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Phenotypic and Molecular Detection of *Acinetobacter baumannii* Strains Producing Carbapenemase from Clinical Specimens in Dezful Teaching Hospital

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Abstract

Nowadays, antibiotic resistance in bacteria has become a global problem. Therefore, the identification of resistant strains of bacteria has attracted special attention in a pursue to choose more effective treatment solutions. One of the most common mechanisms of resistance is the production of carbapenemase enzyme in Acinetobacter baumannii. This present study aimed to detect carbapenemase-producing strains by phenotypic and molecular methods in clinical specimens collected in Ganjavian Hospital of Dezful between June 2021 and May 2022. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion and Etest assay, while the extended-spectrum beta-lactamase production (ESBLs) and Metalobetalactamases (MBLs) were detected by combined disk method using ceftazidime and ceftazidime/clavulanic acid disks and imipenem and imipenem /EDTA disks, respectively. Molecular detection of bla_{IMP}, bla_{SPM}, bla_{OXA-23}, *and* bla_{OXA-24}, bla_{OXA-58}, blaGES genes was performed. Of a total of 54 strains, the highest resistance rate was for cephalosporins (98.1%), and ciprofloxacin (94.2%), in contrast to minocycline (13%). ESBL and MBL producers were 26% and 80%, respectively. All isolates had intermediate resistance against colistin. The most prevalent gene in the carbapenem-resistant *A. baumannii* (CRAB) was bla_{OXA-23}, followed by $bl_{aOXA-24}$, bla_{GES}, bla_{IMP}, and bla_{OXA-58} genes. Present report highlights the existence of CRAB and intermediate resistance to colistin and also the co-existence of several genes from different carbapenemase classes in this region. Therefore, resistant strains should be identified promptly and specific treatment protocols should be devised to control the dissemination of resistance genes in therapeutic settings.

Keywords: *A. baumannii*, carbapenem resistance, carbapenemase, genotyping

Introduction

Acinetobacter strains are non-fermenting gram-negative, oxidase-negative, and catalase-positive coccobacilli that grow at 44°C. In the last three decades, these bacilli are commonly involved in a variety of nosocomial infections, including infections in the lower respiratory tract, urinary tract, skin and soft tissues, and central nervous system, as well as in septicemia, and other diseases, especially in hospitalized patients.[1]

Currently, the emergence of multidrug-resistant *Acinetobacter baumannii* infections in healthcare settings, which is associated with a long period of hospitalization, has become public health challenge.[2] This has led to social and economic impacts as these patients require longer therapy and more expensive antibiotics, among others. Carbapenems have been considered as a significant development in antibiotic therapy for these cases due to their wide range of activity and resistance to hydrolysis by most beta-lactamases, making it the most effective drug for severe multi-drug resistance (MDR) *A. baumannii* infections. This class of antibiotics is bactericidal and act by inhibiting the synthesis of bacterial cell wall peptidoglycans, with cell wall synthesis as their final action.[3] Carbapenems are the last line of defense against penicillins and cephalosporins-resistant gram-negative bacteria. The broad-spectrum effect of carbapenems has made them one of the most widely used antibiotics and the

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primary option for treating MDR strains.

A. baumannii has been included into the urgent threat list of the CDC since 2019, and the emergence of its carbapenem-resistant strain has making it a more concerning threat.[4] Carbapenem-resistant Acinetobacter strains first caused an outbreak of nosocomial infections in 1991, which have been reported in various countries.[5] The extensive use of carbapenems has resulted in heightened resistance, and the horizontal transfer of these genes has escalated mortality and morbidity caused by carbapenem-resistant *A. baumannii* (CRAB) infections globally.[6]

