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"To Study the Molecular Characterization of Invasive Carbapenem-Resistant *Acinetobacter baumannii* with special reference to blaVIM genes in ICU patients at a Tertiary Care Centre, Uttar Pradesh, India".

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Abstract

Introduction: Carbapenem-resistant *Acinetobacter baumannii* (CRAB) is an important health problem for the treatment of infectious diseases. Carbapenems have historically been used as a last resort for treating MDR Gram-negative bacterial infections. There has been a recent surge in carbapenem resistance in *A. baumannii.* The majority of the carbapenem resistance mechanisms *in A. baumannii* are due to enzymatic degradation by -lactamases.

Aim And Objectives: To study the Molecular Characterization of Invasive Carbapenem-Resistant *Acinetobacter baumannii* with special reference to blaVIM gene in ICU patients at a Tertiary care centre, Uttar Pradesh.

Material And Methods: This was a Cross sectional study carried out in the Department of Microbiology for a period of 1 year i.e, August 2022 to August 2023 at a tertiary care centre, Uttar pradesh. A total of 210 non-duplicate, consecutive, carbapenem-resistant isolates recovered from *Acinetobacter* species were included in this study. The isolates were obtained from the clinical

samples. The isolates were identified by the Standard biochemical tests and the Antimicrobial susceptibility testing was performed according to the CLSI guidelines 2022.

Results: In the present study a total of 926 clinical samples were collected out of which 210 Acinetobacter species were isolated. The maximum number of isolates were from the ETA samples with 132 (62.8%), 70 (33.3%) from blood and 8 (3.8%) from the tissue. The ratio of Males 124 (59%) was more as compared to that of the Females 86(40%) with the maximum age of 31-40 being affected the most followed by 41-50 and least in the age group above 61 years of age.
Antimicrobial susceptibility testing revealed that all the isolates were resistant to ceftazidime, cefepime, piperacillin/tazobactam, cefoperazone/sulbactam, aztreonam, imipenem, meropenem, amikacin, netilmycin, tetracycline, tobramycin, levofloxacin and co-trimoxazole. In the present study it was also observed that 208 (98.5%) of the study isolates were susceptible to polymyxin B (colistin).
The Molecular characterization reveals that among 210 isolates tested for MBL, there were 11 isolates positive for the blaVIM gene (5.2%).

Conclusion: There should be continuous further research necessary to monitor the spread of carbapenem-resistant OXA-type lactamase genes from *A. baumannii* in hospital settings since they are becoming a significant cause of carbapenem resistance. . Effective infection control practises and strict guidelines for the use of antibiotics should be in place.

Keywords: CRAB, Resistance mechanism, carbapenem, oxacillinase, multidrug resistance, blaVIM , Molecular characterization , PCR

INTRODUCTION

Acinetobacter spp. are non-fermenting, largely opportunistic Gram-negative bacteria that has the ability to easily acquire antibiotic resistance and to persist in hospital environments [1]. *A. baumannii* is responsible for about 90% of the clinical infections caused by *Acinetobacter* spp. in humans [1].
This organism is considered an opportunistic pathogen responsible for nosocomial infections, especially in intensive care units [2]. *A. baumannii* commonly causes bacteremia, nosocomial-acquired pneumonia or ventilator-associated pneumonia, catheter-related infections, meningitis, peritonitis, skin and wound infections, urinary tract infections, and endocarditis [3].
The ability to survive in dry or moist conditions at various pH levels and temperatures renders it able to grow in the hospital environment [4,5]. *A. baumannii i*s one of the ESKAPE pathogens, along with *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Enterobacter spp*., which are responsible for the majority of nosocomial infections and are capable of "escaping" the bactericidal activity of antimicrobial agents [6]. The bacteria are multidrug-resistant, making infections very difficult to treat.
The preferred medication for *A. baumannii* is considered to be the carbapenems. However, there has been widespread reporting of rising carbapenem resistance. Both carbapenemase-mediated and non-carbapenemase-mediated resistance in A. *baumannii* are possible. Class A (serine proteases), class B (metallo-beta-lactamases), and class D (oxacillinases) carbapenemases are primarily responsible for carbapenemase-mediated resistance, whereas non-carbapenemase-mediated resistance involves upregulation of the efflux pumps and/or loss of outer membrane porins [7].
Infections from *A. baumannii*, in particular carbapenem-resistant *A. baumannii* (CRAB), are of significant public health importance worldwide because of their association with high treatment costs, mortality and morbidity [8]. Worldwide, CRAB has been implicated in several hospital outbreaks of diseases like pneumonia, bloodstream, wound and urinary tract infections, especially among patients with severe morbidities such as those staying in ICU [9,10]. The pathogen is able to

survive adverse environmental conditions, fostering its persistence and spread in the hospital environment [11,12].

