"TO STUDY THE MOLECULAR CHARACTERIZATION OF INVASIVE CARBAPENEM-RESISTANT *ACINETOBACTER BAUMANNII* WITH SPECIAL REFERENCE TO BLAVIM AND OXA-23 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. MDR Gram-negative bacteria infections have traditionally been treated with carbapenems as a last option. *A. baumannii* has lately shown an increase in carbapenem resistance. Enzymatic degradation by -lactamases accounts for the majority of *A. baumannii's* carbapenem resistance mechanisms.

Aim And Objectives: To study the Molecular Characterization of Invasive Carbapenem-Resistant *Acinetobacter baumannii* with special reference to OXA-51 and OXA-23 gene in ICU patients at a Tertiary care centre.

Material And Methods: This was a Cross sectional study carried out in the Department of Microbiology and the Anesthesia Department for a period of 1 year i.e, April 2022 to April 2023 at a tertiary care centre, Kanpur.

A total of 110 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 834 clinical samples were collected in which 110 Acinetobacter species were isolated. The maximum number of isolates were from the ETA samples with 82 (74.5%), 27 (24.5%) from blood and 1 (0.9%) from the tissue. The ratio of Males 76 (69%) was more as compared to that of the Females 34(30.9%) 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 around 108 (98.1%) of the study isolates were susceptible to polymyxin B (colistin).

The Molecular characterization reveals that among the 110 isolates tested for MBL, the seven isolates were positive for the blaVIM gene (6.3%) and blaOXA-23 gene was present in all the 110 isolates (100%).

Conclusion: Further studies are 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. There should be efficient infection control procedures and strict regulation on the use of Antibiotics followed.

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

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INTRODUCTION:

Acinetobacter baumannii is a Gram-negative coccobacillus that has the ability to easily acquire antibiotic resistance and to persist in hospital environments[1].

Carbapenem-resistant *Acinetobacter baumannii* (CRAB) is a type of bacteria commonly found in the environment, especially in soil and water. CRAB can cause various infections like bacterimia, pneumonia, urinary tract infection, wound, lung and other body site infections. The bacteria are multidrug-resistant, making infections very difficult to treat.

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 [1].

*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 [4,5].

The preferred medication for *A. baumannii* is considered to b 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 [6].

Resistance to carbapenem in *A. baumannii* is most frequently due to oxacillinases, which can be intrinsic or acquired.

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 [7].

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 [8]. *A.baumannii* can develop resistance to many classes of commonly used antimicrobial agents [9 ,10] 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 [11,12,13].

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 [3,14,15]. 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 [16].

MATERIAL AND METHODS:

This was a Cross sectional study carried out in the Department of Microbiology and the Anesthetic Department for a period of 1 year i.e, April 2022 to April 2023 at RMCH&RC, Mandhana, Kanpur.

A total of 110 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 and OXA-23 gene was done by the PCR [17].

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. 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) [17] 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 [17] [18]. 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 and the presence of acquired OXA genes namely OXA-23.

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 purchased from "**Saha gene'** 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	OXA-23 FA OXA-23-RA	5'- ATTTCTGACCGCATTTCCAT-3' 5'- GGTTAGTTGGCCCCCTTAAA-3'	501 bp	19
B	blaVIM-FB blaVIM-RB	5'-CCGATGGTGTTTGGTCGCAT -3' 5'-GAATGCGCAGCACCAGGAA -3'	390bp	20

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

Figure No. 3: The blaVIM and the OXA-23 primers from the Saha gene

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 ºC	
Denaturation	30 s	94 ºC	
Annealing	1 min30 s	59 ºC	
Extension	1 min 30 s	72º C	30
Final extension	10 min	72º C	

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

Step	**Program OXA-23**		
	Time	**Temperature**	**Cycles**
Initial denaturation	15 min	95 ºC	
Denaturation	30 s	94 ºC	
Annealing	1min 30 s	52 ºC	
Extension	1 min 30 s	72º C	30
Final extension	1 min 30 s	72º C	

Table No. 2.b : The PCR cycling conditions to amplify OXA-23 gene fragments

For acquired OXA genes, the initial denaturation was at 95 °C for 15 min, 30 cycles of 94 °C for 30 s, 52 °C for 1 min 30 s and 72 °C for 1 min 30 s, followed by extension of 72 °C for 1 min 30 s.

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 [21].

RESULTS

A total of 834 clinical samples were included in this study out of which 110 invasive clinical isolates of Acinetobacter species were studied.

The maximum number of isolates were from the ETA samples with 82 (74.5%) isolates whereas 27 (24.5%) isolates were from blood sample and 1 (0.9%) from the tissue [Table No. 2].

Type of Clinical Isolates	Number of Ioslates	Percentage
Acinetobacter species	110	13.1%
Others clinical isolates	724	86.8%

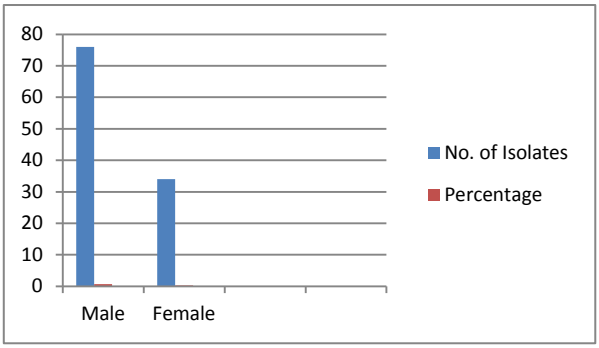
Table No. 3: Total Number of clinical isolates

Type of isolates	Number of Isolates	Percentage
ETA	82	74.5%
Blood	27	24.5%
Tissue	1	0.9 %
Total	**110**	**100%**

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

Gender	Total no. of Cases studies (N=110)	Percentage
Male	76	69%
Female	34	30.9%%

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



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

The ratio of Males 76 (69%) was more as compared to that of the Females 34(30.9%) [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	7	6.3 %
3.	21-30	16	14.5 %
4.	31-40	48	43.6 %
5.	41-50	23	20.9 %
6.	51-60	13	11.8 %
7.	≥61	3	2.7 %

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 108 (98.1%) of the study isolates were susceptible to polymyxin B (colistin.).

