Evaluation of Antibiotic Resistance Patterns and Frequency of Carbapenemase-Producing *Acinetobacter baumannii* Isolates by the Carbacineto NP Test

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ABSTRACT

Background and Objectives: *Acinetobacter baumannii* is an opportunistic pathogen associated with nosocomial infections. Treatment of infections caused by this bacterium has become challenging due to increasing rate of resistance to a wide range of antibiotics such as carbapenems. One of the main mechanisms of resistance to carbapenems is the production of carbapenemase. The objective of this study was to evaluate antibiotic resistance patterns and frequency of carbapenemase-producing *A. baumannii* strains using the CarbAcineto NP Test.

Methods: In this descriptive cross-sectional study, 130 *A. baumannii* isolates were collected from clinical specimens of teaching hospitals in Zahedan in 2016. After determining the antibiotic resistance patterns, all *A. baumannii* isolates were examined using the phenotypic method of CarbAcineto NP test to evaluate production of carbapenemase enzymes.

Results: Based on the antibiogram results, more than 90% of the isolates were resistant to the antibiotics tested in this study. However, the lowest rate of resistance was observed against colistin, minocycline, tigecycline and doxycycline, respectively. Based on the results of the CarbAcineto NP test, 96% of carbapenem-resistant strains were positive for the production of carbapenemases.

Conclusion: Due to the high resistance of *A. baumannii* to carbapenems, they are not currently suitable for the treatment of infections caused by this bacterium. However, since most carbapenem-resistant strains are susceptible to colistin, minocycline, tigecycline, and doxycycline, these antibiotics or their combination are recommended for the treatment of the infections caused by the resistant strains. Rapid identification of carbapenemase-producing bacteria using efficient methods such as CarbAcineto NP test is essential to prevent their spread, particularly in hospitals.

KEYWORDS: *Acinetobacter baumannii*, Carbapenemase, CarbAcinetoNP Test.

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INTRODUCTION

Acinetobacter baumannii is a gram-negative, non-motile, non-fermenting, oxidase-negative, and opportunistic pathogenic coccobacillus. The bacterium can survive on solid and dry surfaces for up to 5 months, mainly because of simple nutritional requirements, ability to grow in a wide range of temperatures and pH values. They also have high degree of resistance to disinfectants and antiseptics, and ability to form biofilm on abiotic and biotic surfaces. *A. baumannii* has a natural ability to acquire antibiotic resistance mechanisms, all of which are essential for spread of hospital infections caused by the bacterium (1). The most common nosocomial infections caused by *A. baumannii* are ventilator-associated pneumonia and septicemia. They are associated with high mortality rate, as well as other infections including skin and soft tissue infections in burn patients, wound infection, urinary tract infection, and (rarely) secondary meningitis (2). The bacterium was the main cause of wound infections among military personnel in the wars in Afghanistan and Iraq, earning the nickname "Iraqibacter". In a study in the US on war-wound infections in Iraq and Afghanistan during 2007-2008, it was found that 63% of all bacterial isolates from biopsy tissues were *A. baumannii* (3). In 2004, the American Centers for Disease Control considered *A. baumannii* accountable for about 80% of all *Acinetobacter* infections.

Treatment of infections caused by these bacteria has become challenging, especially in recent years, due to the ability of these bacteria to obtain resistance markers (4). Carbapenems are a group of beta-lactam antibiotics with extensive activity against gram-negative and gram-positive bacteria, and are used as the last-line treatment. However, in recent years, resistance to carbapenems has increased significantly. Different mechanisms are responsible for the resistance of *A. baumannii* strains to carbapenems, but acquisition of carbapenemase genes is regarded as the most important mechanism (5). There are various molecular and phenotypic methods for detection of carbapenemase-producing strains. The phenotypic method, also known as the CarbAcineto NP test, is simple to use, cheap, rapid, and highly specific and sensitive method of detecting carbapenemase-producing *Actinobacter* spp.(6). It is essential to evaluate prevalence of carbapenemase-producing *A. baumannii* strains in different locations to determine the proper treatment options and methods for preventing the spread of infections caused by these bacteria. The objective of this study was to investigate antibiotic resistance patterns and frequency of carbapenemase-producing *A. baumannii* using the CarbAcineto NP test.

