the disk with AmpC producing strain.

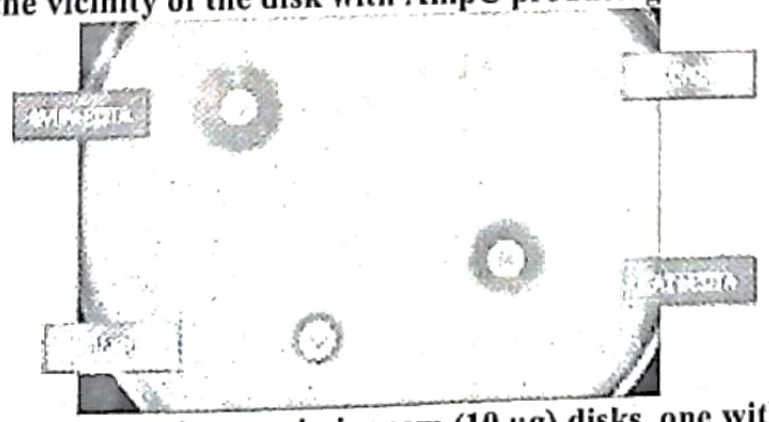

Figure 3. Combined disk test, using two imipenem (10 µg) disks, one with 750 µg EDTA and two ceftazidime (30µg), one with 750 µg EDTA, showing an increase in zone inhibition of ≥7 mm around the disk with EDTA.

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Table (4). Combined disk test results (inhibition zone diameter in mm).

Isolate no.	CAZ	CAZ+EDTA	IMP	IMP+EDTA
46	0	13	11	14
63	0	12	12	14
64	0	16	0	13
122	0	14	9	15
127	0	10	8	11
116	0	14	8	15
154	0	9	19	21
155	0	14	20	20
92	0	13	9	21
135	0	7	10	13
163	0	10	9	12
168	0	16	23	23
170	0	15	0	14
173	0	16	25	25
174	0	8	8	13
191	0	10	12	20
132	0	18	13	14
158	0	9	0	21
184	0	10	0	21
101	0	12	11	21
107	0	15	10	17
109	0	15	10	25
130	0	13	22	22
93	0	11	7	16

An increase in the inhibition zone of ≥7 mm around the disk with EDTA is interpretted as positive MBL producer .

Detection of carbapenem-resistant genes by PCR assay

All 24 isolates (100%) possessed the encoding gene for an intrinsic OXA-51-like carbapenemase and an acquired OXA-23-like carbapenemase (Figure 4).

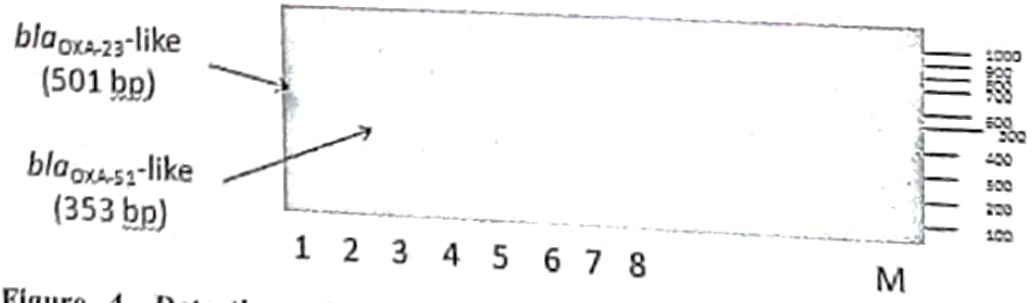

Figure 4. Detection of genes encoding OXA carbapenemase in imipenem resistant *Acinetobacter baumannii* by PCR. Lane M,100 bp Plus DNA Ladder (Bioneer, Daejeon, Korea); lanes 3, 7 isolates carrying bla_{OXA-51}-like gene; lanes 1, 5 isolates carrying bla_{OXA-23}-like gene; lane 2, 6 show no bands for bla_{OXA-24}-like gene and lane 4, 8 show no bands for bla_{OXA-58}-like gene.

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DISCUSSION

The present study aimed to evaluate the susceptibility of isolates to different antibiotics, to investigate the prevalence of imipenem resistance and the most prevalent mechanism of resistance to carbapenems.

Out of 32 isolates, 24 strains were found to be resistant to imipenem and meropenem with a percentage of 75% for both. All isolates were sensitive to colistin. This high percentage of resistance to imipenem come in accordance to results of a study performed by Antonio *et al.* (2011). Another study done by Fonseca *et al.* (2013) show nearly 100% resistant to meropenem but only 50% resistance to imipenem. In the study of Kabbaj *et al.* (2013), all isolates were sensitive to colistin.

