Research Journal of Medical Sciences 8 (1): 20-26, 2014
ISSN: 1815-9346


# Detection of β-Lactamase Genes (*bla$_{TEM}$* and *bla$_{CTX}$*) Resistant to Drugs and Glutaraldehyde in Samples of *Acinetobacter baumannii* Isolated from Surfaces of the Medical Device of Intensive Care Units of Hospitals of Tehran

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**Abstract:** Excessive consumption of antimicrobial materials in hospitals and the community is as the main encoder, led to the emergence, development and acquisition of new bacterial resistance to antimicrobials. According to the lack of the enough information about the mechanism of the resistant genes to disinfectants and receiving no report from the country about this study and with the aim of considering the resistance or sensitivity of the isolates of the *Acinetobacter baumannii* MDR in facing disinfectants, this study was taken place in the selected intensive care units if the hospitals of Tehran. This study which was conducted over a period of 10 months, *Acinetobacter baumannii* species were separated by culture and biochemical tests. The resistance and sensitivity of the isolates to antibiotics is considered according to CLSI guidelines. By multiplex PCR Method bla$_{CTX}$ and bla$_{TEM}$ were detected and finally, MDR strains were treated with 2% glutaraldehyde. In the study 131 isolates (22/28%) of *Acinetobacter baumannii* were isolated. The amount of the resistance to various antibiotics was in the range of the 69/4 to 100%. The percentage of frequency of the bla$_{TEM}$ and bla$_{CTX}$ was 3/2% and 19/4%, respectively. And there was seen no resistance to glutaraldehyde. It seems that beside variety and prevalence of bla$_{TEM}$ and bla$_{CTX}$, enormous mechanisms like porin and leaking systems (efflux Pumps) are responsible in the making of the resistance of *Acinetobacter baumannii* to disinfectants. Also, these results facilitated the study of phenotypic and genotypic resistance patterns of these antimicrobials and disinfectants in different parts of the world.

**Key words:** *Acinetobacter baumannii*, lactamase genes, disinfectants, antimicrobial resistance, PCR

## INTRODUCTION

Acinetobacter families are Gram-negative, obligate aerobic and non-fermentative bacillus (Cunha, 2003) Based on taxonomy and genomic data, 32 species of the organism are known and ten of them such as *Acientobacter baumannii*, *Calcoaceticus*, *Haemolyticus*, *Schindleris*, *Johnsonii*, *Junii*, *Lowffii*, *Radioresistens*, *Parcus*, *Ursngii* are associated to human. The organism can cause different type of disease like Ventilator Associated Pneumonia (VAP), eye infections, urinary tract infections, meningitis, osteomyelitis, peritonitis, endocarditis, ulcerative colitis pancreatitis, infections, burns, wounds and postoperative sepsis (Magnet *et al*., 2001; Bonomo, 2011; Zarrilli *et al*., 2009; Bou *et al*., 2000; Gouby *et al*., 1992; Dijkshoorn *et al*., 1996; Seifert and Gerner-Smidt, 1995; Ecker *et al*., 2006; Maiden *et al*., 1998; Enright *et al*., 2003). Excessive and improper use of antimicrobials (antibiotics and biocide) in both hospitals and community led to development and acquisition of new bacterial resistance to antimicrobials as a main concern and serious threat of today communities.

In this way, antimicrobial resistance genes within mobile genetic elements such as plasmids, transposon and integron which transfer by Horizontal Gene Transfer (HGT) mechanisms play the main role to get and develope such phenomenon between bacteria in different sources and environments. In recent decade resistance to wide range of antimicrobial agents and emergence of MDR and XDR phenotypes between some nosocomial infectious agent such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Acenitobacter* species in some countries especially in Iran has raised and raises concerns. The emergence of PDR, XDR, MDR phenotype of nosocomial pathogen, *Acinetobacter baumannii* during the last three decades has been limited the options and application of

