Research article

Prevalence and molecular characterisation of 16S rRNA methyltransferase genes mediating aminoglycoside resistance in Gram-negative bacteria from cancer patients A tertiary care study in India.

. Murugesan S, Kottarathil K, Samuel S, Keloth Erakkodan S, Rudrapathy P

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Abstract

Introduction: Infections significantly contribute to morbidity and mortality in cancer patients, with Gram-negative bacteria (GNB) posing a major threat due to multidrug resistance (MDR). High-level aminoglycoside resistance, driven by 16S rRNA methyltransferases (16S RMTases) limits treatment options. This study aimed to determine the prevalence of 16S RMTase genes in clinical GNB isolates from a tertiary cancer care centre in North Kerala.

Methods: A prospective cross-sectional study analysed 592 non-duplicate GNB isolates collected from June 2021 to May 2022. Bacterial identification was performed using standard techniques and MALDI-TOF MS. Antimicrobial susceptibility testing was done using the VITEK 2 system. Aminoglycoside-resistant isolates were subjected to PCR for detecting 16S RMTase genes (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF*).

Results: Urinary tract infections (33%) and bloodstream infections (26%) were predominant. *Escherichia coli* (38.3%) and *Klebsiella pneumoniae* (27%) were the most common species. Among 267 aminoglycoside-resistant isolates, 134 (50%) carried 16S RMTase genes, primarily *rmtB* (n = 57), *armA* (n = 42), and *rmtF* (n = 13), with co-occurrence in 22 isolates. No *rmtA*, *rmtC*, *rmtD*, or *rmtE* genes were detected.

Conclusion: The prevalence of 16S RMTase genes, especially *rmtB* and *armA*, underscores the challenge of MDR GNB in cancer patients. Rapid detection and effective therapeutic strategies, along with coordinated surveillance, are essential to mitigate the impact of MDR infections in healthcare settings.

Keywords: *Aminoglycoside resistance, ribosomal, methyltransferases, Gram-negative bacteria, multidrug resistance, cancer.*

Microbiology Division, Department of Clinical Laboratory Services and Translational Research, MCC-PGIOSR, Thalassery, Kannur, Kerala – 670103, India

Address for correspondence: Dr. Parthiban Rudrapathy; Microbiology Division, Department of Clinical Laboratory Services and Translational Research, MCC-PGIOSR, Thalassery, Kannur, Kerala – 670103, India.

Tel: 8056666270 Email: drparthiban@mcc.kerala.gov.in https://orcid.org/0000-0002-1200-5111

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Introduction

Infections continue to be a significant cause of morbidity and mortality in patients with cancer. One of the most frequent and potentially fatal side effects in immunocompromised hospitalised patients is nosocomial infection. Cancer patients are especially vulnerable to hospital-acquired infections because of their compromised immune systems, use of invasive technology, and exposure to chemotherapy and surgery.[1,2] Among cancer patients, bacterial infection is still one of the most harmful side effects of cancer treatment.[3] Treatment complications and significant morbidity and death can result from bacterial infections due to intrinsic and rapidly developing multi-drug resistance (MDR). Aminoglycoside resistance in bacteria is a well-known phenomenon that has been linked to a number of processes, including changes in the 16S rRNA. Gentamicin, amikacin, and kanamycin are examples of aminoglycoside antibiotics widely used to treat a range of bacterial illnesses. Bacterial resistance to aminoglycosides can severely limit the effectiveness of these antibiotics. ß-lactam antibiotics and aminoglycosides are commonly used to treat aerobic Gram-negative infections.[4,5,6] A combination of aminoglycosides and ß-lactam antibiotics is commonly used to treat severe infections in hospitalised patients.[7,8]

This therapeutic strategy was seriously threatened by the emergence of MDR, as organisms can carry multiple resistance determinants, rendering other chemotherapy treatments ineffective.[9] One of the main mechanisms of aminoglycoside resistance is 16S rRNA methylation. Many novel genotypes and subtypes have consistently appeared since the first acquired 16S rRNA methyltransferases (16S-RMTases) gene mediating high-level resistance to numerous aminoglycosides was discovered in 2003. Eleven acquired 16S-RMTases, including *armA*, *npmA*, *npmB*, and *rmtA* through *rmtH*, have been found and reported so far.[10-12] The majority of the 16S-RMTase-encoding genes discovered so far are found within mobile genetic elements such as transposons, integrons, and insertion sequences, or connected to them. Moreover, 16S-RMTase producing genes are frequently linked to other resistance genes in plasmid and mobile genetic elements, resulting in MDR and even pan-drug resistance (PDR).[10] The research focus should therefore be more on pan-aminoglycoside resistance brought on by 16S-RMTase because of the high levels of antimicrobial resistance and mobility provided by plasmids and other mobile genetic elements.

