

Detection of Multidrug-Resistant Integrons Associated with *Acinetobacter baumannii* Isolated from Clinical and Environmental Samples

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Abstract

The emergence of multidrug resistance (MDR) among pathogenic bacteria is a root cause of severe infections. It is threatening to observe that MDR is also found in ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens, which has caused a hike in nosocomial infection. The diminishing spectrum of treatment against these pathogens demands an alternative realm of treatment. One such nosocomial pathogen, *Acinetobacter baumannii* is known to cause pneumonia, blood stream infection, urinary tract infections, especially affecting immunocompromised individuals. Due to indiscriminate use of antibiotics, these pathogens have gained resistance to major classes of antibiotics through mutation and horizontal gene transfer via mobile genetic elements such as plasmids, transposons and integrons. This study mainly aims at identifying integron mediated drug resistance among clinical and environmental strains of *A. baumannii*. In this study, *A. baumannii* strains isolated from clinical and environmental samples were screened for antibiotic susceptibility tests. The multidrug resistant isolates were then checked for the presence of 3 classes of integrons viz *Int1* (Class 1), *Int2* (class 2) and, *Int3* (class 3). The integron region of the positive isolates was sequenced, and the drug-resistance gene cassettes were identified. All the clinical and environmental isolates were multidrug resistant. Three clinical isolates of *A. baumannii* showed positive amplification to *Int1* at 2kb, while none of the environmental isolates carried integrons, though they were multidrug resistant. The sequencing of the integron region of clinical isolates revealed the presence of three antibiotic resistance genes within the integron that encode resistance to chloramphenicol, rifampin, and aminoglycoside. This study prominently highlights the presence of class 1 integrons carrying different antimicrobial resistance determinants among *A. baumannii* isolated from clinical samples.

Keywords: *Acinetobacter baumannii*, Class 1 Integrons, Antimicrobial Resistance Genes

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INTRODUCTION

As an opportunistic gram-negative ESKAPE pathogen, *Acinetobacter baumannii* is responsible for 2-10% of gram-negative clinical infections. It is one of the six most multidrug resistant (MDR) pathogens declared by the Infectious Disease Society of America. It causes disease among immunocompromised individuals with a prolonged hospital stay. Commonly found in colonized form in the esophagus and respiratory secretions. It can cause hospital acquired pneumonia, bloodstream infections, and meningitis.¹ It usually targets moist tissues such as mucous and leading to symptoms such as sepsis, chills, and fever. It can survive for a prolonged period under various environmental conditions. These bacteria rank among the most hazardous nosocomial ESKAPE pathogens due to their resistance to last-resort antibiotics like colistin, tigecycline, and carbapenems.² Since only a few antibiotics have been available for treatment over the past ten years, *A. baumannii* has become one of the most challenging pathogens. A broad spectrum of antimicrobial resistance is seen in these bacteria in addition to its built-in resistance, primarily caused by the low permeability of the outer membrane to specific antibiotics and the constitutive production of efflux pumps on their cell wall.^{3,4} The remarkable ability of *A. baumannii* to build resistance to numerous antimicrobial drugs has led to the identifying numerous resistance pathways. The multidrug resistance of this organism, which can affect all systemic antimicrobials, including carbapenems and even polymyxins, frequently makes it difficult to treat.⁴ The final option in these circumstances is combination therapy.⁵ The multidrug resistance characteristics of *A. baumannii* are primarily associated with its ability to rapidly acquire and incorporate genetic components, including plasmids, transposons, and integrons. The most prevalent mechanism of antibiotic resistance to all beta-lactam classes (apart from monobactams) in *A. baumannii* is the production of metallo-beta-lactamases (MBLs) enzymes. MBLs are typically linked to gene cassettes of integrons and are easily transmitted between bacteria.⁶

Several studies have indicated the presence of integrons carrying antibiotic resistant

genes in *A. baumannii*.⁷ Also, there have been evidences of the prevalence of integrons encoding Extensively drug resistance (XDR) in *A. baumannii*.²² Initially discovered as mobile genetic elements in harmful bacteria, integrons were later found to be a significant source of genes responsible for antibiotic resistance and their transfer.⁷ They are a section of dsDNA that is important in the adaptability and evolution of bacteria. The presence of the three required apparatuses—*IntI* (integrase gene), *attI* (recombination site), and *Pc* (promoter) identifies these genetic determinants. Mobile integrons (MIs) and chromosomal integrons (CIs) are the primary categories found.⁸ They harbor multiple gene cassettes expressed by the promoter, thus making the bacteria express phenotypic resistance toward various antibiotics. Hence, a detailed analysis of bacterial strains containing integrons is the need of the hour. Therefore, our study attempted to evaluate the presence of multidrug-resistant integrons in *A. baumannii* strains isolated from clinical and environmental samples.

