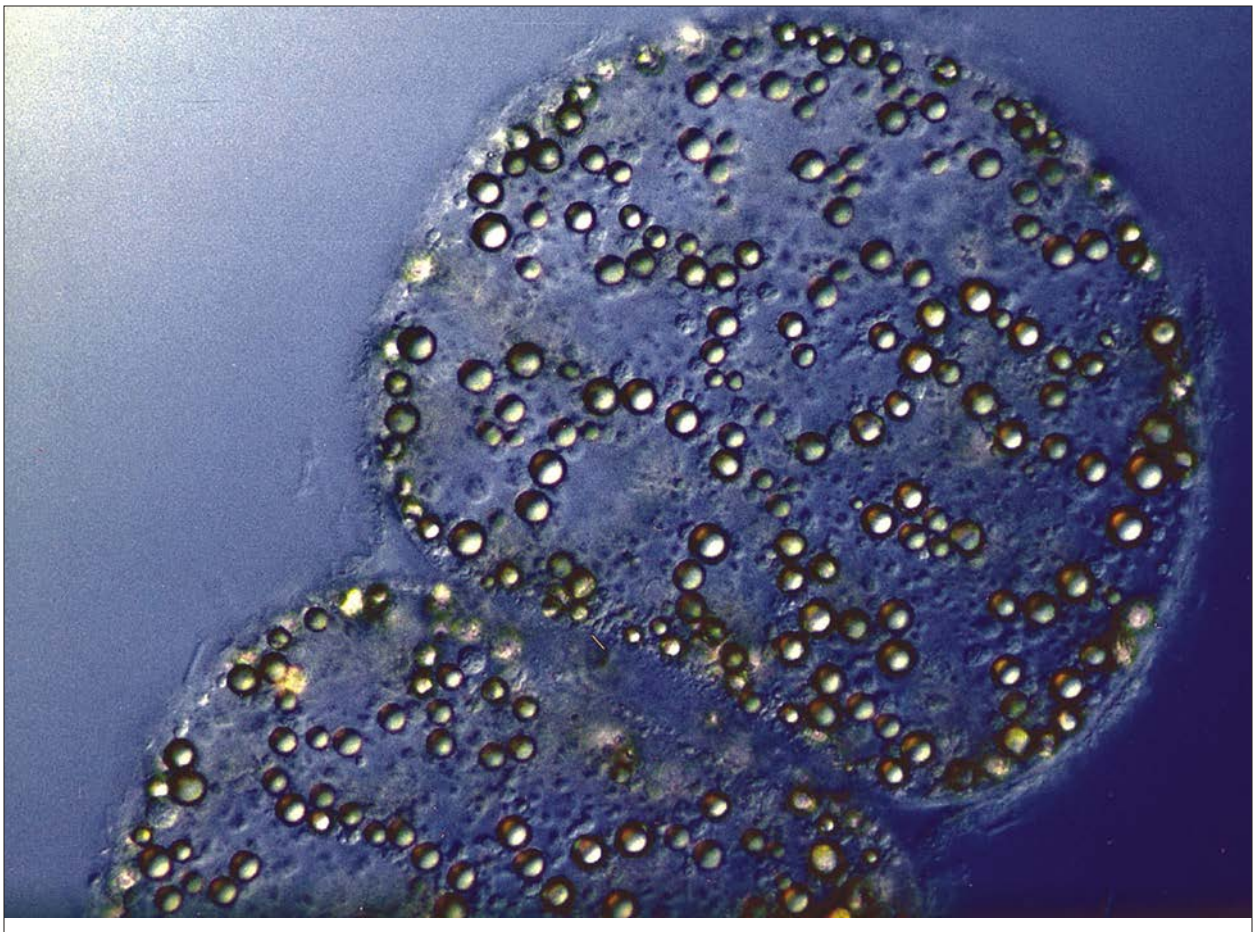

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Plasmid Curing Assay in Clinical Isolates of Antibiotic Resistant *Acinetobacter baumannii*