Numerous studies published in India, have reported 16S-RMTase genes (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *rmtF*) from Gram-negative bacteria.[13-15] The most prevalent and extensively distributed genes in Asia are *armA* and *rmtB*, which encode the enzymes that modify the 16S rRNA's A site.[16,17] These genes are often associated with the growth and horizontal transmission of aminoglycoside resistance in hospital settings, together with other resistance determinants. 16S-RMTases confer resistance to all clinically significant aminoglycosides, with amikacin resistance being particularly potent.[10]

Since their first discovery in several *Enterobacteriaceae* and *Pseudomonas aeruginosa* in the late 1990s, 16S-RMTases have been found to represent emergent aminoglycoside resistance mechanisms in *Enterobacteriaceae*, *Acinetobacter baumannii*, and *P. aeruginosa*. There are currently ten genes known to code for 16S-RMTase (*armA*, *rmtA -rmtH* and *npmA*).[18-20] The genes encoding these enzymes have all been shown to be plasmid-mediated and are frequently linked to ß-lactamases, including carbapenemases, which can facilitate their selection and spread. However,

reports about high-level resistance to aminoglycosides have increased and spread widely in recent years.[14,15,21-23] Understanding the specific mechanisms of aminoglycoside resistance and monitoring their prevalence in bacterial populations is crucial for effective antibiotic stewardship and the development of strategies to combat resistance.

The frequency of 16S RMTases genes varies depending on the region. Remarkably, very few studies have been conducted on Gram-negative bacterial (GNB) genes, including 16S-RMTases, in cancer patients. Early detection of these microorganisms with high levels of aminoglycoside resistance associated with hospital infections is crucial in order to initiate effective antibiotic therapy for these cancer patients.

This study was carried out in a tertiary care cancer centre to determine the prevalence of the 16S RMTases gene acquired by clinical isolates of GNB.

Methods

A prospective cross-sectional study was conducted in a tertiary care cancer centre in North Kerala. All cancer patients diagnosed with infection and admitted to the hospital between 1st June 2021 and 31st May 2022 for the purpose of receiving cancer treatment met the inclusion criteria. A total of 592 consecutive, non-duplicate GNB clinical isolates were collected from various wards. Isolation and identification of the bacterial isolates were performed using standard microbiological techniques.

Microbiological investigations

The clinical samples collected from the cancer patients were processed as follows: Blood samples were processed using an automated blood culture system (BacT/ALERT system - bioMerieux). Positive blood cultures and other clinical samples were stained with Gram stain and inoculated onto blood agar, chocolate agar, and MacConkey agar and incubated aerobically at 37 °C for 18-24 hours. Only one isolate per patient was included. Species identification was confirmed for all selected isolates by matrix- assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a VITEK MS (Biomerieux) following the manufacturer's instructions.

Antimicrobial susceptibility testing

The bacterial antibiotic susceptibility testing (AST) was performed using the VITEK 2 system (BioMerieux, France). AST of all isolates was performed against a panel of antimicrobials as follows: amoxicillin-clavulanate (20/10µg), amikacin (30µg), ceftazidime (30µg), ciprofloxacin (5µg), cefepime (30µg), cefoperazone-sulbactam (15µg), gentamicin (30 µg), imipenem (30µg), meropenem (30µg), piperacillin-tazobactam (100/10µg), trimethoprim-sulfamethoxazole (1.25/23.75µg). GNB resistant to at least one of the aminoglycoside drugs (gentamicin, amikacin) was selected and subjected to molecular characterisation. The minimum inhibitory concentration (MIC) was then used to determine whether the isolates were sensitive or resistant using Clinical and Laboratory Standards Institute (CLSI) 2020 guidelines.[24] Quality control was performed using *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. All quality control results were within specified ranges as published in CLSI documents.