METHODOLOGY

Bacterial strains

The clinical isolates *Acinetobacter baumannii* (n=32) revived from the institutional repository (Nitte University Centre for Science Education and Research, Mangaluru, Karnataka, India) were kindly provided by Dr. Anusha Rohit, Department of Microbiology, The Madras Medical Mission, Chennai, India used in the study. The gene typing of these clinical isolates have been performed using random amplified polymorphic DNA (RAPD), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR), and repetitive extragenic palindromic sequence-based PCR (Rep-PCR).⁹ The environmental samples (15), such as soil and water from Mangaluru, Karnataka, India, were used to isolate environmental strains. The collected samples were then inoculated to Dijkshroom enrichment media and incubated at 37°C for 24 hours, and then they were streaked on Leeds *Acinetobacter* selective media. Specific colonies were identified using a series of biochemical tests. These clinical and environmental isolates were confirmed by PCR using *bla*_{OXA-51} primers.

Table. Oligonucleotides used in the study

Genes	Primer Sequence	Annealing temp. (°C)	Product size (bp)	Ref.
<i>bla</i> _{OXA-51}	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG		343	[12]
Int1 (Class 1) Integrase	F- TCTCGGGTAACATCAAGG R- AGGAGATCCGAAGACCTC		243	[13]
Int12 (Class 2)	F- CACGGATATGCGACAAAAAGG R- TGTAGCAAACGAGTGACGAAATG	63	788	[14]
Int13 (Class 3)	F- AGTGGGTGGCGAATGAGTG R- TGTTCTTGATCGGCAGGTG		600	[14]
Int1 (Class 1) complete sequence	F- GGCATCCAAGCAGCAAGC R- AAGCAGACTTGACCTGAT		>1000	[15]

Antimicrobial susceptibility testing

The confirmed isolates were checked for antimicrobial susceptibility pattern using disc diffusion assay as described by Kirby and Bauer, for a series of antibiotics *viz* piperacillin-tazobactam (100/10mcg), co-trimoxazole (25mcg), cefoperazone/sulbactam (75/10mcg), nalidixic acid (30mcg), nitrofurantoin (300mcg), ampicillin (10mcg), amikacin (30mcg), cefuroxime (30mcg), tigecycline (15mcg) and imipenem (10mcg). Briefly, the young cultures grown up to 0.5 MacFarland unit were swabbed onto Muller Hinton agar (HiMedia Laboratories Pvt Ltd), and the antibiotic discs were placed aseptically using a sterile applicator. The zone of inhibition was measured after incubation at 37°C for 18-24 hrs. The results were interpreted as resistant, intermediate, or susceptible using the Kirby Bauer chart as described by CLSI guidelines.¹⁰ A quality control strain *E. coli* (ATCC 25922), was also used in the study.

Chromosomal DNA and plasmid DNA extraction

The genomic DNA from all the isolates was extracted using CTAB method.¹¹ Briefly, 1ml of culture was centrifuged at 12,000rpm for 10 minutes, and the pellet was resuspended in TE buffer containing 3µl of RNase. The mixture was then incubated for an hour at 37°C. 100µl of 5M NaCl was mixed, and 80µl of CTAB/NaCl was added. The mixture was then incubated at 63°C for 10 minutes. To this mixture, an equal amount of chloroform and isoamyl alcohol was added and mixed. It is then centrifuged for 5min at 10,000rpm. The supernatant was then transferred

into the new tube and an equal amount of phenol chloroform isoamyl alcohol (P:C:I) in the ratio of 24:25:1 was added. It is then centrifuged at 10,000rpm for 5 minutes. The obtained supernatant was transferred into a new vial, and an equal amount of isopropanol was added. The mixture was centrifuged at 10,000 rpm for 5 minutes to pellet the DNA. The obtained pellet was washed with 70% ethanol and centrifuged for 5min at 10,000rpm. The final pellet was dried and resuspended using ultrapure water.