Among the 110 clinical isolates, the CarbAcineto NP test was positive in 102(92.7%) isolates and negative in 8 (7.27%) 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 and OXA Gene.

Figure No. 4: The PCR cycling conditions

Figure No. 5: Electrophoresis unit under run Run of Amplified product

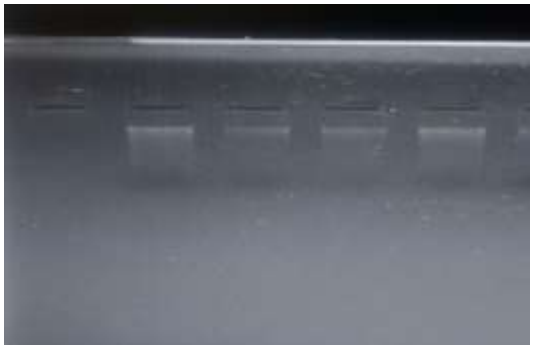


Figure No.6: The DNA Extraction of the MBL resistant blaVIM gene

Figure No. 7: 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 110 isolates tested for MBL, the Seven isolates were positive for the blaVIM gene (6.3%). 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.*

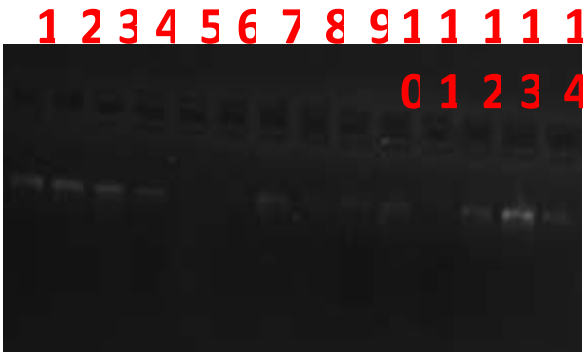


Figure No.8: The DNA Extraction of the OXA resistant OXA-23 gene

Figure No.9 : The Amplified DNA with PCR for OXA-23 gene of *A. baumannii* . Lane -1-9 are positive for OXA-23 gene; Lane 10 is the DNA Ladder; Lane 11-16 and 19, 20 are OXA-23 gene positive; Lane 17 is the Negative control for OXA-23 gene; Lane 18 is the positive control for OXA-23 gene

The acquired OXA carbapenemase, blaOXA-23 gene was present in all the 110 isolates (100%). The molecular detection of the blaoxa-23-like gene revealed a 501 bp band in all clinical isolates, which preliminarily confirmed the identification of the clinical isolates as being *A. baumannii.*

DISCUSSION

Globally, infections linked to healthcare are increasingly being reported as multidrug-resistant *A. baumannii*. As a result, the preferred medication for treating severe infections for MDR *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 [17].

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 [22,23]. 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 [24].

In the present study a total of 834 clinical samples were included out of which 110 clinical isolates of Acinetobacter species were studied. The maximum number of isolates were from the ETA samples with 82 (74.5%) isolates whereas 27 (24.5%) isolates were from blood sample and 1 (0.9%) 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%) [25] .There were other studies which were also parallel to our study stating the rate of Acinetobacter to be similar studies by Fayyaz et al [26] (10.9%) and Goossens [27] (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 [28].

In the present study it was observed that the ratio of Males 76 (69%) was more as compared to that of the Females 34(30.9%) 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 [25]. Another study was also found to be similar in the studies by Fayyaz et al [26] but in contrast with the studies by Tahseen and Talib and Saleem et al. [29, 30].

It was observed that the maximum number of isolates were from the ETA samples with 82 (74.5%) whereas 27 (24.5%) isolates were from blood sample and 1 (0.9%) from the tissue. This study was supported by Sharma RK et al. and L Dortet et al.[25] [19] where similar findings were recorded.

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 [31] and Sharma RK et al.[25].

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 108 (98.1%) of the study isolates were susceptible to polymyxin B. This study was similar to the study by the author authors where polymyxin B was observed to be susceptible [25] [19].

Among the 110 clinical isolates, the CarbAcineto NP test was positive in 102(92.7%) isolates and negative in 8 (7.27%). This study was similar to the study by the other author Vijaykumar S et al in 2016 [20]. It was noted that around 108 (98.1%) of the study isolates were susceptible to polymyxin B and colistin, which was parallel to the study performed by the other author [20].

In the present study among the study isolates, OXA carbapenemases were detected in all 110 (100%) isolates of carbapenem-resistant *A. baumannii*. The blaOXA-23-like oxacillinases were the most common type. This study was supported by the study from East India also showed the OXA-23 genes as the prevalent type of oxacillinase contributing to carbapenem resistance [32].

Among the metallo b-lactamases (MBLs), 7 (6.3%) were positive for the blaVIM gene.However, studies by Saranathan et al. and Amudhan et al. showed IMP-like and blaVIM-like as the prevalent MBL genes [19, 33].

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* [34].

CONCLUSION

Acinetobacter species is emerging worldwide disease that is acquired in hospitals. Since *A. baumannii's* drug resistance trend is extremely concerning in current healthcare settings, efficient infection control procedures and strict regulation on the use of Antibiotics should be followed.

Further studies are 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.

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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