MATERIAL AND METHODS

In this study, 160 strains of *Acinetobacter* spp. were collected from teaching hospitals in Zahedan (Iran) within a 9-month period in 2016. The strains had been isolated from various clinical samples such as endotracheal tube, blood, ulcer, cerebrospinal fluid, urine and other body secretions. Among the 160 strains, 130 strains were determined as *A. baumannii* using biochemical tests and polymerase chain reaction (PCR). First, the bacteria were cultured on MacConkey agar and blood agar to obtain pure colonies. Then, the primary isolation of *A. baumannii* was carried out using multiple tests including triple sugar iron agar test, motility test in SIM medium, oxidase test (negative), growth at 42 °C and oxidative/fermentation glucose test (positive) (7). However, since the phenotypic methods are not able to differentiate and detect *A. baumannii*, the molecular method (PCR) was used for the detection of *blaOXA-51* gene. PCR is a an accurate, reliable and easy method of differentiating *A. baumannii* species (8). For amplification of the *OXA-51* gene, the boiling method was used for extraction of bacterial DNA. First, 24 hours after bacterial culture, a standard loop (10 µL) of bacteria was removed from the culture media and transferred to a 1.5 ml microtube containing 150 µL of normal saline. The content of the tube was mixed well until a uniform and concentrated suspension was obtained. Then, the microtube was placed in boiling water for 20 minutes and then centrifuged at 12500 rpm for 15 minutes. Approximately, 100 µL of supernatant was transferred to a 0.5 ml microtube. Primers used for amplification of the *OXA-51* gene are presented in Table 1.

The PCR reaction solution (25 µL) contained 22.5 µL of buffer mix, 0.5 µL (10 pmol) of each primer, 0.1 µL of Taq DNA polymerase and 1.5 µL of DNA. In order to prepare the buffer mix, 5 ml of deionized distilled water

was mixed with 1 ml of (10X buffer), 300 µL of $MgCl_2$ (50mM) and 70 µL of dNTP (10mM) and later brought to volume of 10 ml using deionized distilled water. The buffer was distributed in 1.5 ml microtube and then kept in freezer.

The amplification program was as follows: primary denaturation at 94 °C for 5 minutes, denaturation at 94 °C for 30 second, annealing at 53 °C for 35 seconds, extension at 72 °C for 1 minute, and 35 cycles of final extension at 72 °C for 10 minutes. PCR products were electrophoresed on 1.2% agarose gel stained with safe stain and then examined with gel doc (Figure 1). Antibiotic resistance patterns of samples containing the *OXA-51* gene (130 strains of *A. baumannii*) were evaluated. For this purpose, 20 antibiotic disks and E-test were used for colistin and imipenem antibiotics. The antibiotic disks used in the study were as follows: Aztreonam (30µg), piperacillin (100µg), piperacillin tazobactam (110µg), ciprofloxacin (5µg), ceftriaxon (30µg), levofloxacin (5µg), ceftazidime (30µg), imipenem (10µg), meropenem (10µg), cefepime (30µg), cotrimoxazole (25µg), rifampin (30µg), amikacin (30µg), tobramycin (10µg), gentamicin (120µg), ampicillin sulbactam (20µg), doxycycline (30µg), minocycline (30µg) tigecycline (15µg) and colistin (10µg).

All disks were purchased from MAST Co. and standard strain of *Escherichia coli* ATCC 25922 was used for quality control. Antibiotic susceptibility testing was done based on Clinical and Laboratory Standards Institute (CLSI) guidelines and criteria for all antibiotics except for tigecycline (10). In this study, resistance of *A. baumannii* to tigecycline was evaluated based on two criteria, American Food and Drug Administration (FDA) criteria for *Enterobacteriaceae*. and the Jones criterion for *Acinetobacter* spp. (11). First, the solutions were prepared according to the CLSI guidelines for the Carba NP Test as described below (10). Solution A: in a 25 to 50 ml container, 16.6 ml of deionized distilled water were mixed with 2 ml of 0.5% phenol red solution and 180 µl of 10Mm $ZNSO_4$-$7H_2O$. In addition, pH of the solution was adjusted to 7.8 using NaoH (0.1N) or HCL (10%). The solution was kept away from light and stored at 4-8 ^oC. The solution remains stable until it is discolored. Solution B: imipenem powder was added to solution A to obtain a 6 mg/ml solution. The solution remains stable for three days in refrigerator.

Note: Phenol red (0.5%) and zinc sulfate (10mM) are kept at room temperature. These two solutions are stable at room temperature for one year.

In a 1.5 ml microtube, 100 µL of NaCl solution (5M) was mixed with 10 µL of bacteria until a uniform and concentrated suspension was achieved. Next, 100 µl of solution B was added to the solution. After through mixing, the tube was incubated for two hours at 37 °C. Color change from red to yellow or orange confirmed production of carbapenemase (6). Metallo-β-lactamase positive *Klebsiella pneumonia* (Pasteur Institute of Iran) was used as positive control, while *E. coli* ATCC 25922 and a blank tube (reagent without bacteria) were used as negative control.