The plasmid DNA from the MDR strains was extracted using the alkaline lysis method.¹¹ Briefly, 1.5ml of overnight grown culture was centrifuged at 10,000 rpm for 5 minutes. The pellet obtained was resuspended in 100µl of alkaline lysis solution 1 (1M Glucose, 1M Tris-Cl, 0.5M EDTA). This bacterial suspension was mixed with freshly prepared 200µl of lysis solution 2 (10N NaOH, 1% SDS), and then 150µl of neutralization solution 3 (5M Potassium acetate, glacial acetic acid) was added. This mixture was then centrifuged at 14,000 rpm for 5 minutes. The supernatant was then transferred to a fresh microcentrifuge tube, the plasmid DNA was precipitated and washed using isopropanol and the pellet was washed with 70% ethanol. The pellets obtained were resuspended in 20µl of TE buffer (10mM Tris-cl, 1mM EDTA). The extracted chromosomal and plasmid DNA concentration was measured using a UV nano spectrophotometer (Bio Spectrometer, Eppendorf, Germany). The extracted chromosomal and plasmid DNA was stored at -20°C for further analysis.

Determination of integrons by PCR

The genomic DNA and plasmid DNA of all the isolates were checked for different classes of integrons (Class 1, 2, and 3) using PCR. PCR was carried out in 30 µl reaction volumes in a thermal cycler (Eppendorf, Germany and Biorad, USA). The composition of the PCR master mix was 22.2 µl of sterile water, 3 µl of 10 x buffer (100 mM Tris-HCl (pH 9), 1.5 mM MgCl₂, 50 mM KCl, and 1% gelatine), 100 µM of four deoxyribonucleotide triphosphates (dNTPs) each (dATP, dGTP, dCTP and dTTP), 10 pmol of each forward and backward primers (Table) and 1.0 U of *Taq* DNA polymerase with 2 µl of template DNA. The amplified products were resolved by agarose gel electrophoresis on a 2% agarose gel stained with ethidium bromide. The gel documentation system (BioRad, USA) was used to visualize the obtained DNA bands.

Sequencing

The amplified PCR products were sent for sequencing to Biokart India, Pvt Ltd. The obtained sequences of integrons were analyzed using NCBI blast programs blastn and blastp (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) was used to determine the presence of gene sequences. A web-based Java program, Molecular Tool Kit (<http://www.vivo.colostate.edu/molkit>) was used to obtain the amino acid sequences from

the nucleotide sequences. The final nucleotide sequences of class 1 integron obtained for each isolate were then submitted to the GenBank. Based on the sequence data, the class 1 integron was mapped for each isolate to reveal the type and the number of gene cassettes present in the integron structure.

RESULTS

Isolation of *Acinetobacter baumannii* from environmental samples

Among the 15 environmental samples of water and soil, 7 showed the presence of *Acinetobacter baumannii* by PCR using *bla*_{OXA-51} primers.

Antimicrobial susceptibility test

Among the 32 *Acinetobacter baumannii* clinical isolates tested for antimicrobial susceptibility, highest resistance was found towards nitrofurantoin (100%), ampicillin (100%), cefuroxime (100%) followed by nalidixic acid (96.87%), piperacillin/tazobactam (96.85%), imipenem (90.62%), amikacin (75%) and cefoperazone/sulbactam (71.87%). While least resistance was found towards co-trimoxazole (53.12%) and tigecycline (3.12%) (Figure 1). Among the 7 environmental isolates, maximum resistance was observed towards cefuroxime (100%)