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antimicrobials to control and treat of the infections cause by the organism. This phenomenon return to the ability of the bacterium to accept resistance genes such as β-lactamase encoded enzymes, $bla_{TEM}$, $bla_{CTX}$ and New-Delhe-metallo-β-Lactamase-1from wide variety of environments (Nordmann *et al*., 2011a, b) also presence of OXA-48, OXA-181, CTX-M-15, SHV, blaoxA-23, 24, 51, $bla_{CTX}$ (oxA-10, 2), TEM ($bla_{TEM}$-1, 2, 13), methylase, ESBL, qnr and six type of NDM (Lacey, 1984; Issa, 2001; Sabath *et al*., 1977; Tseng *et al*., 2011; Nordmann *et al*., 2011b; Chen *et al*., 2012), disinfectant resistance genes, quc (A-E) (Johnston and Dyke, 1969) and detection of new plasmid such as PNDm-1-DOK01 (Chen *et al*., 2012) encoding of different type of Outer Membrane Proteins (OMPA), Siderophores and iron acquisition systems, presence of pili mediated DNA uptake and biofilm formation by fimberia and exopolysaccharides on biotic and abiotic surfaces (Lankford and Byers, 1973) convert the *Acinetobacter baumannii* to a super bug. Hence, researchers were eager to study about *Acinetobacter baumannii* collected from hospital environments and medical equipment surfaces from selected intensive care units of some hospitals located in Tehran. By using molecular techniques, researchers evaluated the resistance genes $bla_{TEM}$, $bla_{CTX}$ frequency among the isolates. In the next step, researchers determined the susceptibility of isolates *Acinetobacter baumannii* to 2% glutaraldehyde solution.

## MATERIALS AND METHODS

The cross-sectional analysis was perform during 4 months from the December to end of March 2012 and 588 samples collected from surfaces of medical equipment from intensive care units of different hospitals including ICU, NICU, RCU, CCU of five hospitals of Tehran by Using standard sampling swabs and transport medium Tryptic soy broth (MERCK company). In this study, out of 588 collected samples 131 samples of *Acinetobacter baumannii* is isolated by phenotypic and biochemical tests. For final confirmation API2ONE (Biomerieux) biochemical commercial kite was used. For long term storage, the isolates suspended in nutrient broth containing 18-15% glycerol and kept at -80°C.

**Antibiotic susceptibility pattern:** Antibiotic susceptibility pattern of the *Acinetobacter baumannii* isolates performed by Kirby-Bauer Disk Diffusion Susceptibility test (Table 1) and the discs were consistently tested for efficacy against standards strains recommended by CLSI guideline. Evaluation of the Minimum Inhibitory Concentration (MIC) was performed by micro broth dilution method briefly microbial suspension was prepared using a McFarland 0.5 standard, equivalent to the concentration of $1\text{-}2\times10^{8}$ cfu $mL^{-1}$ of *E. coli* ATCC25922 bacterium. By a sterile swab, the bacterial suspension, equivalent to McFarland 0.5 standards was cultured in the Mueller-Hinton agar (Merck, Germany) plate then antibiotic disks in an appropriate distance placed in the plate and the plates were incubated at 35°C for 18-16 h. The discs used in this study were imipenem (10 ug ), meropenem (10 ug), ceftizoxime (30 ug), oxacillin (1 ug), gentamicin and lincomycin (2 ug) ceftazidime (10 ug), ceftazidime (30 ug), cefotaxime (5 ug), floxacin (30 ug), ampicillin (10 ug), tetracycline (30 ug), cefixime (5 ug) and Colistin (10 ug). Broth micro tube dilution method was used to determination of minimum inhibitory concentration imipenem and meropenem antibiotics against isolates.

**Multiplex PCR and PCR:** In order to identify the genes encoded β-Lactamase, $bla_{TEM}$ and $bla_{CTX}$ from resistant isolates researchers used MultiplexPCR and PCR according to the following steps.