RESULTS

Table 2 demonstrates the results of antibiotic susceptibility testing for all 130 *A. baumannii* strains. Table 3 represents the results of resistance pattern and susceptibility testing for tigecycline based on the Jones and FDA criteria.

Figure 1- Image of gel electrophoresis for the *OXA-51* gene. Column 4: positive control, column 5: negative control, column 9: Blank and columns 1, 2, 3, 6, 7, 8: strains containing the *OXA-51 gen.*

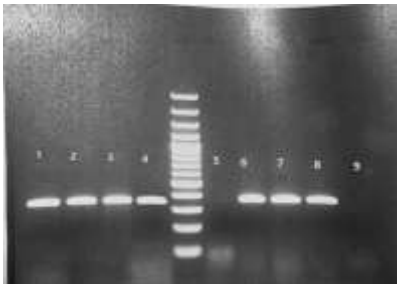


Table 1- Primers used for the amplification of *OXA-51* gene (9)

Gene name	Nucleotide sequence	Product size
bla oxA - 51F	TAA TGC TTT GAT CGG CCT TG	353bp
bla oxA 51R	TGG ATT GCA CTT CAT CTT GG	

Table 2- Frequency of antibiotic resistance for *A. baumannii* strains

Antibiotics	Resistant	Moderate	Susceptible
1. Aztreonam	125 (96.2%)	1 (0.8%)	4 (3%)
2. Piperacillin	125 (96.2%)	0	5 (38%)
3. Piperacillin tazobactam	124 (95.4%)	1 (0.8%)	5 (38%)
4. Ciprofloxacin	124 (95.4%)	0	6 (4.6%)
5. Ceftriaxone	124 (95.4%)	4 (3.1%)	2 (1.5%)
6. Levofloxacin	123 (94.6%)	0	7 (5.4%)
7. Ceftazidime	123 (94.6%)	0	7 (5.4%)
8. Imipenem	123 (94.6%)	0	7 (5.4%)
9. Cefepime	123 (94.6%)	1 (0.8%)	6 (4.6%)
10. Cotrimoxazole	121 (93.1%)	3 (2.3%)	6 (4.6%)
11. Rifampin	121 (93.1%)	7 (5.4%)	2 (1.5%)
12. Meropenem	120 (92.3%)	3 (2.3%)	7 (5.4%)
13. Amikacin	118 (90.8%)	5 (3.8%)	7 (5.4%)
14. Tobromycin	90 (69.2%)	9 (6.9%)	31 (23.9%)
15. Gentamicin	89 (68.5%)	11 (8.5%)	30 (23%)
16. Ampicillin Sulbactam	79 (60.8%)	27 (20.8%)	24 (18.4%)
17. Doxycycline	47 (36.2%)	0	83 (63.8%)
18. Minocycline	16 (12.3%)	8 (6.2%)	106 (81.5%)
19. Colistin	0	0	130 (100%)

Table 3- Resistance patterns against tigecycline among *A. baumannii* isolates based on the Jones and FDA criteria

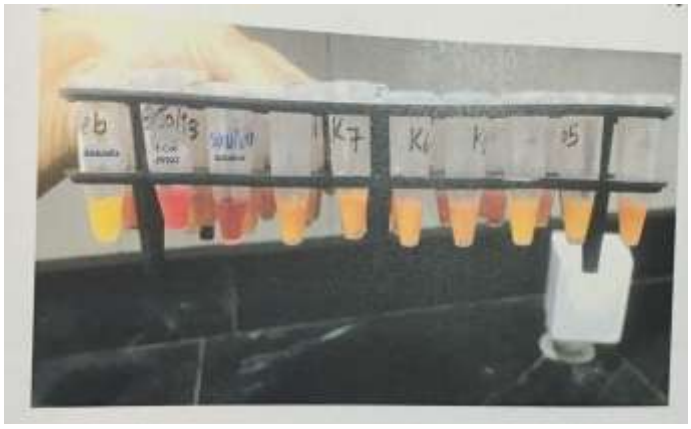
Criterion	Resistant	Intermediate	Susceptible
Jones	14.3%	32.4%	53.3%
FDA	35.2%	49.5%	15.3%

All 130 *A. baumannii* isolates were susceptible to colistin based on the CLSI guidelines. Seven *A. baumannii* strains were susceptible to imipenem. The remaining 123 strains of *A. baumannii* were resistant to imipenem. Similar findings were obtained for susceptibility to colistin and imipenem in the disk diffusion method. Based on both criteria, the lowest rate of resistance was observed against clostin, minocycline, tigecycline, doxycycline, and ampicillin-sulbactam, respectively.

