Relationship between Clinical and Environmental Isolates of *Acinetobacter baumannii* in Assiut University Hospitals

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Abstract: This study was conducted during the period from February 2010 to February 2011 to correlate the *Acinetobacter baumannii* strains isolated from clinical and environmental samples by different methods including biotyping, antibiogram, phenotyping (detection of metallo-B-lactamase enzyme) and also molecular typing throw detection of universal gene of *Acinetobacter species.* We isolated a total of 51 *Acinetobacter species* from clinical and environmental samples from different wards and ICUs of Assiut University Hospitals. Biotyping of the isolates were done using API 20NE Index system which identified all clinical & environmental isolatesas *Acinetobacter baumannii / calcoaceticus* complex. Antimicrobial susceptibility testing was determined by Kirby Bauer disk diffusion method. The highest resistance was to penicillin derivatives (66.7% and 51.9% in clinical and environmental samples respectively). The lowest resistance was to tetracycline (20.8% and 29.6%) and imipenem (29.2% and 33.3% in clinical and environmental samples respectively). Phenotypic detection of Metallo-B-lactamase (MBL) was done by double disc synergy test. All the imipenem resistant *Acinetobacter baumannii* strains isolated from clinical and environmental samples expressed MBL phenotypically. Molecular typing by PCR showed that 49 of *Acinetobacter baumannii* isolated from clinical and environmental samples had positive ITS of 602-622bp with an overall sequence similarity of more than96%.These methods supported a close relationship between clinical and environmental isolates and also indicated the important role of hospital environment in spread and transmissibility of multidrug resistant *A. baumanii* among hospitalized patients.
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Introduction:

Acinetobacter species are saprophytic bacteria found in living organism and inanimate beings. Owing to its scarce virulence, the great majority of infections are in the hospital environment, with great incidence in patients who are seriously ill and even in a critical state (Pedro *et al.*, 2011).

Acinetobacter baumannii, a very common hospital pathogen in ICUs and wards, has been identified as one of the six important and highly drug resistant hospital pathogens by the "Infectious Disease Society of America" (Gant *et al.*, 2007).

A. baumannii has been isolated from various types of opportunistic infections, including septicemia, pneumonia, endocarditic, meningitis, skin and wound infection, and urinary tract infections (Stapleton, 2002; Zurowska *et al.,* 2008).

A number of risk factors have been associated with *Acinetobacter* nosocomial infections. They include sever underlying diseases, immuno-suppressed hosts, exposure to frequent use of invasive devices and advanced age (Manfredi *et al.*,2002; Mahgoub *et al.*, 2002).

Prolonged hospitalization or ICU stay for 5 days or more appear to be another important risk factor for nosocomial *Acinetobacter* infections (Infectious Diseases Society of America, 2005).

During the last two decades, hospital acquired infections involving multi-resistant *A. baumannii* isolates have been reported. Once it enters a hospital ward, *A. baumannii* can spread from the colonized patient to the environment and other susceptible patients. The direct environment of the patient can become contaminated by excreta, air droplets and scales of skin (Zeana *et al.*, 2003).

Since then, strains of *A. baumannii*have also gained resistance to newly developed antimicrobial drugs. Although multidrug resistant (MDR) *A. baumannii* is rarely found in the community isolates, it became prevalent in many hospitals (Poirel and Nordmann, 2006).

Interpreting the significance of isolates from clinical specimens is often difficult, because of the wide distribution of *Acinetobacter* species in the nature and its ability to colonize healthy or damaged tissue (Lahiri *et al.*, 2004).

Nosocomial Acinetobacter infection is commonly acquired through cross transmission

because of its propensity to survive in the hospital environment and persistently contaminate fomites (Borer *et al*., 2005).

The objective of this study was typing and correlation of *Acinetobacter baumannii* strains isolated from clinical and environmental samples by different methods including biotyping, antibiogram, phenotyping (detection of metallo-B-lactamase enzyme) and also molecular typing throw detection of universal gene of *Acinetobacter baumannii*. Typing of *Acinetobacter baumannii* isolated from clinical and environmental sample at the same time are important tools for establishing the sources and modes of transmission of such epidemic strains.