DNA extraction and multiplex polymerase chain reaction (PCR) assay

DNA extraction was done by the modified method of Nagarajan *et al*.[25] A few bacterial colonies were suspended in 100µl of DNase free water and kept in a dry bath (Eppendorf Thermomixer) for 10 min, and subsequently kept in a freezer (-20 ºC) overnight. The suspension was then centrifuged at 6000 rpm for 5 min. Two microlitres of the supernatant were used as templates for the PCR**.**

Table 1: Primers used for detection of 16S rRNA methylase genes by multiplex PCR

Gene	Primer Sequence (5'-3')	Amplicon size (bp)
armA- F	ATTCTG CCTATCCTAATTGG	315
armA - R	ACCTATACTTTATCGTCGTC	
rmtA - F	CTAGCGTCCATCCTTTCCTC	635
rmtA - R	TTGCTTCCATGCCCTTGCC	
rmtB- F	GCTTTCTGCGGGCGATGTAA	173
rmtB - R	ATGCAATGCCGCGCTCGTAT	
rmtC - F	CGAAGAAGTAACAGCCAAAG	711
rmtC - R	ATCCCAACATCTCTCCCACT	
rmtD - F	CGGCACGCGATTGGGAAGC	401
rmtD -R	CGGAAACGATGCGACGAT	
rmtE - F	TGGTTGCAGAGGTTCTGTCGAGC	518
rmtE - R	CGGCGTAACAGACACGGCATCA	
rmtF- F	ATTCATCTGGGCTGCGTGCGAC	338
rmtF- R	ACCAGCTCGTCCGACACCGTAA	

Multiplex PCR targeting seven 16S-RMTase genes *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE* and *rmtF* was carried out according to the method of Doi *et al*. (2007).[19] PCR amplification was performed in a Proflex thermocycler (Applied Biosystems, USA).

The following cycling conditions were used: initial denaturation at 96 °C for 5 min; followed by 30 cycles of 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; followed by a final extension step at 72 °C for 5 min.

The amplified products were separated on a 1.5% low-melting agarose gel (Biorad). They were electrophoresed (0.5X TBE buffer at 150V and 90mA for 30 minutes), stained with 0.5% ethidium bromide, visualized and recorded by using a gel documentation system (BIO-RAD). A 100bp ladder (GeneDirex) was run as a molecular marker. The list of primers used for the detection of 16S rRNA methylase genes is provided in Table 1.

All PCR reactions was carried out in a 25µl reaction volume containing 0.2 µl of Taq Polymerase (5 Units/ µl), 2.5 µl of 10X reaction buffer, 0.5 µl of 10mM dNTP, 0.5 µl of 10µM Forward primer, 0.5 µl of 10µM Reverse primer, 2 µl of template DNA, and 18.8 µl of PCR grade water (PCR reagents were GenetBio, Korea).

Cycling conditions: Initial denaturation of 96 °C for 5 minutes, 30 cycles of denaturation at 96 °C for 30 seconds, annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute, followed by a final extension at 72 °C for 5 minutes.

DNA sequencing

Amplified PCR products from specific genes (*armA*, *rmtB* and *rmtF*) representing a single clinical isolate were subjected to DNA sequencing with the ABI Prism Big Dye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (GeneSpec Pvt. Ltd). The nucleotide sequences analysed were

compared with existing gene sequences in the Genbank using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) tool for confirmation. The DNA sequences were then submitted to the Genbank and the following accession numbers were obtained: *armA* (OK482771), *rmtF* (OK504483) and *rmtB* (OK425847).

Statistical analysis

On the basis of averages and percentage values, the data was examined and assessed. The gathered information was imported into Microsoft Excel and subjected to descriptive statistical techniques for analysis.

Results

Figure 1 displays the age and sex distributions. The maximum number of cases were in the >50 years age group. The oldest patient was 87-year-old and the youngest was 6 months.