Resistance to carbapenem in *A. baumannii* is most frequently due to oxacillinases, which can be intrinsic or acquired. Treating carbapenem-resistant *A. baumannii* infections is very challenging since they are naturally resistant to antibiotics in the WHO “Access” and “Watch” list. They are therefore associated with poor clinical outcomes across many healthcare settings [13].

Since, the intrinsic blaOXA-51 gene is found on *A. baumannii's* chromosome, the organism is thought to be unique to it. In contrast to MBLs encoded by the blaIMP, blaVIM, blaNDM, and blaSIM genes, acquired OXA enzymes, which are produced by the blaOXA-23, blaOXA-40, and blaOXA-58 genes, are more prevalent in *A. baumannii* isolates [9].

The overproduction and spread of OXA genes in *A. baumannii* are largely caused by the insertion sequence. Insertional sequences that act as promoter sequences for OXA gene overexpression are a major factors responsible for the high levels of carbapenem resistance. The resistance genes blaOXA-23, blaOXA-51, and blaOXA-58 have all been linked to the insertion sequence ISAba1, which is a member of the IS4 family [14]. *A.baumannii* can develop resistance to many classes of commonly used antimicrobial agents [15,16] where Carbapenems was considered as a last resort to treat infections caused by MDR, Gram-negative bacteria, but recently, carbapenem resistance has been increasingly common in *A. baumannii.* The Multi and extensively drug-resistant (MDR and XDR) *Acinetobacter baumannii* (*A. baumannii*) are two main causative agents of nosocomial infections leading to increased morbidity and mortality which have been progressively increasing globally over the last decade [17-19].

Several resistance mechanisms of *A. baumannii* against carbapenems have been reported, including antimicrobial-inactivating enzymes, efflux pump, loss of the CarO outer membrane porin, and decreased target access [3,20,21]. One of the most important carbapenem resistance mechanisms is the production of class D β-lactamases (oxacillinase; OXA). This group of enzymes can hydrolyze oxacillin and the third-generation cephalosporins, but possesses weak activity against carbapenems [22].

It is observed from various studies that OXA-23 is the most frequently detected carbapenemase in the CRAB worldwide but recent studies have been reported with an increased prevalence of other carbapenemase genes in various regions over OXA-23 and the importance of various mobile genetic elements. There is the need for active surveillance to understand the exact situation in various countries, to track the spread, to start the appropriate controlling measures, to break the chain of this bug and, most importantly, to start timely proper treatment of patients based on the presence of various genes to avoid therapeutic failure and mortality[18]. Thus, the genotypic detection of the gene is the need of an hour with its global genetic diversity.

Therefore, the present study was undertaken to study the Molecular Characterization of Invasive Carbapenem-Resistant *Acinetobacter baumannii* with special reference to blaVIM gene in ICU patients at a Tertiary care centre, Uttar Pradesh.

MATERIAL AND METHODS

This was a Cross sectional study carried out in the Department of Microbiology for a period of 1 year i.e, August 2022 to August 2023 at a tertiary care centre, Uttar Pradesh. A total of 210 non-duplicate, consecutive, carbapenem-resistant isolates recovered from ICU patients of *Acinetobacter* species were included in this study. The isolates were obtained from invasive clinical specimens including blood ,endotracheal aspirates (ETAs) and the tissue. The isolates were identified up to the species level as *Acinetobacter baumannii* by Standard biochemical tests and the Antimicrobial susceptibility testing was performed according to the CLSI guidelines 2022. The DNA was extracted using the Qiagen DNA extraction kit from the Clinical samples where the confirmation of the gene blaVIM gene was done by the PCR [23].