Material and Methods:
I- Subjects
A) Patient:
In this study, 440 clinical samples obtained from 220 nosocomially infected patients. These samples were 169 blood cultures, 87 urine cultures, 66 sputum cultures, 63 endotracheal tubes, 20 Throat swabs, and 33 wound swabs.

B) Environmental Assessment:
Total numbers of 672 environmental samples were collected from surfaces, walls, furniture, beds, floor and trolleys of ICUs and wards of Assiut University hospital beside the patients from whom positive *Acinetobacter* strains were isolated.

II-Isolation and characterization of *Acinetobacter* species:
Acinetobacter species were isolated from clinical and environmental samples using standard microbiological techniques which included morphological and cultural properties as well as biochemical characteristics (Washington *et al.,* 2006).

III-Biotyping:
Using the commercial test system API 20NE Index system (BioMèrieux, France).

API20NE is a standardized system for the identification of non-fastidious, non-enteric Gram-negative rods, combining 8 conventional tests which include potassium nitrate (NO3), L-tryptophane (TRP), D-glucose (GLU), L-arginine (ADH), urea (URE), esculin ferric citrate (ESC), gelatin (GEL), and 4-nitrophenyl-β-D-galactopyranoside (PNPG), and also the 12 assimilation tests which include D-glucose (GLU), L-arabinose (ARA), D-mannose (MNE), D-mannitol (MAN), N-acetyl-glucosamine (NAG), D-maltose (MAL), potassium gluconate (GNT), capric acid (CAP), adipic acid (ADI), malic acid (MLT), trisodium citrate (CIT), and phenylacetic acid (PAC).

IV-Antimicrobial susceptibility testing:
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 antibiotics which were tested were imipenem 10 μg (IPM), amoxicillin clavulanic acid 30 μg (AMC), tetracycline 30 μg (Te), ceftriaxone 30 μg (CRO), amikacin 30 μg (AK), ciprofloxacin 5 μg (CIP) and chloramphenicol 30 μg (C). *A. baumannii* ATCC 19606 was used as control.

V-Phenotypic detection of Metallo-B-lactamase by Double Disc synergy test: (Franklin *et al*., 2006).
In this test, two disks were used, one contain IPM (10 μg), the other contain 10μl of 0.1 M (292 mg) anhydrous EDTA **(Sigma Chemicals, St. Louis, MO)**, the two disks were placed 20 mm apart from each other. A presence of inhibition zone between the two discs indicates positive results for an MBL enzyme**.**

VI-Molecular typing by PCR: Table (1) showed Oligonucleotides Primers Used for amplification of 16S rRNA-23S rRNA gene by simple PCR method (**Relman, 1993).**

Table (1): Sequence of 16S rRNA-23S rRNA gene:

Primer	Oligonucleotide sequence(5-3)	Amplicon size
1**6S-23S rRNA Gene Spacer Region(1512)– F**	GTCGTAACAAGGTAGCCGTA	607-622 bp*
16S-23S rRNA Gene Spacer Region (1512)– R	GGGTTYCCCCRTTCRGAAAT Where Y is C or T and R is A or G)	

(bp*): base pair

Result:
Nosocomial infection rate during the period of the study was 2.3% (276/11975 patients admitted to different ICUs and wards).

Out of 440 clinical isolates, 24 *Acinetobacter* isolates were detected (5.45%). Out of 672 environmental samples, 27 *Acinetobacter* isolates were detected (4.017%). All *Acinetobacter* species were described as Gm-vecocco-bacilli, non-motile, capsulated, oxidase –ve, not reduce nitrate to nitrite and citrate positive bacilli. *Acinetobacter* grows on blood agar as mucoid colonies; grow on MacConkey agar as non-lactose fermenter colonies, and as purple colonies on Herellea agar.

The highest isolation rate of *A.baumannii from* clinical samples were from Neurology ICU, Trauma

ICU and Internal medicine unit (17.65%, 6.93% and 11, 11% respectively). *A.baumannii* was isolated from the environmental samples in the same units at the same time (0.44%, 1.93% and 0.44% respectively).

Biotyping using the API 20 NE Index system:

The API 20 NE Index system identified all isolates from clinical & environmental samples as Acinetobacter *baumannii / calcoaceticus* complex with seven analytic profile index numbers (0041453, 000473, 0041173, 0041072, 0043073, 0045073, 0040053). *Acinetobacterbaumannii* can be differentiated from A.*calcoaceticus* by ability to grow at 44˚C.