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ABSTRACT

Acinetobacter baumannii an antibiotic resistant bacterium was isolated and identified from clinical samples and assessed for plasmid carrying ability and the locations of resistant gene markers. A total of hundred and sixty six clinical samples were collected from the respiratory tracts and wound infections in the patients hospitalized in the Intensive Care Unit (ICU) of Namaze Hospital, Iran. The isolates were identified into phenotypes and genotypes and their antibiotic susceptibility assessed by disk diffusion method. The antibiotics used were: amikacin 30 μg, gentamicin 10 μg, imepenem 10 μg, meropenem 10 μg, ceftazidime 30 μg, ceftaxime 30 μg, ciprofloxacin 5 μg, polymyxin 300B and tetracycline 30 μg. The resistance strains of *Acinetobacter baumannii* was subjected to plasmid curing to find plasmid mediated resistance markers. The results showed that fifty one *Acinetobacter* spp. were isolated and all of them were identified as *A. baumannii*. In addition, the isolates were resistant to cefotaxime (100%) and gentamicin (90.1%) and susceptible to polymyxin (100%). The results from plasmid curing showed that the acridine orange could cure, the plasmids of *A. baumannii* cured. *A. baumannii* isolates were sensitive to ceftazidime, meropenem, cefotaxime, ceftazidine and amikacin. Therefore, antibiotic resistance markers associated with these antibiotics were plasmid encoded. The present study showed that *A. baumannii* is an important nosocomial infection in the ICU. Many antibiotic resistance markers in the *A. baumannii* were carried by the plasmids. Hence, they might be transmitted easily among the bacteria in the hospitals.

Key words: Plasmid curing, antibiotic susceptibility, *Acinetobacter baumannii*

INTRODUCTION

Genus *Acinetobacter* are gram-negative, obligate aerobic, non-motile, non-spore forming coccobacilli, catalase and oxidase positive bacteria belonging to family Neisseriaceae. It is an opportunistic pathogen associated with a wide spectrum of nosocomial infections (Montefour *et al*., 2008; Peleg *et al*., 2008; Gordon and Wareham, 2010).

The natural habitat of *Acinetobacter* is water and soil and sometimes they live on the skin of the patients and attached to various instruments in the hospitals (Fournier *et al*., 2006). *Acinetobacter* as an opportunistic pathogenic bacteria and is the commonest gram negative isolate from immunocompromized patients, posing risk high mortality (Murray and Baron, 2007). Among all species of *Acinetobacter*, *Acinetobacter baumannii* is associated with several infections such as pneumonia, septicemia, urinary tract infection, meningitis, endocarditic and cutaneous infections

in the hospitals (Go *et al*., 1994; Dijkshoorn *et al*., 2007). On the other hand, this bacterium exhibited multidrug resistance to beta lactam antibiotics, aminoglycosides, carbapenem and fluroquinolone (Henwood *et al*., 2001). Several studies illustrated that the potent activity of *Acinetobacter baumannii* for acquisition the resistant genes culminated in the occurrence of multidrug resistance strains (Song *et al*., 2001). Multidrug resistance character of *Acinetobacter* related to the presence of antibiotic resistance markers in plasmids, transposons and integrons (Devaud *et al*., 1982; Dijkshoorn *et al*., 2007; Cambray *et al*., 2010). Hence, antibiotic resistance genes could horizontally transfer among the bacteria by conjugation, transformation and transduction. It must be noted that acquire of the resistance gene cause increased ability of *Acinetobacter* for surviving in the environment, that of the hospitals (Thomas and Nielsen, 2005; Antunes *et al*., 2011). More than 80% of *Acinetobacter* isolates carry multiple indigenous plasmids (Gerner-Smidt, 1989; Dale and Park, 2004). Based on foregoing evidence the present study was conducted to isolate *A. baumannii* from clinical samples of the patients in the Intensive Care Units (ICU) and evaluate the existence of indigenous plasmid in them. In addition, the relation between antibiotic resistant markers and the existence of plasmids in the isolates were also studied.

MATERIALS AND METHODS

Sample collection: Hundred and sixty six clinical samples were collected from the respiratory tracts and wound infections of the patients hospitalized in the intensive care unit of Namaze Hospital. The samples were collected during the six months from March to August in 2014. All collected samples were taken to the laboratory within 2 h and subjected to microbiological analysis. The samples were cultivated on blood and MacConkey agar and incubated at 37°C for 24 h.

Phenotypic identification of *Acinetobacter* isolates: In order to isolate *Acinetobacter*, microscopic observation was carried out on all isolates. The gram negative isolates were assessed by catalase, oxidase, oxidative/fermentative, nitrate and citrate tests. Triple sugar iron agar and Api 20NE kits were used for phenotypic identification.