**Preparation of template DNA:** DNA was extracted by boiling method briefly, the organism was cultured on blood agar after one overnight a colony was selected and inoculated. In 5 mL of luria Bertoni medium (Applichem GmbH Germany). In the next step, it is incubated at 37°C on shaker incubator for 20 h. After incubation 1.5 mL of the culture medium poured aseptically into micro tubes and was centrifuged for 5 min at 14000 rpm. Then, supernatant remove and 500 mL distilled water added to the pellet. The suspension heated for 10 min at 95°C. The heated suspension centrifuged again for 5 min in 14000 rpm. Supernatant containing DNA was extracted and used as template for PCR techniques (Pitout *et al*., 1998).

***$bla_{TEM}$*, *$bla_{CTX}$* genes amplification by PCR Method:** For this study the PCR primers used for amplification of the *$bla_{TEM}$*, *$bla_{CTX}$* genes shown in the Table 1. The PCR mix consisted of 25 μL containing 7.5 μL distilled water, 12.5 μL 10X PCR Master Mix (Fermentas), 3 μL of DNA template. The 1 μL of forward and reverse primers of the genes TEM and CTX in 10 pmol concentration were used for PCR amplification of corresponding genes. The primer sequences are summarized in Table 1.

Table 1: Characteristics of primers used in PCR

| Genes | Nucleotide sequence | Primers |
|---|---|---|
| *CTX-A* | 5'-CGCTTTGCGATGTGCAG-3' | $Bla_{CTX\ 550\ bp}$ |
| *CTX-B* | 5'-ACCGCGATATCGTTGGT-3' | $bla_{CTX\ 550\ bp}$ |
| *TEM-A* | 5'-GAGTATTCAACATTTCCGTGTC-3' | $Bla_{TEM\ 800\ bp}$ |
| *TEM-B* | 5'-TAATCAGTGAGGCACCTATCTC-3' | $bla_{TEM\ 800\ bp}$ |

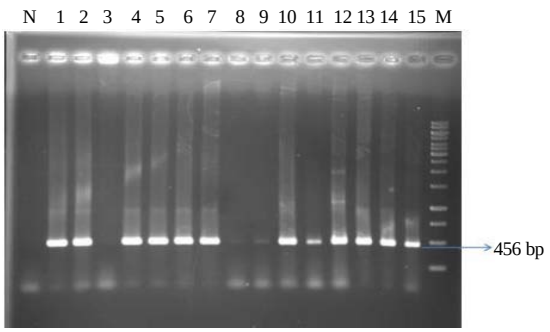


Fig. 1: PCR results of gene $bla_{CTX}$ *Acinetobacter baumannii* strains isolated from surfaces and equipment N: Number of template DNA, M: size marker 1 kb ladder

Table 2: PCR conditions

| | Gene *CTX/TEM* (Factors) | |
|---|---|---|
| Steps | Temp. (°C) | Time |
| Initial denaturation of heat shock | 94/94 | 3/3 min |
| Denaturation DNA | 94/94 | 30/30 sec |
| Annealing (pairing primer) | 63/45 | 1/1 min |
| Extension (primer elongation) | 72/72 | 1/1 min |
| Final extension (final elongation) | 72/72 | 10/10 min |
| Cycle number (number of cycles) | 35 | - |

For sequencing of the *TEM*, *CTX* genes, researchers used http:www.lahcy.orgs site (Song *et al*., 2006). PCR (using a Master cycler Eppendrof and a Palm-cycler) consisted of a 3 min denaturation at 94°C followed by 35 cycles of 30 sec at 94°C, 60 sec at 45°C for $bla_{TEM}$ and 63°C for $bla_{CTX}$ for 1 min at 72°C with a final extension of 10 min at 72°C, ending at 4°C according to Table 2.

**Electrophoresis:** The PCR products were screened by agarose Gel Electrophoresis Method on 1.5% Ultrapure™ Agarose (invitrogen). Electrophoresis was performed by the voltage 90 A during 60 min. Amplified genes were separated according to their molecular size. The gels were stained by ethidium bromide (0/5 g mL) and then were washed within distilled water 2 times. Finally, the stained gels monitored by Gel Doc System (TN) and the correspond images were taken and analyzed for associated bounds according to the location and size in compare to DNA molecular size marker genes (1 kbp DNA Ladder Company Fermentas) (Fig. 1-3). The data were analysis by SPSS Statistical Software and t-test test.