Three primary mechanisms are involved in the resistance to carbapenems and reduced uptake of drugs, including overexpression of efflux pump, mutations in porin, genetic variations in the structure of penicillin-binding proteins, and carbapenemase enzyme production. The phenotypic and genotypic techniques are used to identify these resistance enzymes. The major carbapenemases are divided into three groups according to the Ambler classification: class A, class B, and class D c[7]. Class A is serine carbapenemases and includes KPCs, GES, SME, and NMC, some are encoded by chromosomes ($bla_{IMP,}$ $bla_{NMC,}$ bla_{SME} genes) while the KPC genes are plasmid-encoded.8 Class B includes IMP, SPM, GIM, and SIM, known as Metallo-β-lactamases (MBLs), with strong hydrolysis activity against all beta-lactam antibiotics except for monobactams.[8,9] Another characteristic of these enzymes is the presence of zinc or other heavy metals in their active site, which are inhibited by metal chelators such as the thiol group instead of beta-lactamase inhibitors.[10] The last class, class D, includes Oxacillinase genes that hydrolyze oxacillin faster than benzyl penicillins. bla_{OXA-51} gene is inherent in *A. baumannii*, and genes $bla_{OXA-23,}$ $bla_{OXA-24,}$ $bla_{OXA-48,}$ and bla_{OXA-58} are involved in the hydrolysis of carbapenems. [11]

This is the first study that has been conducted in North Khuzestan using antimicrobial susceptibility testing by disk diffusion method and minimum inhibitory concentration (MIC), phenotypic tests of MBLs and extended-spectrum beta-lactamases (ESBLs), and molecular detection of bla_{IMP}, bla_{SPM}, bla_{OXA-23}, and bla_{OXA-24}, bla_{OXA-58}, bla_{GES} genes in *A. baumannii* strains. In this study, the phenotypic and molecular detection of carbapenem resistance *A. baumannii* was performed in clinical isolates collected in a university teaching hospital.

Methods

This cross-sectional study was carried out using medical records of patients admitted to different wards of the teaching hospital where the study was performed. From a total of 225 bacteria isolated from the clinical samples of inpatients and outpatients referred to the hospital in the period of June 2021 to May 2022, 54 nonduplicate *A. baumannii* strains from tracheal, broncho-alveolar lavage (BAL), urine, blood, and wounds were collected. Isolation of bacteria on sheep blood agar and MacConkey agar was routinely performed in the hospital laboratory as part of their diagnostic and treatment processes. The phenotypic identification of strains was performed by gram-staining and conventional biochemical tests that included oxidase, catalase, citrate utilize, sugars fermentative oxidative test, growth at 44 ° C, motility, growth on MacConkey agar, and inoculation on Triple Sugar Iron agar (Pronadisa) .[12]

The polymerase chain reaction (PCR) amplification was employed to recognize an intrinsic bla_{OXA-51} gene as the indentified marker of the *A. baumannii* species using primers (Table 1) for the amplification of 353-bp fragment (Bioneer, Daejeon, Korea).[13] Bacterial DNA obtained from the boiling method was subjected to PCR. The PCR process was performed with a final volume of 20 µl, consisting of 10 µl Taq PCR, Master Mix 2x (Qiagen), 1 µl in each primer in the concentration of 0.2 mM, and distilled water. The PCR amplification process consisted of initial denaturation (95 °C, 30 sec), 35 cycles of denaturation (95 °C, 30 sec), annealing (54°C, 30 sec), extension (72 °C, 30 sec); final extension (72°C, 10 min). which was performed using a Labnet 85 MultiGene™ OptiMax thermal cycler. The PCR products were investigated by electrophoresis on 1% w/v agarose gel (Sigma-Aldrich) in 1X TBE (Tris-borate-EDTA) containing SafeStain and visualized on a UV transilluminator on Gel Documentation System (DAIHAN Scientific, Korean).

A fresh pure colony of bacteria was prepared on equal turbidity to 0.5 McFarland standard, inoculated on *Mueller Hinton Agar* (*MHA*) (Pronadisa, Madrid, Spain). and testing was done by Kirby-Bauer disk diffusion method for various antibiotics including amikacin (30 µg), gentamicin (10 µg), imipenem (10 µg), meropenem (10

µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), doxycycline (30 µg)), and minocycline (30 µg) that were obtained from MAST company. Data were interpreted according to the method from the *Clinical and Laboratory Standards Institute (CLSI).* Minimal inhibitory concentrations (MIC) to meropenem (0.016 to 256 µg) and colistin (0.64 to 1024 µg) were determined by Etest assay (Liofilchem). Extensively drug resistant (XDR) *A. baumannii* strains are defined as strains that are resistant to at least 3 different classes of antibiotics (Aminoglycosides, Cephalosporins, and Fluoroquinolones) and one of the carbapenems in antimicrobial susceptibility testing.