Authentication of *Acinetobacter* isolates: Identification of *Acinetobacter* isolates was verified by gene fragment sequence of blaOXA-51, DNA of the isolates was extracted by DNA gene kit (Tajhez gostar, Iran); amplification of gene was done by the Polymerase Chain Reaction (PCR) method. The PCR products were electrophoresed and the purified bands were cut and sent to 1st step company in Malaysia for DNA sequencing. The sequence data for bioinformatic applications were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/).

Antibiotic susceptibility of *Acinetobacter baumannii* isolates: Antibiotic susceptibility of the isolated *A. baumannii* was performed by disk diffusion method according to CLSI and EUCAST guidelines (Bauer *et al*., 1966). The overnight growth cultures (equal to turbidity of 0.5 McFarland tube) of the isolates were diluted with sterile normal saline. Then 0.1 mL of the suspension was fully cultivated on Mueller Hinton agar and antibiotic disks placed on the seeded medium plate. The antibiotic disks used were as follows: amikacin (30 μg), gentamicin (30 μg), ciprofloxacin (5 μg), imipenem (10 μg), meropenem (10 μg), ceftazidime (30 μg), cefotaxime (30 μg) and polymyxin B (300 μg) (Hi-media., India). The plates were incubated at 37°C for 18-24 h, the inhibition zone around each antibiotic disk was measured and recorded.

Plasmid isolation: Occurrence of the plasmids in *Acinetobacter* isolates was verified by DNA plasmid extraction kit (Yekta tajhez-Iran). The extracted DNA plasmids were mixed with 1 µL of loading dye and added into the wells of gel agarose. The electrophoresis apparatus was adjusted on 85 V for 40 min. After finishing the electopharsis, the visible bands were evaluated by Alpha imager gel documentation system (Syngene, UK), under Ultra Violet at 260 nm.

Plasmid curing: Plasmid curing of *A. baumannii* isolates was carried out using acridine orange and elevated temperature methods. Elevated temperature was followed by adjusting the temperature for growth of *A. baumannii* isolates at 44°C. In addition, to perform plasmid curing by acridine orange, serial double dilution of 0.05 µg mL^{-1} of acridine orange was made, then 200 µL of bacterial culture (turbidity equal to 0.5 Mcfarld tube) was added into the different dilutions and the suspension tubes were incubated at 37°C. After 24 h the Minimal Inhibitory Concentration (MIC) and Subminimal Inhibitory Concentration (SIC) of acridine orange was determined based on inhibition growth dilution and a dilution under minimal inhibitory concentration, respectively.

To perform plasmid curing of *A. baumannii* isolates, 0.1 mL of SIC suspension of acridine orange was cultivated onto nutrient agar and incubated at 37ºC for 24 h. The colonies were picked up separately by sterile tooth stick and transferred into the Mueller Hhinton agar with and without antibiotics and the plates were incubated at 37ºC for 24 h. The antibiotics used were meropenem (10 µg), amikacin (30 µg), cefotaxime (30 µg) and ceftazidime (30 µg). Observation of *A. baumannii* colonies on the medium without antibiotics and absence, of the same colonies on the medium containing antibiotics considered cured strain.

Statistical analysis: To verify significant relation between the antibiotic resistance and plasmid carriage of clinical *Acinetobacter baumannii* isolates , the data was subjected to statistical analysis using SPSS version 22.

RESULTS

Isolation and Identification of the isolates: Out of all collected samples, fifty one strains of *Acinetobacter* were isolated and subjected for molecular identification by gene fragment sequence of *blaOXA-51*. The results indicated that all isolated strains were *A. baumannii*.

Antibiotic susceptibility of *Acinetobacter baumannii* isolates: The results obtained from antibiotic susceptibility of clinical isolated *Acinetobacter baumannii* indicated that all isolates were resistant to cefotaxime (100%) followed by gentamicin (91/1%). However, all isolates were susceptible to polymyxin B (100%). The rest of the antibiotics have shown different responses against the isolates (Table 1).

Plasmid isolation and curing: Out of all *Acinetobacter baumannii* isolates, 20 strains exhibited resistant character to 90% of antibiotics. These strains were subjected for plasmid isolation and curing. The result obtained from plasmid isolation illustrated that out of all antibiotic resistant *Acinetobacter baumannii*, 15 strains harbored plasmid.

In addition, plasmid curing of *Acinetobacter bagman* was performed only by SIC of acridine orange. Table 2 showed Minimal Inhibitory Concentration (MIC) and Subminimal Inhibitory Concentration (SIC) of acridine orange against clinical *Acinetobacter bagman* isolates.