**Evaluation of 2% glutaraldehyde sensitivity of the antibiotic-resistant isolates:** Bacterial suspension perpetrated by Direct Colony Suspension Method (DCS) in a McFarland 0.5 standards of the resistant isolates. The suspension exposure to 2% glutaraldehyde (neodisher septo 3000). At 5, 10 and 15 min after exposure time, the

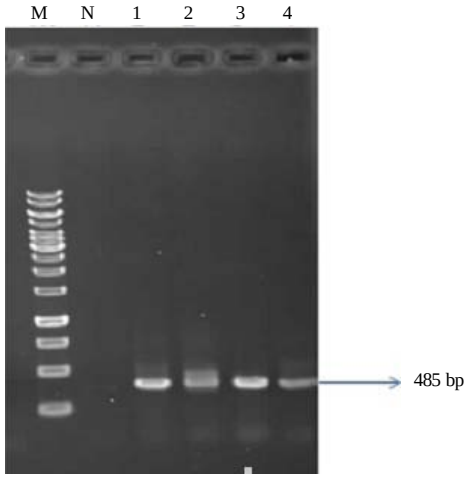


Fig. 2: Results of PCR gene $bla_{CTX}$, M: size marker 1 kb ladder

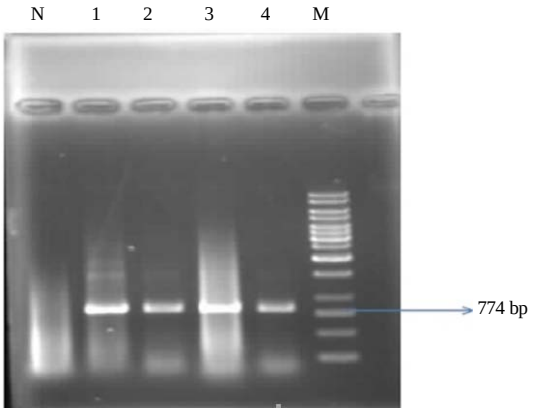


Fig. 3: Results of $bla_{TEM}$ gene was PCR N: Number of template DNA, M: Size marker 1 kb ladder

suspension were cultured on blood agar and incubated 18-16 h in 37°C. After incubation, the plates were screened for colony formation. In the next step by adding sodium hydroxide, researchers neutralized 2% glutaraldehyde and raised the pH to 12 finally low concentration of 2% glutaraldehyde removed by inorganic acids (e.g., hydrochloric, etc.). In this solution the pH level is neutral. By adding sterile PBS the solutions were washed and again were cultured on blood agar plates and were incubated for 18-16 h at 37°C after that the result compared with previous step.

## RESULTS

During 3 months period from January to end of March 2013 the number of 588 samples collected from surfaces of medical equipment of intensive care units from selected hospitals of Tehran as shown in Table 3.

Table 3: Distribution of *Acinetobacter baumannii* isolates in intensive care units

| Section | Hospital | Number of samples | Percentage of isolated |
|---|---|---|---|
| ICU I | Fayazbakhsh | 115 | 19/56 |
| ICU II | Fayazbakhsh | 33 | 5/61 |
| NICU | Fayazbakhsh | 23 | 3/91 |
| RCU | Labbafinejad | 40 | 6/80 |
| ICU | Labbafinejad | 42 | 7/15 |
| NICU | Vali Asr | 94 | 15/92 |
| CCU | Vali Asr | 34 | 5/78 |
| ICU set | Imam Khomeini Hospital | 128 | 20/58 |
| NICU | Taleghani | 43 | 7/31 |
| ICU | Taleghani | 43 | 7/31 |
| | Total | 588 | 100 |

**Isolation of bacteria:** In this study, the 588 samples collected from surface of medical instruments of intensive care units from 5 selected hospitals, during 4 months from December 2012 to March 2012. Of the total cultures processed, the number of *Acinetobacter baumannii* isolated was 131 (22.27%). The isolate were confirmed by phenotypic and biochemical standard tests and Biochemical Commercial kit. In the next step, evaluation of sensitivity of isolates to the antibiotic was performed by Kirby-Bauer Disk Diffusion Susceptibility test and according CLSI guideline. MIC for selected antibiotics against isolates was determinated.