Antibiotic Resistance Pattern of Clinical *Acinetobacter baumannii*

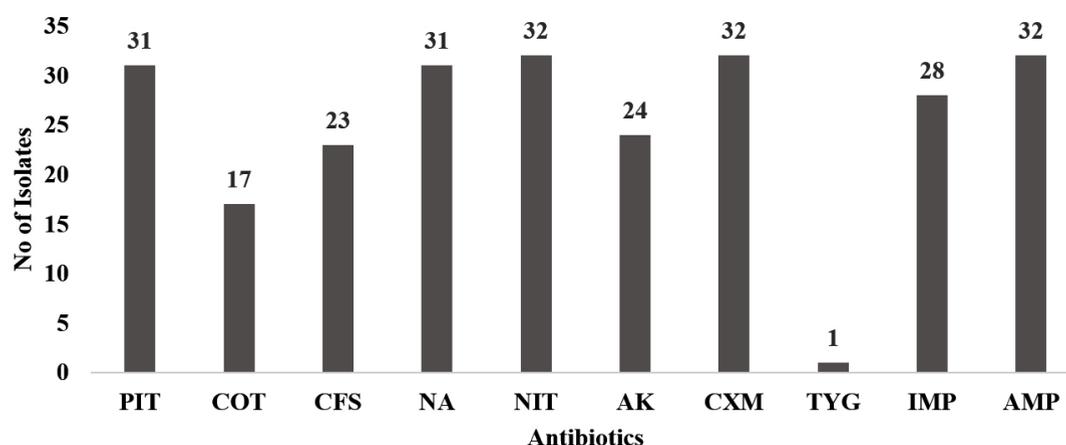


Figure 1. Antibiotic Resistance pattern of *Acinetobacter baumannii* isolated from clinical samples

followed by cefoperazone/sulbactam (71.42%), nalidixic acid (57.14%), nitrofurantoin (57.14%), piperacillin/tazobactam (42.85%), ampicillin (42.85%), co-trimoxazole (28.57%) and imipenem (14.28%). In contrast, all the environmental isolates were found to be sensitive to amikacin and tigecycline (Figure 2).

Detection of integrons in clinical and environmental samples

Among 32 clinical *Acinetobacter baumannii* isolates analyzed for *int1*, *int2*, and *int3*, only 3 isolates (AB34, AB36, and AB41) harbor class 1 integron. All the 3 isolates carried class 1 integrons in the genomic DNA. None of the clinical isolates showed positive for class 2

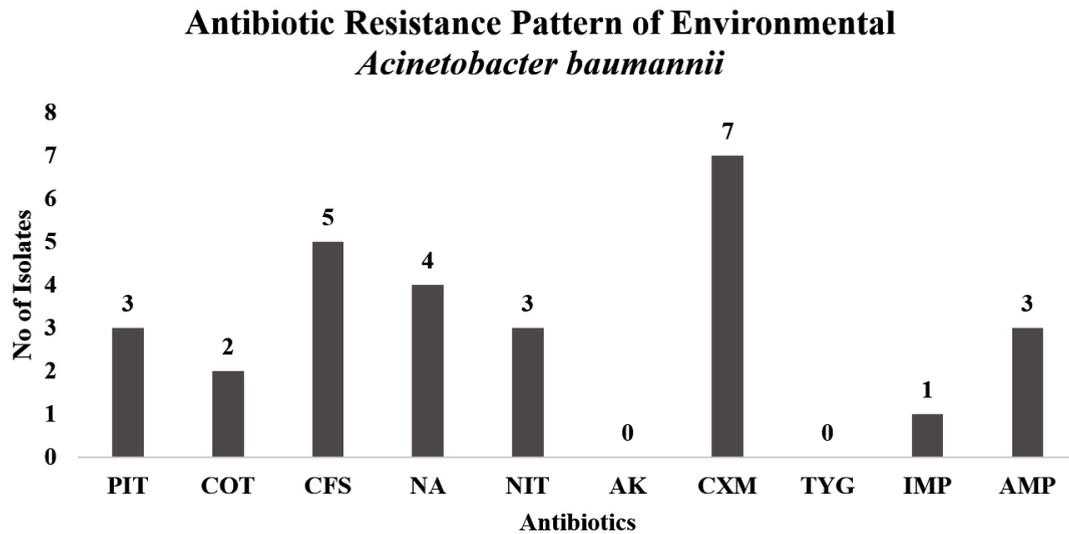


Figure 2. Antibiotic Resistance pattern of *Acinetobacter baumannii* isolated from environmental samples

