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A study on the resistance and outbreak marker genes by multiplex polymerase chain reaction (PCR) in University Hospitals

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There is mounting evidence that *Acinetobacter baumannii* has a naturally occurring carbapenemase gene intrinsic in this species. Presence of class 1 integrase gene in *Acinetobacter* isolates is a useful marker for causing outbreaks in hospitals and for being epidemic strains of *A. baumannii.* The goal of the present study was to detect the resistance and outbreak marker genes by multiplex polymerase chain reaction (PCR) (*blaOXA-51*-like gene and class 1 integrase gene). Also to detect the correlation between imipenem susceptibility and detection of *blaOXA -51*-like gene. For these purposes, 51 consecutive, non-duplicate, *A. baumanii* strains were isolated from various clinical and environmental specimens from the Intensive Care Units (ICUs) of Assiut University Hospitals, Egypt. All the isolates were identified by conventional standard methods. The antibiotic sensitivity pattern was determined by Kirby Bauer disc diffusion method. For imipenem, the minimum inhibitory concentrations (MICs) were determined using Epsilometer (E test). Multiplex PCR was performed for the detection of the *blaOXA-51*-like and Class I integrase genes. The *blaOXA-51*-like gene was detected in (95.8%) and (96.3%) in clinical and environmental isolates, respectively. Class I integrase gene was detected in (75%) and (70.3%) in clinical and environmental isolates, respectively with statistically significant difference (P value of clinical samples = 0.041 and P value of environmental samples =0.011). This means that these strains have metallo-beta-lactamase (MBL) gene (cause outbreak in hospital at any time). Also (67.35%) of *A. baumanii* isolates are imipenem sensitive and positive for *blaOXA-51*-like gene and this means that these isolates contain hidden metallo beta lactamase MBL gene.

Key words: *Acinetobacter baumanii*, *bla*OXA-51-like genes, Class I integrasegene, MBL gene.

INTRODUCTION

Acinetobacter baumannii is an important opportunistic pathogen responsible for severe nosocomial infections, especially in intensive-care-unit patients (Takagi et al., 2009). The majority of infections are of epidemic origin, and treatment has become difficult because many strains are resistant to a wide range of antibiotics, including broad-spectrum β-lactams, aminoglycosides, and fluoro-quinolones (Renu et al., 2010).

Carbapenems are the drugs of choice for *A. baumannii* infections and are often used as a last resort. However, decreased susceptibility to carbapenems has been recently observed worldwide (Peleg et al., 2008; Valenza et al.,

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2010).

There are several carbapenem resistance mechanisms described in *Acinetobacter* species (Peleg et al., 2008). Many carbapenem hydrolyzing beta- lactamases have been identified, amongst which are the metallo-beta-lactamases (MBLs). Most of the MBL -encoding genes reside on integrons and plasmids which in turn allows for the widespread dissemination of these genetic elements (Walsh et al., 2005; Perez et al., 2007).

Also, there is mounting evidence that *A. baumannii* has a naturally occurring carbapenemase gene intrinsic in this species. The first report of this gene described *blaOXA-51* (Brown and Amyes, 2005), but since then a large number of closely related variants have been found (*OXA* numbers 64, 65, 66, 67, 68, 69, 70, 71, 75, 76, 77, 83, 84, 86, 87, 88, 89, 91, 92, 94 and 95) and we have referred to them collectively as "*blaOXA-51*-like" genes (Brown and Amyes, 2006; He´ritier et al., 2005).

The *blaOXA-51*-like genes are unique to the species, and then their detection could provide a simple and convenient method of identifying *A. baumannii.* This method could more easily be carried out than current definitive methods, such as amplified rRNA gene restriction analysis and biochemical identification which is most commonly used (Vaneechoutte et al., 1995; Woodford et al., 2006).

In recent years, a novel mechanism of resistance gene dissemination among bacteria has been described (Stokes and Hall, 1989). This mechanism is based on the location of these genes on integrons. The majority of integrons belongs to class 1 and has been found predominantly in clinical isolates of Gram-negative bacteria, including *Acinetobacter* species (Martinez-Freijo et al., 1998). Presence of class 1 integrase gene in *Acinetobacter* isolates is a useful marker for causing outbreaks in hos-pitals and being epidemic strains of *A. baumannii* (Koeleman et al., 2001; Turton et al., 2005).

Identifying MBL carrying isolates has been challenging due to the emergence of carbapenem-susceptible MBL carrying organisms which may be missed in daily laboratory practice, compromising the sensitivity of detection methods. These carbapenem susceptible organisms with hidden MBL genes can spread unnoticed in hospitals if such isolates are reported as sensitive without screening for the presence of MBLs. The treatment of these organisms poses a serious therapeutic challenge as these strains are most often resistant to multiple drugs (Walsh et al., 2005).