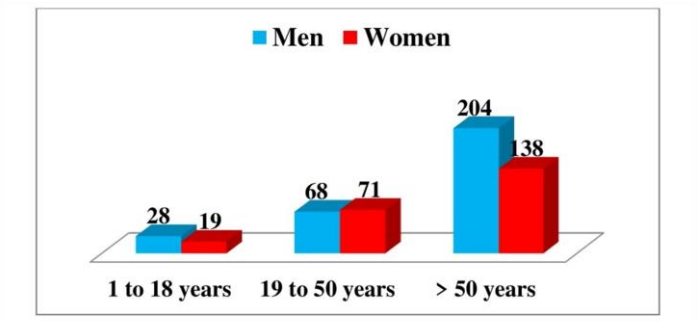


Figure 1: Age and gender-wise distribution of the cancer patients

A total of 592 isolates of non-duplicate GNB were isolated from 528 samples. The most commonly encountered type of clinical infection was urinary tract infections (n=175;33%), followed by blood stream infections (n=137;26%), and skin and soft tissue infections (n=103;19.2%) as shown in Table 2.

Table 2: Types of infections caused by various organisms

Isolated Organisms		Types of infections				
	Total number of organisms	Blood stream	Respiratory tract	Urinary tract	Skin and soft tissue	Others
E. coli	227	53	14	102	27	31
K. pneumoniae	159	37	16	45	32	29
P. aeruginosa	99	33	9	17	31	9
A. baumannii	34	9	8	6	10	1
Enterobacter spp.	16	5	2	5	3	1

In this study, polymicrobial growth was detected in 28 samples. Automated methods were used to identify the species of all isolates. *E. coli* (n=227;38.3%) was the predominant isolate followed by *Klebsiella pneumoniae* (n=159;27%), *P. aeruginosa* (n=99;17%) and *Acinetobacter baumannii* (n=34; 6%) (Figure 2).