Antibiotic Susceptibility pattern of *Acinetobacter baumannii* isolated from clinical and environmental samples showed in table (2). The highest resistance was to penicillin derivatives (66.7% and 51.9% in clinical and environmental samples respectively). The lowest resistance was to tetracycline (20.8% and 29.6%) and Imipenem (29.2% and 33.3%in clinical and environmental samples respectively).

Phenotypic detection of Metallo-B-lactamase(MBL) by Double Disc synergy test: showed that (94.66 % and 81.48%) of *Acinetobacter baumannii* isolated form clinical and environmental samples respectively gave increase in zone of inhibition of about 4mm or more around EDTA (0.1M)-IPM disc compared to IPM disc alone. All the Imipenem resistant *Acinetobacter baumannii* showed positive results for detection of MBL. This means that Imipenem resistant *Acinetobacter* isolates from clinical and environmental samples expressed MBL phenotypically, while Imipenem susceptible isolates contain hidden metallo-β-lactamases enzymes (Table 3).

Detection of 16S rRNA-23S rRNA gene (intergenic spacer (ITS) region):

Several genotypic methods have been developed for species identification of *A. calcoaceticus-A. baumannii* complex. These methods include 16S-23S rRNA gene intergenic spacer (ITS) region. The 16S rRNA gene sequences of members of the genus *Acinetobacter* have an overall sequence similarity of more than 96%.

Twenty three (95.83%) and 26 (96.3%) of *Acinetobacter baumannii* isolated from clinical and environmental samples have 16SrRNA-23SrRNA gene respectively (Table 4 & Figure 2).

Discussion:

Nosocomial *A.baumannii infection* is commonly acquired through cross-transmission because of its propensity to survive in the hospital environment. Data from published studies have shown that *A.*

baumannii can survive for long periods of time on inanimate surfaces (Borer *et al*., 2005). Environmental contamination is important as the organism can be directly transmitted from environmental surfaces to patients or from the hands of health care workers to patients. Majority of cases have been described in medical and surgical wards and intensive care units, which are the area most frequently colonized by *A. bauman*nii (Cisneros *et al.,* 2002).

During period of study from October 2009 to February 2011, nosocomial infection rate was 2.3 %. However this result was different from that detected by (Mona *et al.,* 2006) who reported that nosocomial infection rates in Assiut University hospital was 23%. A likely explanation for the relatively low level of nosocomial infection rate at Assiut University hospitals is the strict isolation of patients with multi-resistant isolates, and the immediate beginning of infection control measures when several patients are infected.

Out of 440 different specimens, 24 (5.45%) *Acinetobacter* isolates were identified. Our findings were less than that reported by (Hanna *et al.,* 2010) that identified 29 *Acinetobacter* isolates (10.6%) from 273 clinical specimens. This difference may be attributed to different patient population with different underlying diseases and different environment.

Out of 672 different environmental samples, 27(4.017%) *Acinetobacter* isolates were identified. Similar results were reported by *(Paavilainen et al.,* 2001) who showed that the isolation of *Acinetobacter baumannii* from the environmental samples collected from floor, showers, sink trap, bed rail, and wash basin was 4%. However these results disagreed with results of (Banerjee *et al.,* 2005 and Hanna *et al.,* 2010) that showed that isolation rate *of Acinetobacter sp*. from environmental samples were 23.33% and 22% respectively. Variations of the environmental contamination levels may be due to the difference in the application of strict hygienic measures and the level of cleaning of the general hospital environment.

Biotyping of *Acinetobacter sp* isolated from clinical and environmental samples was done by API20NE. This agreed with (Loubinoux *et al.,* 2003) who reported that the identification of non-fermentative gram negative rods is usually carried out by using identification system such as the API 20 NE (bioMerieux). Seven different profiles numbers were identified *Acinetobacter baumannii-Colaecaeticus complex* ((0041453, 000473, 0041173, 0041072, 0043073, 0045073, and 0040053).