The present study aimed to detect the*blaOXA-51*-like gene which can be carried out as part of a multiplex PCR, which detects both *blaOXA-51*-like gene (resistance gene) and class 1 integrase gene (marker for outbreak). Also to detect the correlation between imipenem suscepti-bility and detection of *bla OXA-51* like gene.

MATERIALS AND METHODS

Bacterial strains

A total of 51 consecutive, non-duplicate, *A. baumanii* strains were isolated from various clinical and environmental specimens from the ICUs of Assiut University Hospitals during period of February 2011 to February 2012. Regarding the clinical specimens, *A. baumanii* strains (24 strains) were isolated from urine (n= 5), sputum (n= 8), swabs from endotracheal tubes (n= 6), blood cultures (n=1), throat swabs (n=3) and wound swabs (n=1) that were submitted for bacteriological testing from patients admitted to the ICUs. A total of 27 isolates were obtained from environmental swabs from the ICUs. Swabs were taken from call bells, bedrails, and bedside tables, bedside equipments, commodes, doorknobs and faucet handles.

Identification of strains

Using MacConkey agar and Herellea agar (Dijkshoorn et al., 2005), also using simple biochemical reactions as oxidase test, nitrate test, growth on TSI, Citrate test, Urease test, motility test and growth at 44°C (Collee et al., 1996).

Biochemical identification of the isolates

Usingthe analytical profile index procedure (API 20NE system; bioMe´rieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was done for all isolates using commercially available discs (HiMedia, Mumbai, India) by Kirby Bauer disk diffusion method and interpreted as recommended by Clinical Laboratory Standards Institute (CLSI, 2010).

The following antimicrobial discs were used, Ampicillin (10 µg), Amoxacillin-Clavulanic acid (20-10 µg), Cefaclor (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg), Amikacin (30 µg), Gentamicine (10 µg), Tobramycin (10µg), Netlimicin (30 µg), Tetracycline (30 µg), Ciprofloxacin (5 µ g), Imipenem (10 µg), Chlo-ramphenicol (30 µg) andAztreonam (30 µg).

Determination of imipenem minimal inhibitory concentration (MIC) by IPM E-Test

AB Biodisk, Solna and Sweden E-test strips were placed over-culture streaked over Muller Hinton agar. After overnight incubation in incubator at 35°C, the MIC was read as intersect where the ellipse of growth inhibition intersects the strip. It was used at a cut-off point of ≥16 µ g/ml to define imipenem resistance and a cut-off point of ≤ 4 µg/ml to define imipenem susceptibility (CLSI, 2006).

Multiplex PCR for detection of *blaOXA-51*-like gene & Class 1 intgrase gene (Turton et al., *2005)*: a- DNA extraction

The boiling method was used to extract the DNA from the bacteria (Vaneechoutte et al., 1995). Briefly, one colony of a pure culture was suspended in 50 µl of sterile water and heated at 100°C for 15 min. After centrifugation in a micro centrifuge (6,000 x *g* for 3 min), the supernatant was stored at -20°C for further use.

b-PCR Amplification and detection

This was carried out in 25 µl reaction volumes with 3 µl of extracted DNA, 12.5 pmol of each primer as shown in Table 1 and 1.5 U of Taq DNA polymerase in 1X PCR buffer containing 1.5 mM $MgCl_2$ (QIAGEN) and 200 µM of each deoxynucleoside triphosphate. Conditions for the multiplex PCR were as following: 94°C for 3 min,

Table 1. Primer sequences of *blaOXA-51*-like gene and Class 1 integrase gene (Koeleman et al., 2001; Woodford et al., 2006).

Primer	Sequence	Target gene	Amplicon size (bp)
OXA-51-likeF	5_-TAA TGC TTT GAT CGG CCT TG-3_	*blaOXA-51*-like	353
OXA-51-likeR	5_-TGG ATT GCA CTT CAT CTT GG-3_	*blaOXA-51*-like	
Int1F	5_-CAG TGG ACA TAA GCC TGT TC-3_	Class 1 integrase	160
Int1R	5_-CCC GAG GCA TAG ACT GTA-3	Class 1 integrase	

and then 35 cycles at 94°C for 45 s, at 57°C for 45 s, and at 72°C for 1 min, followed by a final extension at 72°C for 5 min. Amplified products from the isolates were analyzed by electrophoresis on 1.2% (w/v) agarose gels, stained with ethidium bromide. *A baumannii* ATCC 19606 was used as positive control.