It was found that all strains are carbapenemase producers having intrinsic AmpC β-lactamase. The *bla*$_{AmpC}$-like gene was detected in 74 (97.3%) out of the 76 strains analysed by Ruiz *et al.* (2007) in a study performed in spain. In another study, AmpC β-lactamase was detected in 99% of strains by PCR amplification (Hujer *et al.*, 2006) also the *bla*OXA-23-like carbapenemase gene was detected in 11% of strains and the *bla*OXA-58-like carbapenemase gene was found in an additional 12% of the isolates.

Also it was found that all strains produce OXA-51 like carbapenamase which come in accordance with previous findings of Turton *et al.* (2006)showing its intrinsic nature to *Acinetobacter baumannii* . In the present study, only the acquired OXA-23 like carbapenemase was detected in all imipenem resistant strains so it is the most prevelant oxacillinases and neither *bla*$_{OXA-24}$-like nor *bla*$_{OXA-58}$-like genes are detected in PCR amplification.

Worldwide Dissemination of the *bla*$_{OXA-23}$-like Carbapenemase Gene of *Acinetobacter baumannii* has been detected by Mugnier *et al.*(2010)

Jeon *et al.*(2005) show similar result that OXA-23 like carbapenemases are responsible for the outbreak that occur in Korea in 2005. OXA-23 has been reported in Brazil, French Polynesia, Spain, South Korea and England (Bou *et al.*, 2000; Héritier *et al.*, 2003; Da Silva *et al.*, 2004; Dalla-Costa *et al.*, 2003; Naas *et al.*, 2005; Marqué *et al.*, 2005; Poirel *et al.*, 2005) .

Pournaras *et al.* (2006) obtained negative results regarding *bla*$_{OXA-24}$-like genes but *bla*$_{OXA-58}$-like and *bla*$_{OXA-51}$-like genes were detected with high percentage. In the same study no genes detected to OXA-23-like carbapenemases and metallo enzymes (S. Pournaras *et al.*, 2006).

It was observed that MBLs are one of the enzymatic resistance mechanism to imipenem because all 24 resistant isolates show positive result in the combined disk method. Kabbaj *et al.* (2013) found that 74% of imipenem resistant isolates were MBL producers.

The increased MBL production is a serious epidemiological risk for at least two reasons. First of all, the MBL does not confer resistance to carbapenem only, but to all β-Lactams and other classes of antibiotics such as aminoglycoside and fuoroquinolone. Second, the genes encoding these enzymes spread easily on plasmids, by that causing nosocomial infections
(Maltezou, 2009) also *bla*$_{OXA-23}$ like gene could be located on the chromosome or a plasmid facilitating its worldwide dissemination (Mugnier *et al.*, 2010).

These results indicate that the available choices for appropriate treatments for infection, caused by *Acinetobacter baumannii*, are currently limited. *In vitro*, studies reveal that tigecycline, and colistin are the only antibacterial agents with consistent activity against MBL producing strains . Random controlled trials are required in order to evaluate the available

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therapeutic regimens, including treatment combinations (Maltezou, 2009).

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التعيين الوراثي والظاهري لانزيمات تكسير عقارات الكاربابينم في عزلات الاسنيتوباكتر بوماني المقاومة للاميبينم والمعزولة من وحدات العناية المركزة

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يعتبر ميكروب اسنيتوباكتر بوماني مسبب هام لعدوى المستشفيات حيث لديه قدرة عالية على مقاومة العديد من المضادات الحيوية و خاصة للكاربابينم (ادوية الملاذ الاخير) ولذلك فان علاج عدوى المستشفيات الناتجة عن هذا الميكروب كثيرة التعقيد.
في هذه الدراسة تم تعيين انزيمات الخاصة بتكسير الكاربابينم والمسؤلة عن المقاومة لهذا المضاد الحيوي في العينات المعزولة من غرف العنايات المركزة وذلك على اساس وراثي و ظاهري.
من بين ٣٢ عينةمن اسنيتوباكتر بوماني المعزولة من العنايات المركزة في مستشفيات جامعة الزقازيق، شرقية، مصر، كان هناك ٢٤ عينة مقاومة للاميبينم واستخدمت هذه العينات المقاومة للكشف عن قدرتها لانتاج انزيمات تكسير الاميبينمز
تم استخدام طريقة هودج المعدلة، اختبار قرص امب سي ، اختبار القرص المدعم بالاديتا. اظهرت كل العينات مقاومة ١٠٠%لتلك الاختبارات. للتعرف على انزيمات من نوع الاوكسا تم استخدام طريقةPCR.
كانت النتيجة النهائية ان هذه المقاومة لاميبينم (١٠٠%) نتيجة لانتاج الانزيمات المعدنية وانزيم تكسير الكاربابنم اوكسا ٢٣ المكتسب.