Difference in profile numbers in isolated *Acinetobacter sp* depended on utilization of carbon source as N-acetyle-glucosamine (NAG), D-maltose

(MAL), potassium gluconate (GNT), carponic acid (CAP), malic acid (MLT) and phenylacetic acid (PAC). This finding was in agreement with (Loubinoux *et al.,* 2003) who reported that biotyping of *Acinetobacter* isolates causing outbreak depending on cluster analysis of carbon source growth assays and identify a major grouping of isolates that was related to the epidemiological origin of the strains.

Antibiogram typing of *A. baumannii* was done depending on the degree of relatedness between the organisms in term of antibiotic sensitivities. In the present study the majority of *A. baumannii* isolates were multidrug resistant (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. MDR *A.baumannii* was also defined as an isolate with intermediate or complete resistance to at least 3 of the following classes of antibiotics: betalactam, aminoglycoside, carbapenem and fluoroquinolone (Zapantis *et al.,* 2007).

Resistance against carbapenems is, in itself, considered sufficient to define an isolate of *A. baumannii* as highly resistant (Poirel and Nordmann, 2006). We found that 31.4% of all *A. baumanni* isolates were imipenem resistant.

The highest resistance was to penicillin derivatives (66.7% and 51.9% in clinical and environmental samples respectively). The lowest resistance was to tetracycline (20.8% and 29.6%) and Imipenem (29.2% and 33.3%) in clinical and environmental samples respectively. This result was in accordance with (Hashem *et al.,* 2011) who showed that Tetracycline was the most effective antimicrobial agent against *A. baumannii*.(100%). Our results were lower than that reported in another Egyptian study where resistance rates approached nearly 100% against many antibiotics among carbapenem resistant *Acinetobacter* isolates. In that

study all imipenem resistant isolates showed very high resistance to amikacin (100%), 3rd and 4th generation cephalosporins (100%), ampicillin/ sulbactam (100%). Lower rates of resistance were reported against tetracycline (73.9%) and ciprofloxacin (69.6%) (Mohamed and Raafat, 2011).

Carbapenem resistance in *Acinetobacter 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 (Poirel *et al*., 2003and Anil *et al.,* 2011).

The phenotypic method used for detection of metallo-β-lactamase in *Acinetrobacter* isolates was double disc synergistic test (DDST). It showed that (91.66% and 81.48%) of *Acinetrobacter* isolated from clinical and environmental samples gave synergistic zone between IPM and EDTA discs. Our results were comparable to those reported by (Uma *et al*., 2009) by using DDST (70.9%) and, (Shaheda and Ruhul, 2011) by using DDST (51.75%).

MBL producing organisms pose significant risks, particularly due to their role in unnoticed spread within institutions and their ability to participate in horizontal MBL gene transfer with other pathogenic hospital-related organisms (Peleg *et al.,* 2005).

In this study, 95.83% and 96.3% of clinical and environmental *Acinetobacter* isolates were detected by presence of 16srRNA -23srRNA gene (universal gene present in all *Acinetobacter species*). The genus *Acinetobacter* currently contains up to 33 described named and unnamed (genomic) species. Of these, *A calcoaceticus*, *A. baumannii* and genomic species 3 and 13 are genetically and phenotypically very similar. *A. baumannii* had the shortest ITS fragment (607 bp), whereas *A. calcoaceticus* yielded the longest ITS fragment (637 to 638 bp) (Carr *et al.,* 2003).

Table (2): Resistance patterns of *A. baumannii* isolates to different Antibiotics

Members	Strains from clinical samples. No=24		Strains from environmental samples No=27		Total No= 51	
	Resistance		Resistance		Resistance	
	No	%	No	%	No	%
Amoxacillin-Clavulinic acid	16	66.7	14	51.9	30	58.8
Ceftriaxone	15	62.5	14	51.9	29	56.9
Amikacin	16	66.7	14	51.9	30	58.8
Tetracycline	5	20.8	8	29.6	13	25.5
Imipenem	7*	29.2	9*	33.3	16	31.4
Chloramphenicol	13	54.2	14	51.9	27	52.9
Ciprofloxacin	16	66.7	17	63	33	64.7

*All imipenem resistant strains had MICs ranging from 16- 256 μg/ml by the IMP -E test.