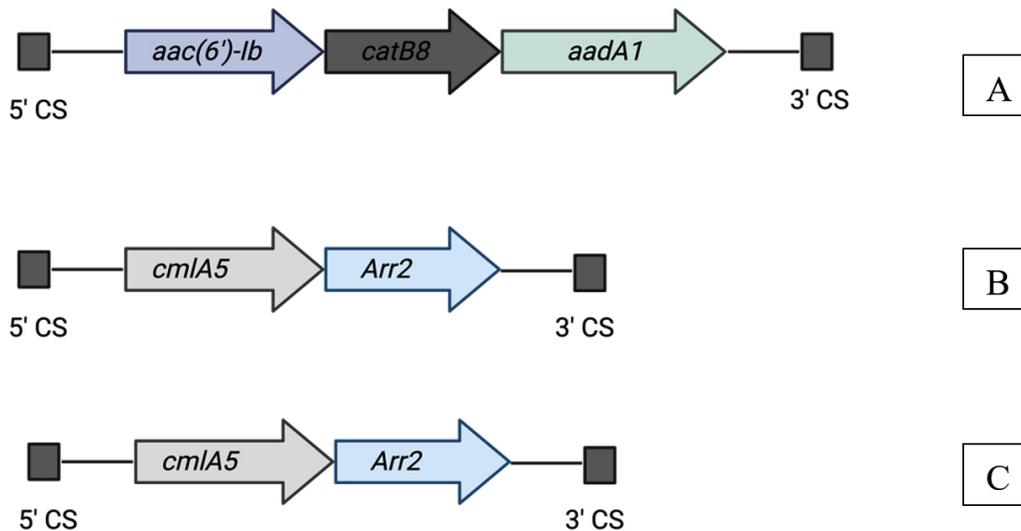


Figure 3. Map of class 1 integron of *Acinetobacter baumannii* AB34 (A), AB36 (B) and AB41 (C)

and 3 integrons. Similarly, all 7 environmental *Acinetobacter baumannii* isolates were negative for all the 3 classes of integrons.

DNA sequencing

Among the 3 clinical isolates that showed positive for class 1 integrons, they harbored different antibiotic gene cassettes within the integron structure. The isolate AB34 showed an amplicon of 2314 bp, while isolates AB36 and AB41 showed an amplicon of 2244 bp for the primer *int1*. The integron of AB34 contained 3 antibiotic resistance genes *viz.* aminoglycoside N-acetyltransferase AAC(6')-Ib, type B-3 chloramphenicol O-acetyltransferase CatB8 and ANT(3'')-Ia family aminoglycoside nucleotidyltransferase AadA1 coding for amikacin, chloramphenicol and streptomycin respectively. While the isolates AB36 and AB41 contained 2 antibiotic resistance genes *viz.* chloramphenicol efflux MFS transporter CmlA5 and NAD(+) rifampin ADP-ribosyltransferase Arr-2 coding for chloramphenicol and rifampin respectively. All the 3 integron sequences were submitted to GenBank and assigned accession numbers (AB34: OR238880, AB36: OR296598, AB41: OR296599) (Figure 3).

DISCUSSION

Multidrug-resistant *A. baumannii* shows high resistance to many classes of drugs. These isolates are immune against major antibiotics largely because of horizontal gene transfer, including integrons.¹⁶ Treatment of conditions caused by these pathogens is challenging due to the transmission of ARGs through mobile genetic elements in MDR *A. baumannii* isolates. Among different mobile genetic elements, integrons play a crucial role in acquiring a large number of drug resistance gene cassettes within them.¹⁷ There have been several studies that demonstrated the presence of integrons in *Enterobacteriaceae*.¹⁸ However, studies on integrons carrying clinical and environmental isolates of *A. baumannii* needs more attention.^{19,20} Therefore, their prevalence in the different sources must be identified as they can be a potential reservoir of ARGs. Following this, there have been several studies isolated *A. baumannii* from various sources,

including clinical and environmental. Sampling has been done from different clinical specimens in many studies.²¹⁻²⁴ Whereas the reports on the isolation of *A. baumannii* from the environment are very less.^{25,26} However, in our study, we could isolate 7 *A. baumannii* strains from the soil and water samples. Most of the clinical isolates were found to be multidrug resistant, exhibiting high resistance towards ampicillin, nalidixic acid, piperacillin/tazobactam, imipenem, etc. A similar trend was observed in a study by Salehi *et al.*, wherein more than 85% of the isolates showed resistance towards amikacin, nalidixic acid, ceftazidime, imipenem, and meropenem.²⁰ Interestingly, *A. baumannii* from both the clinical and environmental sources showed a similar trend of resistance for cefuroxime irrespective of their source of isolation. However, the environmental isolates did not show any resistance towards tigecycline, which is in accordance with the study of Suresh *et al.* wherein multidrug-resistant *A. baumannii* strains isolated from soil samples were found to be sensitive to tigecycline.²⁶