According Kirby-Bauer Disk Diffusion Susceptibility test, the study shown that all *Acinetobacter baumannii* isolated were resistant (100%) to three antibiotics, imipenem and meropenem and lincomycin. The other resistance patterns of the *A. baumannii* isolates were gentamicin 85.97%, oxacillin 90% and ciprofloxacin 70.2% were obtained. The highest sensitivity related to the antibiotic colistin (100%) was shown. Minimum inhibitory concentration related to antibiotics imipenem and meropenem measured by $\geq$64 ug $mL^{-1}$.

***bla*$_{CTX}$ and *bla*$_{TEM}$ gene amplification by PCR Method:** According the PCR amplification and agarose gel electrophoresis analysis of PCR products, the frequency of the genes, *bla*$_{CTX}$ and *bla*$_{TEM}$ among 131 *Acinetobacter baumannii* isolated were 19/4 and 3/2%, respectively.

**Evaluation of sensitivity of 2% glutaraldehyde disinfectant on resistant isolates:** The previous data recommended that the 2% glutaraldehyde disinfectants (glutaraldehyde solution concentrating according to EC neodisher septo 3000 No.: 20048164) at least in 10 or 15 min have inhibitory effect on growth of *Acinetobacter baumannii* suspension equivalent McFarland 0.5 standard (NCCLS, 1990).

In the experiment upper and lower time of exposure was evaluated. The results cleared, 5 min (lower the recommended time) exposure to 2% glutaraldehyde disinfectant had no inhibitory effect on the bacterial growth. In contrast 10 and 15 min exposure time completely inhibited the growth of resistant isolates.

## DISCUSSION

In this study, researchers evaluate the emergence of drug-resistant and sensitivity to 2% glutaraldehyde disinfectant in *Acinetobacter baumannii* strains isolated from medical equipments of intensive care units from five hospitals in Tehran city. *Acinetobacter baumannii* long term survival property in harsh condition of hospital environments by sophisticate mechanisms such as biofilm production and attachment to wide range of materials and the ability of the organism to capture resistance genes. And therefore, emergence of PDR, XDR and MDR phenotypes causes global health concerns. However, *Acinetobacter baumannii* antibiotic resistance due to defects or reduction in expression of outer membrane proteins such as porins, presence of leakage system (efflux pumps) (Bonomo and Szabo, 2006), mutations in topoisomerase, occurrence of RND (resistance-nodulation cell division) which expose the genes *AdeAB*, *AdeBE* to discussion and other hundreds unknown mechanism, further encourage scientists to research about antibiotic resistance in this organism (Bonomo and Szabo, 2006). Due to potent capability of the organism to acquire resistance mechanisms and the ability to survive on dry inanimate and animate surfaces for long time is the main reason to study on the bacterium in Iran. In earlier studies the frequency of multidrug-resistant *Acinetobacter baumannii* species 30% were reported in the world (Kempf and Rolain, 2012) but today in some references announced this rate increased by 50% (Prata-Rocha *et al*., 2012). Ferreira *et al.* (2011) found that 68% of the Brazilian isolates are multidrug resistant. In Greece in 2012, the number of MDR phenotype of *Acinetobacter baumannii* 83.9% have been reported (Maraki *et al*., 2012). Allies in Tabriz in 2012 found that 80% of isolates are multidrug resistant (Peymani *et al*., 2012). Based on the study of Ghalavand in 2013 in Tehran, frequency of multiple drug-resistant isolate *Acinetobacter baumannii* were 83 and 44.8% were XDR. In the study, 87.3 of the isolates were multidrug resistant which is in according to the before findings. As yet no resistant isolate to colistin antibiotic have been reported in Iran which corresponded with other reports from different parts of the world. Ferreira and colleagues in a study in Brazil reported that 79% of isolate were resistant to carbapenems antibiotics in contrast in Ghalavands study this rate was reported as 92%. In the present study, the all