The fresh culture of bacteria grown according to the Kirby Bauer disk diffusion method was inoculated on MHA, and imipenem (10 µg) and imipenem (10 µg) plus 750 µg of EDTA disks (HiMedia) were used for the detection of metalobetalactamases (MBLs). The extended-spectrum beta-lactamases (ESBLs) were detected by ceftazidime (30 µg) alone and in combination with clavulanic acid (10 µg) (MAST). The interpretation of results was recorded based on an increase of the formation of inhibition zone diameter of ≥7 mm and 5 mm in combination disks for MBLs and ESBLs, respectively.

DNA extraction of *A. baumannii* isolates was obtained through boiling, with pure and fresh colonies of carbapenems resistance *A. baumannii* isolates on Tryptic Soy Agar medium were harvested, suspended in 500 µl of sterile deionized water, and boiled for 10 minutes. After centrifugation at 19,000 g for 5 minutes, the extracted DNA was aliquoted and preserved at -20 °C until the PCR analysis was performed. A total of 14 primers were applied to determine the carbapenemase genes. The primers sequence of carbapenemase genes is listed in Table 1. PCR amplification process was performed under the following conditions: initial denaturation (94 °C, 4 min); 35 cycles of denaturation (94 °C, 30 sec), annealing (50 °C, 30 sec), extension (72 °C, 30 sec); followed by an additional 10 min at 72°C whereas bla_{IMP} PCR conditions consisted of annealing (55 °C, 30 sec). The PCR products were visualized in a 1% (w/v) agarose gel electrophoresis.

Data obtained were entered in SPSS v.21 software, and statistical analysis was performed. performed. A value of <0.05 was considered a statistically significant difference. The data obtained were entered into SPSS v.21 software, and Fisher's Exact Test was utilized to analyze the results. A p-value of <0.05 was considered indicative of a statistically significant difference.

This study was approved by the grants from Dezful University of Medical Sciences and performed following the principles of the Declaration of Helsinki (Grant/Ethic Cert. No: IR.DUMS.REC.1397.039).

Results

A total of *A. baumannii* strains were isolated from 40 male and 14 female inpatients. Using the PCR

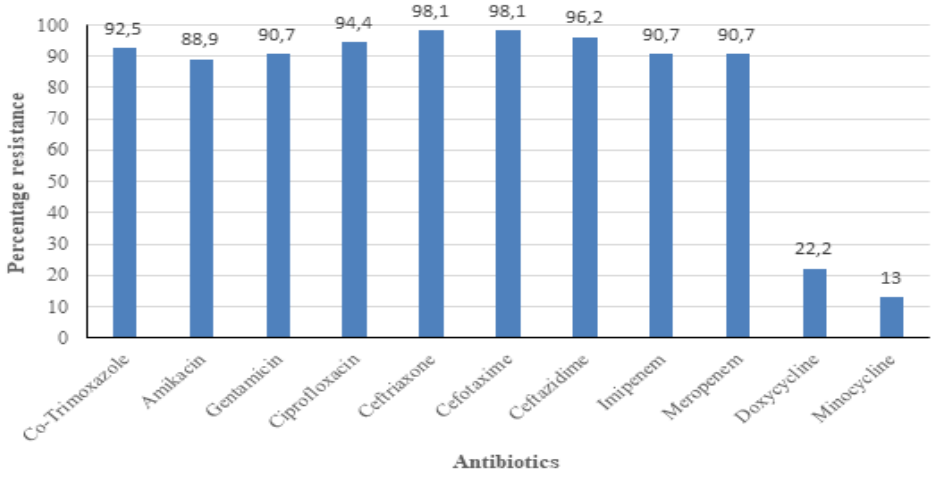

Figure Antibiotic-resistant Pattern of *A. baumannii* Isolates by Disk Diffusion Method

Table 1 Oligonucleotide primers Used in This Study

Genes	Primer Sequence (5' 3')	Product size (bp)	Reference
bla_{OXA-51}			
F	TAATGCTTTGATCGGCCTTG	353	[13]
R	TGGATTGCACTTCATCTTGG		
bla_{OXA-23}			
F	GATCGGATTGGAGAACCAGA	501	[13]
R	ATTTCTGACCGCATTTCCAT		
bla_{OXA-24}			
F	GGTTAGTTGGCCCCCTTAAA	246	[13]
R	AGTTGAGCGAAAAGGGGATT		
bla_{OXA-58}			
F	AAGTATTGGGGCTTGTGCTG	599	[13]
R	CCCCTCTGCGCTCTACATAC		
bla_{IMP}			
F	ACCGCAGCAGAGTCTTTGCC	585	[30]
R	ACAACCAGTTTTGCCTTACC		
bla_{SPM}			
F	GCGTTTTGTTTGTTGCTC	786	[30]
R	TTGGGGATGTGAGACTAC		
bla_{GES}			
F	ATGCGCTTCATTCACGCAC	640	[30]
R	CTATTTGTCCGTGCTCAGG		