Statistical analysis

All data were analyzed using the computerized statistical analysis (SPSS, version 16). Descriptive statistics was used. The P value <0.05 was considered statistically significant. The percent difference of each antimicrobial agent versus Imipenem was calculated at 99% confidence interval.

RESULT

Fifty one strains of *Acinetobacter* sp. were isolated in infection control laboratory at Assiut University Hospitals from February 2011 to February 2012 .They comprised of two sets of isolates, The first set consisted of 24 isolates recovered from clinical samples, the second set consisted of 27 isolates isolated from environmental samples .

All *Acinetobacter* strains were described as Gram negative cocco-bacilli, non-motile, non-spore forming, capsulated, oxidase negative, not reduce nitrate to nitrite, not ferment sugar and citrate positive bacilli.

Acinetobacter grow on blood agar showing mucoid colonies, on MacConkey agar showed non -lactose fermenting colonies, on Herellea agar showed purple colonies. API20NE showed that these strains belong to *Acinetobacter baumannii/calcoaceticus* complex, and isolates identified as *Acinetobacter baumannii* by its ability to grow at 44°C.

Resistance of *A. baumannii* to pencillin derivatives, cephalosporines, monobactam **(**Aztronam) carbapenam (imipenem), quinolones (ciprofloxacin), tetracycline, aminoglycosides (netlimicin, tobramycin, gentamicin and amikacin) and chloramphenicol were 61.82, 61.8, 60.6, 31.24, 64.18, 25.2, 56.48 and 53.01%, respectively. Tetracycline and imipenem were the most active antimicro-bial agent against *A. baumannii* (Table 2).

The susceptibility of *A. baumannii* to different antimicrobial agents was compared to imipenem. Imipenem resistant *A. baumannii* are not susceptible to penicillin derivative or cephlosporine, but these strains are susceptible to tetracycline (more active), chloamphenicol (moderate active) and aminoglycosides (less active) in descending manner. But susceptibility of *A. baumannii* to quinolones is variable (Figure 1).

Phenotypic detection of metallo-B-lactamase by IPM E-Test showed that (31.37%) of *A. baumannii* isolates of environmental and clinical samples contain metallo-β-Lactamase enzyme (MIC>16), while (68.6%) of the isolates show MIC below 4 µg/ml (Table 3).

Detection of *blaoxa-51*-like gene and Class I intgrase gene showed that (95.8%) and (96.3%) of *A. baumannii* isolated from clinical and environmental samples respectively gave positive result for *blaoxa-51*-like gene (intrinsic carbapenamase gene) while (75%) and (70.3%) of *A. baumannii* isolated from clinical and environmental samples respectively gave positive result for Class I intgrase gene (Table 4 and Figure 2).

Relation between imipenem susceptibility and detection of *blaOXA-51*-like gene showed that 67.5% of *A. baumannii* isolates are imipenem sensitive and positive for *blaOXA-51*- like gene and this means that these isolates contain hidden MBL gene (Table 5).

DISCUSSION

A. baumannii infections present a global medical challenge. The interest in this organism has been growing rapidly because of the emergence of multi-drug resistant strains (MDR), some of which are pan resistant to antimicrobial agents (Muthusamy and Boppe, 2012). In the present study the majority of *A. baumannii* isolates were MDR showing resistance to three or more classes of antibiotics. There has been a lot of debate concerning the definition of multidrug resistance (MDR). Renu et al. (2010) defined MDR as resistance to 4 or more classes of antimicrobials. Others defined MDR as resistance to two or more drugs or drug classes of therapeutic rele-vance (Navon-Venezia et al., 2005). Resistance against carbapenemsis, in itself, considered sufficient to define an isolate of *A. baumannii* as highly resistant (Poirel and Nordmann, 2006).

In our study, the results of antimicrobial susceptibility test shown resistance to penicillin derivatives (61.82%), Cephalosporine derivatives (61.82%), Quinolones (64.18%), Monobactam (60.6%), Aminoglycosides (56.48%) and Chloramphenicol (53.01%). The lowest rate of resistance

Table 2. Resistance patterns of *A. baumannii* to different antibiotics.

Sample	Resistance pattern (%)						
	B-lactam						
	Penicillin derivative	Cephalosporine	Monobacam aztronam	Carbapenam imipenam	Quinolone	Tetracyclines	Amin
Clinical sample	66.67	62.5	58.33	29.16	66.67	20.83	
Environmental sample	56.97	61,11	62.96	33.33	62.96	29.6	
Total main resistance	61.82	61.8	60.6	31.24	64.18	25.2	

Figure 1. Forest representation of the 99% confidence interval comparing imipenem activity against other antimicrobials.