The Antimicrobial Susceptibility Testing

The Susceptibility to different classes of antibiotics was determined by the Kirby Bauer disc diffusion method and interpreted according to the Clinical Laboratory Standard Institute guidelines 2022. Antibiotics tested were ceftazidime (30 µg), cefepime (30 µg), piperacillin/tazobactam (100/10 µg), cefoperazone/Sulbactam (75/30 µg), amikacin (30 µg), netilmycin (30 µg), tobramycin (10 µg), aztreonam (30 µg), levofloxacin (5 µg), tetracycline (30 µg), co-trimoxazole (1.25/23.75 µg), imipenem (10 µg), meropenem (10 µg) and polymyxin B (10 µg) [23] were used as per the CLSI guidelines.

The Phenotypic Detection method

CarbAcineto NP test was used for carbapenemase phenotypic detection. All of the research isolates that needed to be evaluated were cultivated for 24 hours on a Mueller-Hinton agar plate, and the isolated colonies were then re-suspended in two 1.5 ml centrifuge tubes (A and B) containing 100 l NaCl (5 M). 100 l of solution A (phenol red solution with zinc sulphate) and 100 l of solution A with imipenem (6 mg/ml) were added to tubes A and B, respectively. Maximum 2 hours were allowed for the tubes to be incubated at 37 °C. The hydrolysis of imipenem caused a pH value reduction, which caused a colour shift in tube B, indicating the presence of carbapenemase [23] [24]. BAA-1705 and BAA-1706 were simultaneously listed as positive and negative controls, respectively.

The Molecular Characterization of the Genes by phenotypic method

The DNA was isolated using the Qiamp DNA Blood Mini Kit (QIAGEN, Germany) as per the manufactures guidelines. The extracted DNA and the gene was confirmed by the PCR to detect the presence of the MBL gene blaVIM .

The DNA was eluted in 60 µl elution buffer and preserve at -20 °C till PCR analysis. For amplification of the target gene, PCR was carried out in a 50 µL reaction mixture with 30 no. of cycles. The primers were synthesized by Chromous Biotech. Pvt. Ltd. (Bangaluru) and was reconstituted with sterile double distilled water based on the manufacturer's instruction.

Figure No.1: The DNA Extraction kit

Figure No.2: The Reagents used for the DNA Extraction

Fragment	Gene	Primer sequence	Length (bp)	Reference
A	blaVIM-FB blaVIM-RB	5’-CCGATGGTGTTTGGTCGCAT -3’ 5’-GAATGCGCAGCACCAGGAA -3’	390bp	[25]

Table No. 1 : Primers used to amplify OXA-23 and blaVIM gene fragments.

Figure No. 3: The blaVIM primers from the Chromous Biotech.

Polymerase Chain Reaction (PCR)

For the PCR amplification, 2 µl of template DNA was added to 18 µl reaction containing 10 µl of Qiagen master mix, 2 µl of primer mix (1 µl each of the respective forward and reverse primers) and 6 µl of molecular-grade water.

The cyclic conditions for MBL genes, initial denaturation at 95 °C for 15 min, 30 cycles of 94 °C for 30 s, 59 °C for 1 min 30 s and 72 °C for 1 min 30 s were followed by extension of 72 °C for 10 min.

The PCR cycling conditions

Step	Program blaVIM Time	Temperature	Cycles
Initial denaturation	15 min	95 ℃	
Denaturation	30 s	94 ℃	
Annealing	1 min30 s	59 ℃	
Extension	1 min 30 s	72º C	30
Final extension	10 min	72º C	

Table No. 2 : The PCR cycling conditions to amplify blaVIM gene fragments.

The Agarose gel preparation and visualized by Gel Doc™ EZ Gel Documentation System

The Agarose Gel Electrophoresis was performed in order to identify the Purified PCR Product which was previously identified by its amplified DNA fragments. The resulting PCR product was subjected to 1 % agarose gel electrophoresis and visualized by Gel Doc™ EZ Gel Documentation System (Bio-Rad Laboratories Inc., Hercules, CA, USA). A 1 kb DNA Ladder (Thermo Fisher Scientific ™, Waltham, MA, USA) was used as the marker to evaluate the PCR product of the sample [26].

Figure No. 4: Agarose

Figure No. 5: TAE Buffer

RESULTS

A total of 926 clinical samples were included in this study out of which 210 invasive clinical isolates of Acinetobacter species were studied [Table No. 3]. The maximum number of isolates were from the ETA samples with 132 (62.8%) isolates whereas 70 (33.3%) isolates were from blood sample and 8 (3.8%) from the tissue [Table No. 4].