Notes: OXA: Oxacillinase, IMP: Imipenemas, SPM: Sao Paulo metallo-β-lactamases, GES: Guiana extended-spectrum β-lactamase. All primers are manufactured by Metabion International AG, Germany

method of bla_{OXA-51} gene, it was confirmed that all 54 bacteria investigated by phenotypic methods were *A. baumannii*. The highest frequency of *A. baumannii* strains was found in ICUs (82.7%), internal (nephrology and gastroenterology) (7.7%), and orthopedic (3.8%) wards. The frequency of clinical samples was as follows: 37 lower respiratory tract secretion samples (68.5%), 9 urine (16.6%), 6 wound (11.1%), and 2 blood (3.7%).

The antimicrobial-resistant pattern of *A. baumannii* strains is displayed in Figure 1. The highest and lowest resistance rates were observed in third-generation cephalosporins (98.1%) and minocycline (13%), respectively. There was a correlation between meropenem disk diffusion and MIC, with 40 (74%) resistant strains were detected (MIC ≥ 8 µg/mL). Ninety percent of CRAB strains were isolated from ICU patients, endotracheal tubes (n=25, 65%), and BAL (n=5, 12.5%). The majority of patients experienced pulmonary disorders (80%), with mortality was identified in 22.5% of hospitalized patients in the ICU wards. All CRAB had a colistin MIC of less than 2 µg/mL, which was interpreted as the intermediate breakpoint. In phenotypic detection methods, 14 (26%) and 43 (80%) were ESBL and MBL producers, respectively.

The molecular findings of carbapenemase genes showed that bla_{OXA-23} was the most prevalent gene in the carbapenem-resistant *A. baumannii* (57.4%), followed by bla_{OXA-24} gene (51.9%), bla_{GES} gene (11.1%), bla_{IMP} gene (9.3%), and bla_{OXA-58} gene (3.7%). None of the strains carried the bla_{SPM} gene. Overall, the carbapenemase genes were diagnosed in 35 carbapenem-resistant *A. baumannii* in eight different patterns (Table 2). According to the antimicrobial susceptibility testing profile, the strains in all of these patterns were extensively drug-resistant

Table 2 The Patterns of Carbapenemase Genes in Carbapenem Resistance *A. baumannii* Isolates

Gene Pattern Number	Class A	Class B	Class D	Number of isolates (%)	Specimen type	Ward	Gender	Death outcome no. (%)
1	*GES*	-	*OXA-23, OXA-24, OXA-58*	1 (2.86)	Wound (1)	Orthopedic (1)	M (1)	0 (0)
2	*GES*	-	*OXA-23, OXA-24*	4 (11.43)	Tracheal (2), BAL (1), Urine (1)	ICU (3), Internal * (1)	F (2), M (2)	3 (75)
3	*GES*	-	*OXA-24*	1 (2.86)	Tracheal (1)	ICU (1)	M (1)	0 (0)
4	-	*IMP*	*OXA-23, OXA-24*	4 (11.43)	Urine (1), Tracheal (3)	ICU (4)	M (3), F (1)	1 (25)
5	-	*IMP*	*OXA-24*	1 (2.86)	Tracheal (1)	ICU (1)	F (1)	0 (0)
6	-	-	*OXA-23, OXA-24, OXA-58*	1(2.86)	Tracheal (1)	ICU (1)	F (1)	0 (0)
7	-	-	*OXA-23, OXA-24*	17 (48.56)	BAL (3), Tracheal (10), Ascites fluid (1), Urine (2), Wound (1)	ICU (15), Internal (1), Orthopedic (1)	M (14), F (3)	4 (23.5)
8	-	-	*OXA-23*	6 (17.14)	Tracheal (4), Urine (1), Wound (1)	ICU (5), Internal (1)	M (4), F (2)	1 (16.6)