Table 3. Detection of Metallo-B-lactamase in *A. baumannii* by IPM E-test.

Sample		MIC (below 4 µg/ml)		MIC (16-256 µg/ml) MBL-producing strain	
Type	Number of total isolate	Number of isolate	Percentage (%)	Number of isolate	Percentage (%)
Clinical	24	17	70.83	7	29.16
Environmental	27	18	66.7	9	33.33
Total	51	35	68.6	16	31.37

Table 4. Detection of *blaoxa-51*-like gene & Class 1 integrase gene by Multiplex PCR

Result		*blaoxa-51*-like gene				Class I intgrase gene			
Sample		Positive		Negative		Positive		Negative	
Type	No	No	%	No	%	No	%	No	%
Clinical	24	23	95.8	1	4.17	18	75	6	25
Environmental	27	26	96.3	1	3.7	19	70.3	8	29.63
Total	51	49	96.1	2	3.9	37	72.5	22	27.5

P value of clinical samples = 0.041, P value of environmental samples = 0.011, P value of < 0.05 indicates significant results. No = number.

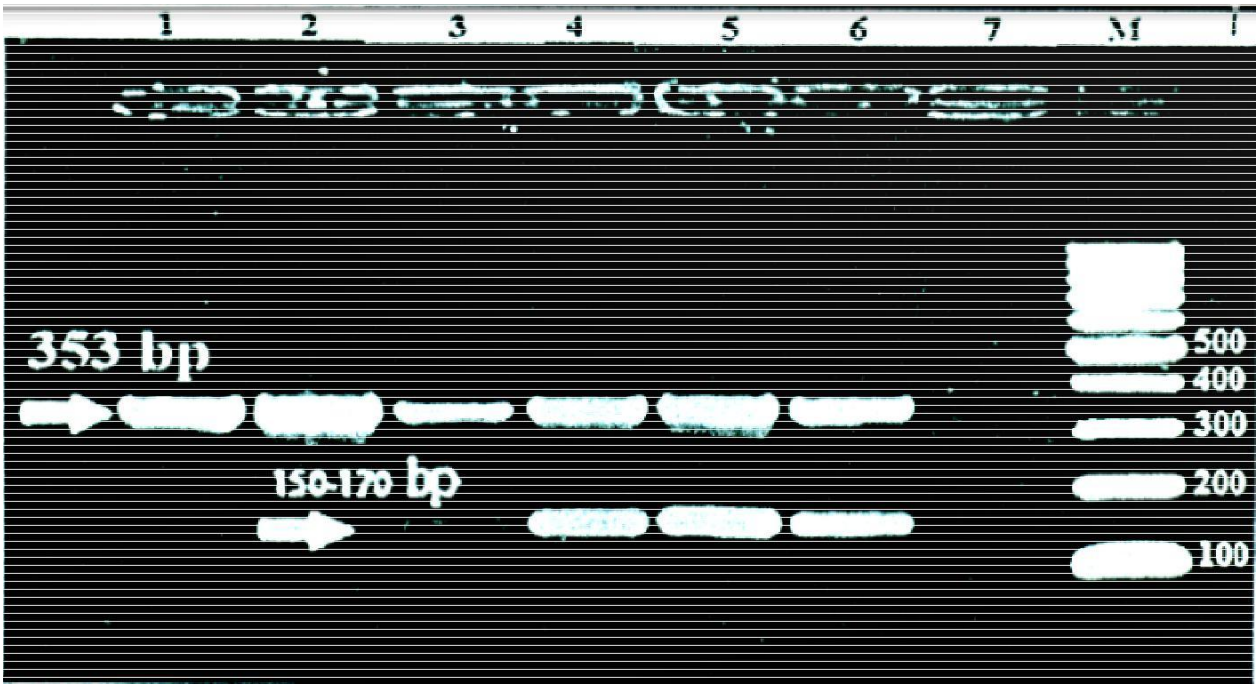

Figure 2. Multiplex PCR for detection of *blaOXA-51*-like gene and Class I integrase gene. Lane 1 to 5: positive results for *blaOXA-51* like gene. Lane 4 and 5: positive results for class I integrase gene. Lane 6: positive control- Lane 7: negative control -M: DNA marker (100 bp).

was toImipenem (31.24%) and tetracyclines (25.2%). This agreed with Hashem et al. (2011) who showed that tetracycline was the most effective antimicrobial agent against *A. baumannii* derivatives and cephalosporins were the least active agents against *Acinetobacter* when compared with Impinem. Similar results reported by Hanaa et al. (2010) who found that sensitivity of *Acinetobacter* to imipenem. The findings of the present study confirm that penicillin was 64.2% while susceptibility to penicillin derivatives and cephalosporines was 0%.