The incidence of *A. baumannii* being positive for integrons has been a critical observation in a few studies.^{23,27-30} In our study, only 9.4% (3) of the isolates harbored class 1 integron, and none of the isolates carried class 2 and 3 integrons. However, a study by Goudarzi *et al.* reported that 74.1% and 12.5% of *A. baumannii* isolates were positive for class 1 and 2 integrons, respectively.³¹ No correlation was observed between the isolates carrying integrons and their drug resistance pattern. However, the 3 of isolates that carried class 1 integrons were found to be resistant to most of the tested antibiotics except tigecycline. A study by Huang *et al.* stated that the integron positive isolates were resistant to more antibiotics than integron negative isolates.²³ The size of the integrons was in the range of 0.4 kb to 3kb in those isolates. Similarly, in our study, all the 3 isolates harbored integrons of more than 2 kb. The most common drug resistance genes carried by these integrons were *aadA1*, *aacA4-catB8*, *aadA2*, *dfrA5*, *dfrA25*, *aadB*, *blaCARB-2*, *aadB-catB3*, *dfrA1-aadA1a*, *ampC*, *ISAbal-bla_{OXA-23}-GES-14*, *Arr-3-aacA4*, *dfrA17-aadA5*, *aadA2-cm1A6-GES-14-qaF*, *VIM-25-GES-24-qaF*, *dfrA5-ISAbal-bla_{OXA-51}-bla_{OXA-40}*, *aadB-GES-11-IMP-1*, *AadB-cat-blaOXA10-aadA1*, *AadA2-HP-*

dfrA, *aacC1-ORF1-ORF2- aadA1*.^{21,28,29} However, our study demonstrated that the 2.3kb sized integron of the strain AB34 contained *AAC(6')-Ib-CatB8-ANT(3'')-Ia -AadA1*. The gene cassette exhibited 100% identity with aminoglycoside N-acetyltransferase, type B-3 chloramphenicol O-acetyltransferase and ANT (3'')-Ia family aminoglycoside nucleotidyltransferase coding for amikacin, chloramphenicol and streptomycin respectively, which co relates with one of the studies, wherein the authors could isolate integron containing chloramphenicol, aminoglycoside gene cassettes.³² In the present study, the cassette array is identical to the integrons of other *A. baumannii* strains (CP054302, CP050916, MK541903, and APO19685). Similarly, our report also demonstrated that the 2.2kb sized integron of the strains AB36 and AB41 contained a rare gene cassette *CmlA5-Arr2* coding for chloramphenicol and rifampin, which showed 100% identity with *A. baumannii* (CP050386, CP021344) and *E. coli* (LC542972) integrons. This is in accordance with the study of Azizi *et al.* wherein 30% of the *A. baumannii* isolates harbored *Arr2-cmlA5* cassette.³³ The variations in the integron sizes between AB34 and two other strains (AB36 and AB41) indicate the presence of more gene cassettes in AB34 compared to the other two isolates. This highlights that there is always a high probability of integrons acquiring new gene cassettes into their system, resulting in frequent acquisition and dissemination of AMR determinants among *A. baumannii*.

The integrons are usually present in plasmid and are one of the prominent mechanisms of drug resistance dissemination in the environment. However, our study identified the presence of class 1 integrons in *A. baumannii* within the genomic DNA. This contrasts earlier studies wherein the majority *A. baumannii* isolates harbored integrons in the conjugative plasmids.^{33,34} Nevertheless, the presence of integrons even in the organism's genome is worrisome since these integrons are usually associated with a transposon, which can easily move from genomic DNA to plasmid DNA, thereby causing *A. baumannii* to become resistant to the broad spectrum of antibiotics, such as the emergence of carbapenem-resistant integrons.³⁵ In conclusion, the study detected new variants of resistance genes within the integron of *A. baumannii*. Investigating

these integrons in MDR *A. baumannii* is very important since it would enable us to develop new intervention strategies to combat AMR and to reduce the disease progression.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

DVK conceptualized the study and experimental design the study. VPS performed data curation and contributed in data revision. CR performed the experiments. VPS and CR wrote the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee, Nitte University Centre for Science Education and Research, Mangalore, India, with reference number INST.EC/2022-23/001 dated 29.08.2022.

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