Type of Clinical Isolates	Number of Isolates	Percentage
Acinetobacter species	210	22.6%
Others clinical isolates	716	77.3%

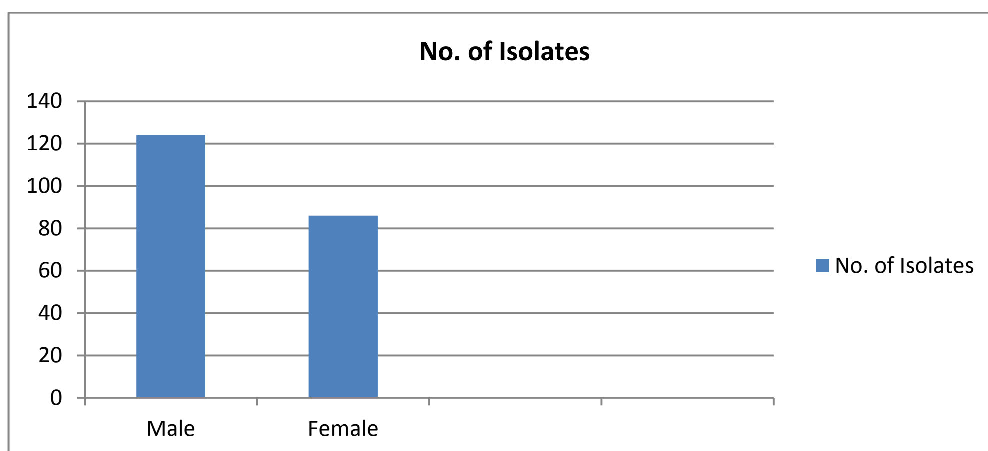
Table No. 3: Total Number of clinical isolates

Type of isolates	Number of Isolates	Percentage
ETA	132	62.8%
Blood	70	33.3%
Tissue	8	3.8 %
Total	**210**	**100%**

Table No. 4: Total Number of clinical isolates of Acinetobacter species

Gender	Total no. of Cases studies (N=210)	Percentage
Male	124	59%
Female	86	40%

Table No. 5 : Genderwise distribution of the *Candida albicans*



Graph No. 1: The graphical Representation of the Genderwise distribution

The ratio of Males 124 (59%) was more as compared to that of the Females 86 (40%) [Table No. 5] with the maximum age of 31-40 being affected the most followed by 41-50 and least in the age group above 61 years of age [Table No. 6]. There was no *Acinetobacter baumannii* isolated in the age group of 0-10 years of age.

S.No.	Age (in years)	No. of Cases	Percentage
1.	0- 10	-	-
2.	11-20	9	4.2%
3.	21-30	32	15.2%
4.	**31-40**	**88**	**41.9 %**
5.	41-50	51	24.2 %
6.	51-60	26	12.3 %
7.	≥61	4	1.9 %

Table No.6 : Age wise distribution of *A.baumannii* patients from the study

Antimicrobial susceptibility testing revealed that all the isolates were resistant to ceftazidime, cefepime, piperacillin/tazobactam, cefoperazone/sulbactam, aztreonam, imipenem, meropenem, amikacin, netilmycin, tetracycline, tobramycin, levofloxacin and co-trimoxazole. In the present study it was also observed that around 208 (98.5%) of the study isolates were susceptible to polymyxin B (colistin.).

Among the 210 clinical isolates, the CarbAcineto NP test was positive in 197 (93.8%) isolates and negative in 13 (6.1%)

The Molecular characterization for the detection of the genes in Acinetobacter was performed where the DNA was isolated using the Qiagen DNA extraction kit as per the manufacture's guidelines. The PCR was run for the detection of MBL Gene.

Figure No. 6: The PCR cycling conditions

Figure No. 7: Electrophoresis unit under run Run of Amplified product

Figure No.7: The DNA Extraction of the MBL resistant blaVIM gene

Figure No. 9: The Amplified DNA with PCR for blaVIM gene of *A. baumannii* . Lane 1 is the positive control; Lane 2 is the sample negative for blaVIM; Lane 3 is the Negative control; Lane 4-7 are sample positive for blaVIM gene; Lane 8 is the DNA Ladder

Among the 210 isolates tested for MBL, there were 11 isolates positive for the blaVIM gene (5.2%). The molecular detection of the blaVIM -like gene revealed a 390 bp band in all clinical isolates, which preliminarily confirmed the identification of the clinical isolates as being *A. baumannii.*