Table 5. Relation between Imipenem Susceptibility & Detection of *blaOXA-51*-like gene (intrinsic carbapenamse gene).

Sample		Imipenem susceptible and positive blaOXA-51-like gene(Hidden Metallo-B-lactamase)		ImipenemResistant and positive blaOXA-51-like gene(Expressed Metallo-B-lactamse)	
Type	**Number**	**Number**	**Percentage**	**Number**	**Percentage**
Clinical	23	16	70	7	30
Environmental	26	17	65	9	35
Total	49	33	67.5	16	32.5

The predominant *Acinetobacter* sp. in clinical settings are the members of the *A. calcoaceticus-baumannii* com-plex which are multi drug resistant and are responsible for causing outbreaks. Carbapenem resistance in *A. calcoaceticus-baumannii* complex is very high and is predominantly due to carbapenemase production, metallo-β-lactamases, oxacillinases, mobile genetic elements, and reduced expression of outer membrane proteins (Limansky et al., 2002; Poirel et al., 2003; and Anil et al., 2011).

E-test results were showed that (34/51 or 66.67%) of *Acintobactersp* isolates were *imipenem* susceptibile (MIC below 4 µg/ml), while (17/51 or 33.33%) were imipenem resistant (MIC above 8 µg/ml). This agreed with (Livermore, 2002) who found that the high levels of imipe-nem MIC (16-256 mg/L) observed in these *A. baumannii* isolates suggested the presence of a metallo-β-lactamase (MBL) or an oxacillinase, since these carbape-nemases were considered the major mechanism of carbapenem resistance in these organisms.

The resistance of *A. baumannii* to antimicrobial agents is mediated by all of the major resistance mechanisms that are known to occur in bacteria. β-Lactamases are the most diverse group of enzymes that are associated with resistance, and more than 50 different enzymes, have been identified so far in *A. baumannii*. OXA-51-like carbapenemases are class D β-lactamases which are intrinsic to *A. baumannii* and confer resistance to carbapenems (Turton et al., 2006a; Brown et al., 2005).

In this study, (96%) of *A. baumannii* were showed band of *blaOXA-51*-like genes. This agreed with Turton et al. (2006b) who found that all *A. baumannii* gave a band in the blaOXA-51-like PCR, but they remain alert to the possibility of non-detection of some variants. A further potential problem is that these genes are sometimes associated with *ISAba1*, which may render them mobile. We currently encounter also results of Hanna et al. (2010) that showed that detection of *blaOXA-51*-like genes is the most specific, simple and reliable method for detection of *A. baumannii* as carbapenamse gene is intrinsic to this species.

Among imipenem-susceptible and resistant *A. baumannii* which were screened by PCR for different β-lactamases. The *blaOXA-51*-like gene was the only one detected, even in imipenem-susceptible strain (Takagi et al., 2009). It has been reported that among *A. baumannii* isolates with *blaOXA-51* -like as sole carbapenemase gene, imipenem and/or meropenem resistance was associated only with isolates in which *ISAba1* was upstream of *blaOXA-51*-like, suggests that *ISAba1* is providing the promoter for this gene (Turton et al*.,* 2006a).

In this study, (72.5%) of *A. baumannii* showed positive bands for class I integrase gene (gene responsible for outbreaks in hospitals). The analysis of *A. baumannii* strains with known epidemic behavior demonstrates that early identification of epidemic strains may be possible by detection of integrons or multiple antibiotic resistances. The integrase gene PCR identified almost 75% of the epidemic *A. baumannii* strains. Multiple antibiotic resis-tances, defined as resistance to five or more antibiotics, showed good correlation with the presence of integrons and epidemic behavior of the strains.

This result agree with (Dijkshoorn et al., 1996) who showed that strains may vary considerably in their epide-miological potential, and those strains that have been known to spread widely and rapidly among hospitalized patients have been designated epidemic *A. baumannii* strains. Antibiotic resistance has been shown to be one of the factors which can influence the nosocomial dissemi-nation of *A. baumannii.* Few reports credit outbreak con-trol to reduced prescribing of broad spectrum antibiotics, such as fluoroquinolones or carbapenems (Villegas and Hartstein, 2003).

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Conflict of Interests

The author(s) have not declared any conflict of interests.

Conclusion

The detection of *bla OXA-51*-like gene is the most specific method for detection of *Acinetobacter baumannii* carba-penamase gene which is intrinsic in this species. Also de-

tection of class I integrase gene is very important in the rapid epidemiologic investigation of an outbreak.

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