Notes: M: Male, F: Female, Internal: Nephrology and gastroenterology wards

(XDR), except for two strains in pattern 7, one of which was sensitive to fluoroquinolones and the other has intermediate susceptibility to imipenem and meropenem. Most deaths were observed in pattern 2 (75%), with the co-existence of bla_{GES}, bla_{OXA-23}, and bla_{OXA-24} genes. No statistically significant difference was observed among the eight gene patterns and variables such as XDR, ESBL, resistance to carbapenems, and fluoroquinolones (p-value>0.05) (Table 3). All 35 carbapenem-resistant strains were resistant to aminoglycosides, cephalosporins, and trimethoprim-sulfamethoxazole.

Discussion

In recent decades, an increase in MDR, XDR, and CRAB infections has become a life-threatening clinical problem, limiting therapeutic options and increasing mortality. In this study, the highest level of resistance observed was to the third-generation cephalosporins (98.1%). This result is consistent with the findings reported by Amin[14] in Ahvaz and ultimately limits patients' access to the most effective antibiotics available. The prevalence of CRAB was observed at 74% with an approximate mortality rate of 22 %, and 40 strains were intermediate sensitivity to colistin. In comparison, in Joshi's study, 97.7% were MDR and all strains were sensitive to colistin[15]. Observation of carbapenem resistance raises concerns about the spread of antibiotic resistance in hospitals, especially in ICUs. In contrast to the present study, 5.5% of strains in Turkey were reported to be resistant to colistin.[16] Thus, in our patients, colistin can be used as an option in XDR infections treatment. However, despite the sensitivity of all strains to colistin, the observation of intermediate resistance (i.e., MIC more than 2) requires special attention to the fragility of the situation in the study hospital and the side effects of colistin. This could be a wake-up call for the spread of colistin resistance and the need to provide newer generations of antibiotics.

In present study, minocycline was identified as the most effective therapeutic option, especially in MDR strains like Mohajeri [17], but in another one resistance rate was 40 percent.[18] Considering that fluoroquinolone can also be a treatment option for MDR infections, the ciprofloxacin resistance

Table 3 The Comparison of XDR, ESBL, Antibiotic Resistance Profile, and Patient Outcomes Among Gene Patterns of *A. baumannii* Strains

Valuables	Type	Gene patterns							
		1	2	3	4	5	6	7	8
XDR	Positive (%)	1 (100)	4 (100)	1 (100)	4 (100)	1 (100)	1 (100)	15 (88.3)	6 (100)
	Negative (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (11.7)	0 (0)
ESBL	Positive (%)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)	0 (0)	1 (5.9)	0 (0)
	Negative (%)	1 (100)	4 (100)	1 (100)	3 (75)	1 (100)	1 (100)	16 (94.1)	6 (100)
Fluoroquinolones	Resistance (%)	1 (100)	4 (100)	1 (100)	4 (100)	1 (100)	1 (100)	16 (94.1)	6 (100)
	Susceptible (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (5.9)	0 (0)
Carbapenems	Resistance (%)	1 (100)	4 (100)	1 (100)	4 (100)	1 (100)	1 (100)	16 (94.1)	6 (100)
	Intermediate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (5.9)	0 (0)
	Susceptible (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Outcomes	Death (%)	0 (0)	3 (75)	0(0)	1(25)	0 (0)	0 (0)	4 (23.5)	1 (16.6)
	Life (%)	1 (100)	1 (25)	1(100)	3(75)	1 (100)	1 (100)	13 (76.5)	5 (83.4)

Notes: XDR: Extensively drug-resistant, XDR *A. baumannii* strains include resistance to at least 3 different classes of antibiotics (Aminoglycosides, Cephalosporins, and Fluoroquinolones) and resistance to one of the carbapenems in antimicrobial susceptibility testing. ESBL: Extended-spectrum beta-lactamases. ESBL-positive *A. baumannii* strains were detected by combined disk method using ceftazidime and ceftazidime/clavulanic acid disks.

of 94.2% creates a significant limitation in the treatment of patients, especially in opportunistic nosocomial infections.