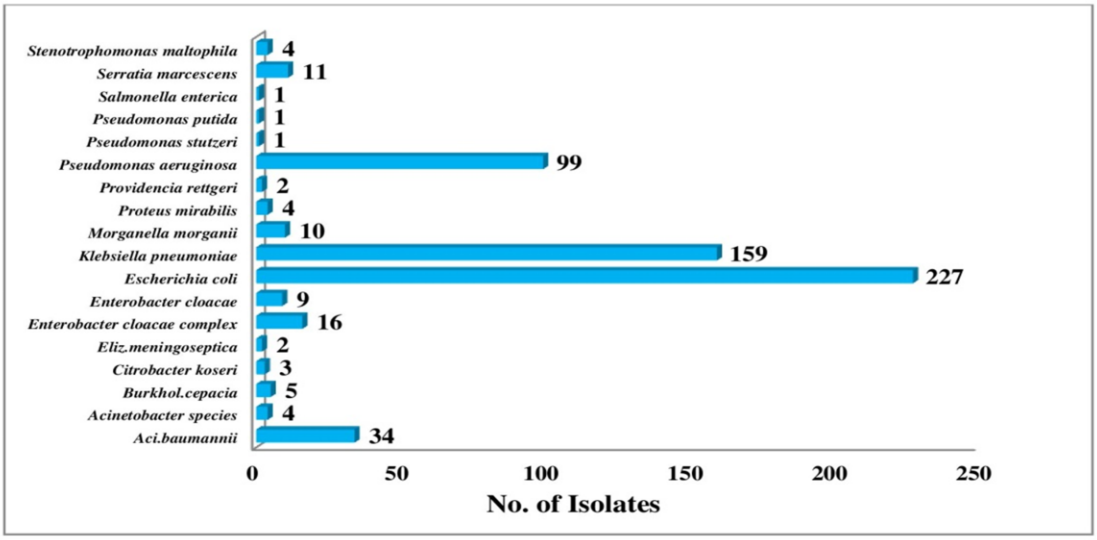


Figure 2: Distribution of Gram-negative bacterial isolates from cancer patients

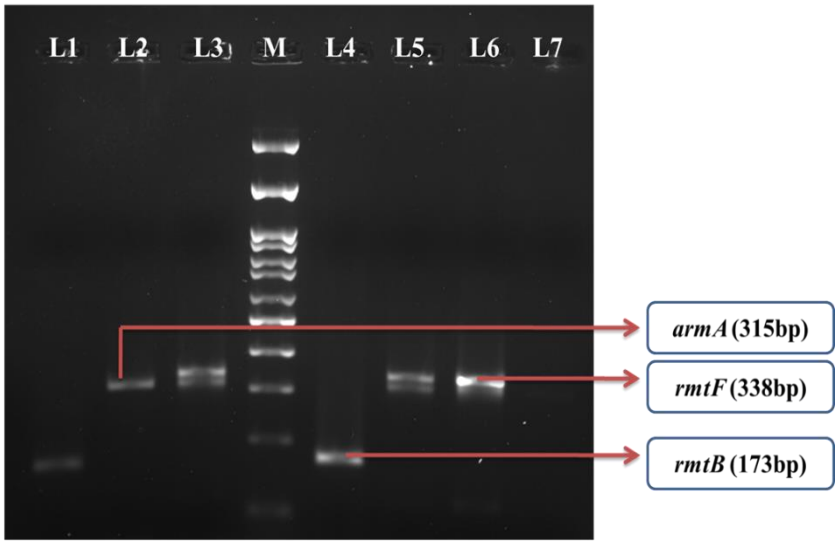
A. baumannii exhibited the highest levels of resistance to the tested drugs. Overall, the lowest resistance was noted for carbapenem antibiotics, piperacillin-tazobactam and cefoperazone-sulbactam across all isolated microorganisms assessed for AST. When comparing the susceptibility profiles of the isolates to various antibiotic classes, it was found that *P. aeruginosa*, a non-fermenting bacterium, displayed a significantly higher sensitivity to all antibiotic groups than *E. coli*, *K. pneumoniae*, and *A. baumannii*. (Table 3).

Table 3: Antibiotic resistance pattern of Gram-negative isolates

	Total No	ESBL		Cefoperazone-sulbactam		Quinolone resistance		Imipenem		Meropenem		Piperacillin-tazobactam	
		n	%	n	%	n	%	n	%	n	%	n	%
A. baumannii	34	23	68	15	44	23	68	24	70	22	65	23	68
E. coli	227	135	59	58	25.5	176	77.5	59	26	65	29	72	32
***E. cloacae* complex**	16	6	37.5	3	19	5	31.2	2	12.5	1	6.25	5	31.2
K. pneumoniae	159	87	55	55	34.5	110	69	47	29.5	48	30	65	41
P. aeruginosa	99	11	11	14	14	28	28.2	25	25.2	18	18	17	17

Among the 592 GNB isolates studied, 267 (45%) were found to be resistant to amikacin (MIC>=64) and gentamicin (MIC>=16). Aminoglycoside non-susceptible isolates were subjected to PCR to detect the genes encoding 16S-RMTases (Table 1).

Of the 267 isolates, 134 (50%) were positive for one or more 16S RMTase genes. PCR assays revealed the presence of different 16S-RMTases genes, of which *rmtB* (n=57) was predominant, followed by *armA* (n=42), *rmtF* (n=13). Combinations of different genes were also observed in a few isolates, i.e., *armA* and *rmtB* (n=14) and *armA* and *rmtF* (n=8). No *rmtA*, *rmtC*, *rmtD* and *rmtE*, genes were detected in this study. The 16S RMTase genes were found in the following species: *E. coli* (n=67), *K. pneumoniae* (n=42), *P. aeruginosa* (n=14), *A. baumannii* (n=9) *M. morganii* (n=2) (Figure 3; Table 4).



L1 – *rmtB*, L2 – *armA*, L3 – *armA* & *rmtF*, L4 – *rmtB*, L5 - *armA* & *rmtF*, L6 – *rmtF*, L7 – Negative Control; M – 100bp ladder

Figure 3: Representative gel picture showing 16s RMTases (*rmtA* - *rmtF*)

Table 4 shows the number of isolates harboring RMTases genes among 267 isolates with resistance to at least one of the 2 aminoglycosides - amikacin and gentamicin.