Table 1: Susceptibility of *Acinetobacter baumannii* isolates to antibiotics

Antibiotics	Susceptible (%)	Resistance (%)
Amikacin	11.7	88.2
Gentamicin	9.9	90.1
Ciprofloxacin	19.7	80.3
Ceftazidime	1.9	98.3
Cefotaxime	0	100.0
Meropenem	21.6	78.4
Imipenem	15.7	84.3
Polymyxin B	100	0.0
Tetracycline	37.2	62.7

Table 2: Determination of minimal inhibitory concentration and subminimal inhibitory concentration of acridine orange against *Acinetobacter baumannii* isolates

Acinetobacter of Isolates	SIC (μg mL^{-1})	MIC (μg mL^{-1})
Nu2	15.6	31.2
Nu6	15.6	31.2
Nw3	31.2	62.5
Nh1	15.6	31.2
Nh4	62.5	125
Nh8	15.6	31.2
Nr5	31.2	62.5
Nr7	15.6	31.2
Nb1	15.6	31.2
Nb4	15.6	31.2
Nb9	31.2	62.5
Nb11	15.6	31.2
Nb17	15.6	31.2
Nb18	31.2	62.5
Nb20	15.6	31.2

SIC: Subminimal inhibitory concentration, MIC: Minimal inhibitory concentration

Table 3: Sensitivity of cured strains of *Acinetobacter bagman* to the antibiotics

Antibiotics	Susceptibility of cured strains (%)
Amikacin	65.2
Cefotaxime	82.6
Meropenem	78.2
Ceftazidime	100.0
Gentamicin	86.9

Plasmid curing: Our finding showed that plasmids of all 15 isolates were cured. Therefore, plasmid bands were observed after plasmid curing in the gel of electrophoresis. On the other hand, elevated temperature failed to cure the plasmids. Plasmids of *A. baumannii* isolates were cured only by acridine orange.

Antibiotic susceptibility of the cured *Acinetobacter baumannii* isolates: Determination of antibiotic susceptibility of the cured *A. baumannii* isolates was carried out in order to achieve information concerning to the change of antibiotic susceptibility character of cured strain compared to non cured strains of *A. baumannii* isolates.

The results obtained indicated that cured strains of *A. baumannii* isolates were sensitive to ceftazidime (100%), cefotaxime (82.6%), meropenem (78.2%), amikacin (65.2%) and gentamicin (86.9%). It means some antibiotic resistance markers in *A. baumannii* isolates are plasmid-mediated (Table 3).

DISCUSSION

Overuse and misuse of antibiotics culminate in the occurrence of high frequency of antibiotic resistant bacteria. On the other hand, the presence of antibiotic resistant markers in the bacterial

chromosome, plasmids, integration and transposons are the reason for transferring genes among the bacteria (Chopade *et al*., 1985; Montefour *et al*., 2008). In the hospital, this phenomenon will happen often because of the existence of drug resistant bacteria. Hence, opportunistic and low virulence microorganisms can cause severe disease in the hospital. *Acinetobacter* is an opportunistic pathogen found to be associated with a wide spectrum of nosocomial infections. Recently multidrug resistance character of *Acinetobacter* considered as a major problem for commercially available antibiotic (Bergogne-Berezin, 2007; Dijkshoorn *et al*., 2007). Among all species of *Acinetobacter*, *A. baumannii* has become a significant pathogen, especially in the intensive care unit (Bauer *et al*., 1966; Brisson-Noel *et al*., 1988; Courvalin, 1994; Beige *et al*., 2015). Survival of this bacterium in the hospital, especially in the ICU seems to be related to their ability of acquiring antibiotic resistant genes. Our study showed a relation between the antimicrobial resistance and the occurrence of plasmid in *A. baumannii*. The results obtained illustrated that ceftazidime, meropenem, cefotaxime, ceftazidine and amikacin resistant marker genes in clinical isolated *A. baumannii* were plasmid mediated. Transfer of antibiotic resistant genes by plasmids to other nosocomial pathogens can create complications in the treatment of patients (Beige *et al*., 2015). Therefore, the existence of *A. baumannii* in the hospital, especially in the ICU might be encountered as a major problem. Thus, *A. baumannii* needs to be considered as an important multidrug resistant pathogen and accordingly to be associated with many nosocomial infections.

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