DISCUSSION

There is a worldwide emergence of *Acinetobacter baumannii* as one of the most prominent nosocomial pathogen. Multidrug resistant phenotypes emerged as a result of misuse of broad-spectrum antibiotics against this bacterium. Natural resistance mechanisms of *Acinetobacter spp.* to several β-lactam antibiotics also aids in its resistance profile. Long term survival in the health care setting is also a contributing factor that acts synergistically with emerging resistance profiles. Resistance nodulation cell-division (RND), Multidrug and toxic compound extrusion (MATE) and Major facilitator super family (MFS) are the efflux systems usually associated along with carbapenemase production in *A. baumannii* for antimicrobial resistance [27].

Globally, infections linked to healthcare are increasingly being reported as multidrug-resistant *A. baumannii*. Carbapenem resistant isolates are generally resistant to most other classes of antibiotics, while usually retaining susceptibility to tigecycline and colistin. Resistance against carbapenem in and on itself is considered sufficient to define an *A.baumannii* isolate as highly resistant.

A. baumannii is carbapenems. There are numerous resistance mechanisms evolving making of class D ,Class B metallo-b-lactamases and OXA carbapenemases which play a major impact in the development *of A. baumannii* carbapenem resistance globally [23].

Due to therapeutic challenges, hospital-acquired infections (HAIs) caused by *Acinetobacter baumannii* (HA-AB), particularly carbapenem-resistant strains (HA-CRAB) pose a serious health threat to patients worldwide. *Acinetobacter baumannii* is an opportunistic pathogen of emerging importance in the clinical settings and responsible for up to 20% of infections in ICUs around the globe. The majority of reported clinical cases involved ventilator-associated pneumonia/pulmonary infections, bloodstream infections, skin and soft tissue infections, including burn and surgical wound infections, endocarditis, meningitis, and urinary tract infections. Furthermore, infections caused by Acinetobacter are not limited to the hospital settings but reports have emerged unfolding cases involving otherwise healthy individuals of all age groups, occurring in community settings, following natural disasters and during wars [27]. Treatment of infections due to this pathogen is becoming a serious clinical concern, since *A. baumannii* shows extensive resistance to many of the

currently used antibiotics, including cephalosporins, aminoglycosides, quinolones, and carbapenems. *Acinetobacter baumannii* is of particular concern due to its predilection to acquire antibiotic resistance determinants [28].

In the present study a total of 926 clinical samples were included out of which 210 clinical isolates of Acinetobacter species were studied. Therefore the prevalence was observed to be 22.6%. This study was in accordance to the present study where the prevalence was observed to be 25.1 % [29]. There was another study by Dhasarathi Kumar *et al.,* in 2023 where in total, 248 (19. 3%) *Acinetobacter* species were isolated [30].

The maximum number of isolates were from the ETA samples with 132 (62.8%) isolates whereas 70 (33.3%) isolates were from blood sample and 8 (3.8%) from the tissue. This study was similar to the study performed by Sharma RK et al., where the percentage of Acinetobacter isolates was found to be (6.42%) [31] . The study by Vijayan Sivaranjani *et al.,* was also in support with the present study where the majority of the isolates, 47 (38.52%), were from pus samples followed by 25 (20.49%) from endotracheal tube aspirate [32]. It was also noted that the percentage of isolation from blood was more with 33.3% but was in contrast with the other study where Percentage of isolation from blood was only 5.73 per cent [32], but in support with the study by Mastofi et al, which showed high isolation rates from blood [33].

There were other studies which were also parallel to our study stating the rate of Acinetobacter to be similar studies by Fayyaz et al [34] (10.9%) and Goossens [35] (4.9%) but in contrast with the study by Sabir et al, where the percentage of positive culture was found to be 87.17%, which was much higher than the present study [36].

In the present study it was observed that the ratio of Males 124 (59%) was more as compared to that of the Females 86(40%) with the maximum age of 31-40 being affected the most followed by 41-50 and least in the age group above 61 years of age .There was no *Acinetobacter baumannii* isolated in the age group of 0-10 years of age. This study was in support with the study performed by the other authors, where the rate of male (75.36%) and female (24.28%) was observed [31]. Another study was also found to be similar in the studies by Fayyaz et al [34] but in contrast with the studies by Tahseen and Talib and Saleem *et al.,* [37,38].