Table (3): Detection of metallo-B-lactmase enzymes in both Imipenem susceptible and Imipenem resistant by Double Disc Synergy test (DDST):

Samples		Imipenem susceptible			Imipenem resistant			Total	
Type	No. of total isolate	No of isolate	Positive	%	No of isolate	Positive	%	Positive	%
Clinical	24	17	15	88%	7	7	100%	22	91.66%
Environmental	27	18	13	72%	9	9	100%	22	81.48%
Total	51	35	28	80%	16	16	100%	44	86.27%
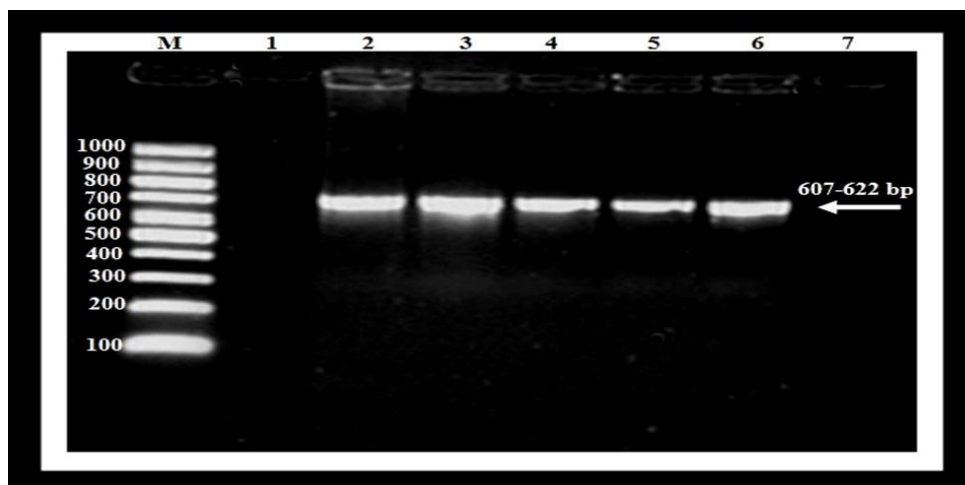
Fig 1: PCR for detection 16S rRNA-23S rRNA gene(intergenic spacer (ITS).
M: DNA marker (100 bp); Lane 1: Negative control; Lane 2: Positive control; Lane 3 to Lane 6: show positive result for the gene; Lane 7: show negative result

Table (4): Detection of 16S rRNA-23S rRNA gene (intergenic spacer (ITS) in environmental and clinical samples:

Result	16S rRNA-23S rRNA gene					
Study	Sample collected	Positive			Negative	
		No	%	ITS length (bp)	No	%
Clinical Sample	24	23	95.83%	607-622	1	4.167%
Environmental Sample	27	26	96.3%	607-622	1	3.7%
Total	51	49	96.08%		2	3.92%
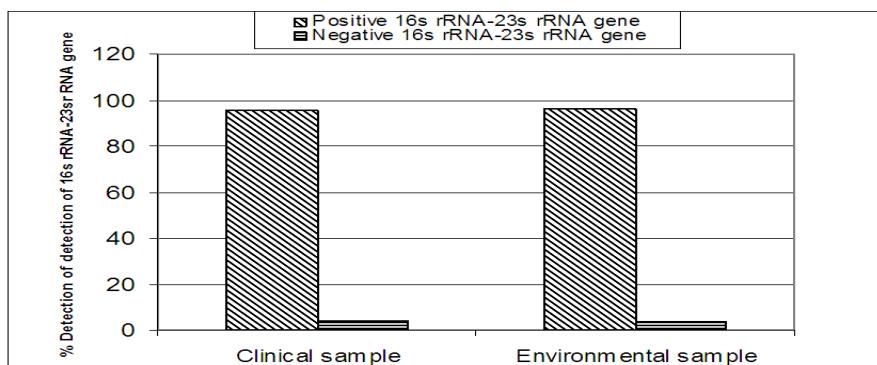
Figure (2): Detection of 16S rRNA-23S rRNA gene (intergenic spacer (ITS) in environmental and clinical samples.

Conclusion:

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, Egypt during period of October 2009 to February 2011. Typing of these strains was done by different methods including biotyping, antibiogram, phenotypic and genotypic method. These methods supported a close relationship between clinical and environmental isolates. It also indicated the important role of hospital environment as a source of infection and in spread and transmissibility of multidrug resistant *A. baumanii* among hospitalized patients, so application of strict infection control measures is very important to reduce the transmission of infection.

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