*Acinetobacter baumannii* isolates (100%) were resistant to carbapenems antibiotics which in compare to other previous data are the high incidence of carbapenem resistance in *Acinetobacter baumannii* isolates. Ghalavand in their study reported the incidence of meropenem, ciprofloxacin and cefepime in *Acinetobacter baumannii* isolates, 99, 98 and 90%, respectively which are same to the results. Vahdany *et al.* (2011) reported the frequency of imipenem resistant isolate by 19% while in the Boroumand *et al.* (2009) study it was 24/6%. In the Faizabady's study this rate was 50.9% (Vahdani *et al.*, 2011) according the results the frequency of imipenem resistant *Acinetobacter baumannii* was in accordance with the results of Faizabady study in contrast, the Imipenem and Meropenem frequency in Hujer research reported by 40% and Imipenem resistant *Acinetobacter baumannii* incidence in Vevencia study was 43% (Hujer *et al.*, 2006). Also, in the study the minimum inhibitory concentration was ≥64 ug $mL^{-1}$. The results showed that *Acinetobacter baumannii* can be isolated and are detachable from the medical equipment in intensive care units of hospitals. Researchers realized that *Acinetobacter baumannii* have asymetric distribution in the world as the frequency of the isolates from surface of medical equipments of intensive care units have been reported by Merick in 2005 in Turkey 26.8%, in India 43% and in Spain 1.7%. The study of Sahrifi in Iran cleared that distribution of *Acinetobacter baumannii* in a city is more homogeneous. They reported the following results of percentages of *Acinetobacter baumannii* isolation from the intensive care unit of surface of medical equipments from some hospitals of Qazvin city. The Shahid Rajaee Hospital 5.93%, the Qods Hospital 4.04%, the Kosar Hospital 1.8% and the Bu Ali Hospital 3.3%. This high percentage of resistance between *Acinetobacter baumannii* isolate from surface of medical equipments from intensive care unit is due to arbitrary and indiscriminate use of antimicrobials in the intensive care units. Since, respiratory tract is one of the main targets for *Acinetobacter baumannii*, adequate and accurate sterilization of equipments associated to respiratory tract in intensive care unit is essential to prevent of spreading of resistant strains of *Acinetobacter baumannii* from hospital to patient and to community. However, in a study in India by 28% of *Acinetobacter baumannii* isolated harbored the genes $bla_{CTX}$ and $bla_{TEM}$ while in the study this frequency was 22.6%. It's important to note that other mechanisms such as efflux pumps, production of β-lactamase enzymes and presence of outer memberan channels (porin) can be involved in resistance to antimicrobials and disinfectants. Moreover, this study showed that 2% glutaraldehyde is the effective disinfectant for sterilization of medical equipment in different hospital units, especially intensive care units as yet.

## CONCLUSION

The results of this study showed that the genetic patterns of particular genes $bla_{TEM}$ and $bla_{CTX}$ of *Acinetobacter baumannii* isolates have wide diversity. In other hand, analysis of genetic pattern of Acinetobacter isolates from hospitals showed close relevance together and same origin. Hence, based on rapid detection, identification and analysis such genes among *Acinetobacter baumannii* isolates and evaluation of new antibacterial and disinfectant agents efficacy and proper management. About the appropriate use of the antimicrobial agents can control and prevent of *Acinetobacter baumannii* infection and spreading of resistant strains from hospital to patient and to community. Also, precise and comprehensive study, scrutiny and research about phenotypic and genotypic resistance pattern of *Acinetobacter baumannii* against different classes of antibiotics and disinfectant agents in other countries in particular in Iran is recommended.

## ACKNOWLEDGEMENTS

Research Laboratory of Medical Microbiology Faculty of genuine cooperation, colleague and respected authorities intensive Imam Khomeini Hospital, Vali Asr, Labbafinejad, Fayaz Bakhsh and Taleghani sectors which have helped us in this study it is gratitude.

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