The maximum frequency of *A. baumannii* isolates was recovered from ICUs (79%), which was found to be similar with the studies by Xia et al [39] and Sharma RK *et al.,* [31].

In the present study antimicrobial susceptibility testing revealed that all the isolates were resistant to ceftazidime, cefepime, piperacillin/tazobactam, cefoperazone/sulbactam, aztreonam, imipenem, meropenem, amikacin, netilmycin, tetracycline, tobramycin, levofloxacin and co-trimoxazole. In the present study it was also observed that around 208 (98.5%) of the study isolates were susceptible to polymyxin B (colistin.). This study was similar to the study by the other authors where polymyxin B was observed to be susceptible [31] [40]. The study by Vijayan Sivaranjani *et al.,* was also in support to the present study where more than 90 per cent of isolates displayed resistance to ampicillin, amoxicillinclavulunic acid, ceftazidime, and amikacin. Resistance to gentamicin, co-trimoxazole and ciprofloxacin were also common [32].

Among the 210 clinical isolates, the CarbAcineto NP test was positive in 197 (93.8%)
isolates and negative in 13 (6.1%). This study was in accordance to the study by the other investigator Vijaykumar S *et al.* in 2016 [20].

In the current study b*la*$_{\text{VIM}}$ exhibited high levels of resistance to commonly used antibiotics especially ceftazidime, gentamicin , ciprofloxacin , cefepime , piperacillin/tazobactam and amikacin .

In the present study among the study isolates, 210 isolates tested for MBL, there were 11 isolates positive for the blaVIM gene (5.2%) .However, studies by Saranathan *et al.,* and Amudhan *et al., showed* IMP-like and blaVIM-like as the prevalent MBL genes [40,41] but contrast with the study by Dickson Aruhomukama *et al.,* in 2019 where *bla*$_{\text{VIM}}$ was the most prevalent carbapenemase gene

and it was detected in all CRPA and CRAB isolates,. Of note, the other MBL encoding genes i.e. *bla*$_{IMP}$, *bla*$_{SPM,}$ and *bla*$_{NDM}$, were not detected; as well, carriage of the OXA-type carbapenemase genes was not detected in CRPA. Also, in agreement with activity assays, none of our isolates was positive for *bla*$_{KPC}$ carriage. We however found the OXA-type carbapenemase genes to be prevalent among CRAB i.e. 29% & 24% for *bla*$_{OXA-23}$ & *bla*$_{OXA-24}$, respectively [42].
There was another study which was in accordance to the present study where the prevalence of blaVIM gene was observed to be 15 (17.44%), which was similar to the current study with the prevalence of blaVIM gene was found to be 5.2% [43].
Studies have reported that carbapenem resistance in *A. baumannii* is mainly due to carbapenemase mediated . However, non-carbapenemase-mediated resistance mechanisms such as reduced membrane permeability due to porin changes and overexpression of efflux pumps make a trivial contribution toward carbapenem resistance in *A. baumannii* [44].

A significant increase in antibiotic resistance is one of the main problems in the treatment of infections caused by *Acinetobacter baumannii*. Efflux pumps play a key role in the development of multiple resistance to antimicrobial drugs. It is important to evaluate the presence of efflux pump genes in preventing the spread of antibiotic resistance and to suggest an appropriate treatment model for patients infected with this bacterium. So the high prevalence of genes encoding efflux pumps in this bacterium is one of the important factors in the spread of antibiotic resistance between isolates in different geographical regions. Of course, the role of other factors and mechanisms involved in the development of *Acinetobacter baumannii* resistance should not be ignored [45].

CONCLUSION

The prevalence of β-lactamase-producing isolates and their isolation from life-threatening infections is dramatically increasing worldwide. Intensity pressure for usage of antimicrobial drugs by patients resulted in eradication of normal flora and situation of MDR isolates substitution. This study showed that β-lactamase producing *A. baumannii* strains are an emerging threat in ICUs and should be supervised by implementation of timely identification and strict isolation methods that will help to reduce their severe outcomes and mortality rate of patients.

Declarations:
Conflicts of interest: There is no any conflict of interest associated with this study
Consent to participate: We have consent to participate.
Consent for publication: We have consent for the publication of this paper.
Authors' contributions: All the authors equally contributed the work.

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