In this study, all *A. baumannii* strains were evaluated by Carb AcinetoNP test.

None of the carbapenem-susceptible strains (7 strains) showed color change from red to yellow or orange, but of the 123 carbapenem-resistant strains, 118 strains showed color change from red to yellow or orange, and therefore found positive for production of carbapenemase. In other words, 96% of carbapenem-resistant strains produced carbapenemase (Figure 2).

Figure 2- Image of CarbAcineto NP test results



DISCUSSION

Carbapenems were drugs of choice for treatment of multidrug-resistant (MDR) *A. baumannii* infections, but the widespread use of these antibiotics in recent years has led to a rapid increase in frequency of carbapenems–resistant *A. baumannii* strains (12). Resistance of *A. baumannii* to carbapenems could be attributed to decreased permeability of the outer membrane following the loss or modification of porins, modification of penicillin-binding proteins (PBPs), increased expression of efflux pumps, and production of carbapenemase. Most carbapenemases identified in *Acinetobacter* spp. are located on mobile genetic elements that may be transferred to other clinically relevant species (6). Therefore, in order to prevent the spread of carbapenemase-producing bacteria, it is essential to separate them from strains whose carbapenem resistance is dependent on non-transferable mechanisms (loss of porines, overexpression of efflux pumps or modification of PBPs (13). Due to the intrinsic low permeability, the evaluation of carbapenemase production in *Acinetobacter* is more difficult compared to *Enterobacteriaceae* and *Pseudomonas* spp. Several phenotypic techniques have been proposed for the detection of carbapenemase-producing *Acinetobacter* spp. (14). For instance, modified Hodge test is based on the in vitro detection of carbapnemase activity, but may produce false negative results in some cases (15). Another method is the Carba NP test, which is based on colorimetry and pH change due to hydrolysis of the beta-lactam ring by carbapenemase. Although this test is valid for the detection of carbapenemase producers among *Enterobacteriaceae* and *Pseudomonas* spp., it has not been validated for the detection of carbapenemase-producing *Acinetobacter* spp., mainly because of low sensitivity (11.9%). The CarbAcineto Np test was adapted from the updated version of the Carba NP test, and has been used as a rapid and reproducible method of detecting all carbapenemase types in *Acinetobacter* spp. with high sensitivity (94.7%) and specificity (100%)(6).

In our study, 123 *A. baumannii* strains (94%) were resistant to carbapenems (imipenem and meropenem) in the CarbAcineto NP test. Of these strains, 118 strains (96%) were found positive for the production of carbapenemase, which highlights the necessity of considering infection control criteria in hospitals. Various studies in Iran (16-19) and other countries (20-23) have shown the high rate of carbapenem-resistance in Acinetobacters, which indicates these drugs are no longer effective for treatment of *Acinobacter* infections. Another noteworthy finding was the high rate of susceptibility to minocycline. Early studies in the 1970s showed that minocycline was an effective antibiotic for the treatment of *Acinetobacter* infections. At that time, most *A. baumannii* strains were susceptible to more potent available antibiotics, and minocycline was not considered as the drug of choice. However, the global emergence of MDR *A. baumannii* strains led to reconsideration of old but safe therapeutic options, such as minocycline. In 2014, the CLSI re-located minocycline for *Acinetobacter* species in own box in test report group B. In a study by Adib Hesami et al. during 2011-2013, it was shown that minocycline is still an effective therapeutic option for the treatment of *Acinetobacter* infections (24). According to a study in the US in 2017, minocycline is the only available therapeutic option and active antibiotic following colistin for the treatment of infections caused by MDR *A. baumannii* (25). In a 6-year study, Denys et al. reported that 72% of *Acinetobacter* isolates were susceptible to minocycline (26). We also found that 81.9% of the isolates are susceptibility to minocycline. However, a study in China in 2017 revealed that minocycline is not commonly used in China due to toxicity. Inconsistent with the mentioned studies, a study on 2917 *A. baumannii* isolates in 2004-2014 showed that susceptibility to minocycline has increased in China from 13.4% to 64.5% (27)

CONCLUSION

Due to the high resistance of *A. baumannii* to carbapenems, they are not currently suitable for the treatment of infections caused by this bacterium. However, since most carbapenem-resistant strains are susceptible to colistin, minocycline, tigecycline, and doxycycline, these antibiotics or their combination are recommended for the treatment of infections caused by the resistant strains. Rapid identification of carbapenemase-producing bacteria using efficient methods such as

CarbAcineto NP test is essential to prevent their spread.

In addition, due to the high prevalence of carbapenemase-producing *A. baumannii* strains, it is crucial to consider infection control criteria in hospitals.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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