Table 4: Multiplex PCR assay results of 16SrRNA methyltransferase genes

Isolated organisms	*armA* (n = 42)	*rmtB* (n = 57)	*rmtF* (n = 13)	*armA* and *rmtB* (n = 14)	*armA* and *rmtF* (n = 8)	Total
A. baumannii	3	1	2	1	2	9
E. coli	17	32	4	10	4	67
K. pneumoniae	15	19	5	2	1	42
P. aeruginosa	6	5	2	1	0	14
M. morganii	1	0	0	0	1	2
Total genes	42	57	13	14	8	**134**

Discussion

Aminoglycoside resistance can be caused by various resistance mechanisms that include AME-modified aminoglycoside molecules, target modifications by spontaneous mutations or 16S-

RMTases, and efflux and permeability issues. In hospital settings, the spread of pathogenic GNB organisms resistant to aminoglycosides may increase the mortality rate and complicate treatment options. Aminoglycosides are often administered in combination with β-lactam antibiotics to treat severe infections in hospitalised patients. In this study, a subset of isolates resistant to both gentamicin and amikacin were selected for 16S RMTase gene screening. Other aminoglycoside agents such as, tobramycin, kanamycin, netilmicin, as well as neomycin and apramycin for NpmA, which are needed to infer the high-level phenotype indicative of producing 16S-RMTases were not tested in this study.

The majority of isolates that produced 16S-RMTases were also producers of carbapenemases and extended spectrum β-lactamases (ESBL's), suggesting a substantial correlation between these resistance mechanisms and offering a potential reason for the rise of 16S RMTases. Epidemiology of 16S-RMTases show variations in different countries. They are rare in certain places, while in others, they are reported with increasing frequency.[14,20,21] In the present study, the predominant aminoglycoside resistance gene was *rmtB* followed by *armA* and *rmtF*. These genes are located on plasmids and other mobile genetic elements such as transposons; they may therefore spread among GNB through horizontal gene transfer.[15] Among 16S RMTase genes, *armA* was the most widely distributed gene.[14,18,21-23] In line with our study, a study from Northern India and Saudi Arabia also found that the prevalent gene is *rmtB*.[10,24] However, a study from Eastern India by Wangkheimayum *et al*. (2020) found that the predominant gene was *rmtC*, followed by *armA*.[11] In contrast to reports from other countries, our study revealed *rmtB* was predominant, followed by *armA* and *rmtF* .[14]

We observed different types of acquired 16S-RMTases and the coexistence of two different methylase gene types in 22 isolates along with β-lactamase resistance and carbapenem resistance. The same was reported in earlier studies.[11,12,14,21,24] The presence of co-existing ESBLs, carbapenemases, 16S-RMTase genes and other resistance determinants is a major concern, as they could significantly compromise the efficacy of combination therapy. This association restricts available treatment options for Gram-negative infections, leaving only last-resort antibiotics such as colistin or tigecycline.

The absence of a 16S-RMTase gene in isolates exhibiting pan-aminoglycoside resistance may be attributed to other resistance mechanisms, such as the action of aminoglycoside-modifying enzymes, or the overexpression of efflux pumps. Alternatively, it can be caused by novel 16S-RMTase genes or gene variations that are not detectable by the PCR tests currently in use. To investigate these isolates further and uncover underlying resistance mechanisms, whole genome sequencing (WGS) is being employed. The rapid identification of 16S-RMTase genes is essential for ensuring appropriate treatment. Different molecular techniques, including PCR and whole-genome sequencing, are being developed to detect these resistance genes in clinical samples.

Conclusion

The increasing prevalence of 16S rRNA methylase genes among GNB poses a significant challenge in treating MDR infections, particularly in hospital environments. The lack of detectable 16S RMTase genes in certain pan-aminoglycoside-resistant isolates indicates the possibility of novel resistance mechanisms or gene variations that current detection methods, such as PCR, may

not capture. Rapid and accurate detection of 16S rRNA methylase genes is critical for guiding treatment decisions and improving patient outcomes.

On-going surveillance programs are necessary to monitor the prevalence of these resistance genes and inform the development of standardised treatment protocols in healthcare settings. Addressing the spread of MDR GNB and their associated resistance genes requires coordinated efforts in surveillance, prevention, and the development of new therapeutic strategies to preserve the effectiveness of existing antibiotics.

Declaration

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