In *A. baumannii* strains, the bla_{OXA-51} gene is used to confirm strains, which was also used in our study. Eight molecular patterns were recognized in this study (Table 2), with pattern 7 (48.56%) as the pattern of the highest frequency with the co-existence of bla_{OXA-23} and bla_{OXA-24}, followed by patterns 2 and 4 with 11.43% frequency. Although bla_{GES} and bla_{IMI} genes were detected in 6 and 5 strains, respectively, their co-existence with other genes can increase their severity of antibiotic resistance and ease of transmission[19]. Accumulation of different genes from population clones of bacteria in one strain can contribute to the simultaneous expression of resistance genes. Therefore, the effective treatment options in infectious diseases are greatly reduced[20]. The highest co-existence was observed in pattern 1 with bla_{OXA-23}, bla_{OXA-24}, $bla_{OXA-58,}$ and bla_{GES} genes isolated from the wound secretions of patients from the orthopedic ward. The co-existence of carbapenemase genes has been reported in Gozalan and Kock's studies for bla_{OXA-23} and bla_{OXA-58} genes,[9,21] also in Mohajeri's studies for bla_{OXA-23} and bla_{OXA-24} genes.[17]

In present study, the bla_{OXA-58} gene was observed in 2 (5%) CRAB strains, which is unlike Ramadani's study that does not detect in any of the strains[22]. Nevertheless, the highest prevalence of bla_{OXA-58} and bla_{OXA-24} genes are reported in Mexican hospitals with 28.3% and 83.3%, respectively[23]. These differences can be due to the diversity in antibiotic regimens used in the therapeutic settings and circulating strains. MBL phenotype was detected in 80% of *A. baumannii* strains of clinical specimens, similar to the findings in a study by Noori[24]. Here, the bla_{IMI} gene was reported to be 12.5%,

which was similar to Amin's findings on isolates circulating in the Ahvaz[14] but in contrast to Joshi and Ramadan's studies that do not detect this gene in any isolates[15,22] Frequent phenotypic and genotypic differences of MBLs in ICUs are observed in the studies, showing that some strains had the MBL phenotype without bla_{IMI} genes. Therefore, the role of other B-class genes should be further evaluated.

The bla_{GES} gene was identified in 6 (11.1%) strains which was inconsistent with the investigations of Hazhir Kamal and the report of bla_{GES} in 14 centers in Lithuania.[25] The dominant molecular pattern in CRAB strains was pattern 7, with the co-existence of bla_{OXA-23}, and bla_{OXA-24} genes (48.56%). Two non-XDR strains were identified, a bacterium with intermediate susceptibility to meropenem and imipenem, with sensitivity to fluoroquinolones was observed in others (Table 3). Therefore, apart from the limited access to molecular techniques in many hospitals, the most effective treatment should be determined with standard phenotypic tests, especially in severe pulmonary infections in ICUs. In this study, some XDR *A. baumannii* strains were resistant to third-generation cephalosporins without ESBL phenotype (Table 3), which could be due to the AmpC cephalosporinase. In this chromosomally mediated cephalosporinase, the insertion of ISAba1 upstream of the *ampC* gene leads to third-generation cephalosporin resistance.[26] The accumulation of several drug resistance mechanisms, in addition to the survival potential of *A. baumannii* strains and evasion mechanisms from host immune responses, has made the treatment of Acinetobacter infections an important challenge worldwide.[27] The selective pressure of excessive antibiotic administration and the presence of drug-resistance genes in the environment have an essential effect on the horizontal transmission of carbapenem-resistance genes and various genotypic and phenotypic patterns. Other carbapenem resistance mechanisms such as reduced drug absorption, overexpression of efflux pumps, mutations in porins, and genetic variations in penicillin-binding proteins should be considered.

This study emphasizes the fact that the presence of resistance strains due to rapid and horizontal transfer of carbapenem resistance genes increase the concern of public healthcare and mortality worldwide.[7] Yet, this sudy has its limitations as the MIC value of all antibiotics cannot be utilized. Additionally, the study was conducted with limited time and number of samples. Therefore, subsequent studies with a larger sample size and a longer period are recommended. In conclusion, Class D carbapenemases (bla_{OXA-23} and bla_{OXA-24}) play an important role in the carbapenem resistance in the study hospital. Due to the co-existence of four genes in one strain and the highest number of deaths in some gene patterns, a serious alarm has been sounded to disable the most effective antibiotics. Effective use of these results in academic infection control and stewardship committees can lead to the rational antibiotic prescription of antibiotics and the control and monitoring of CRAB strains. It is suggested that other carbapenemase genes be studied in future studies.

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