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EMERGENCE OF *armA* AND *rmtB* GENES AMONG VIM, NDM, AND IMP METALLO-β-LACTAMASE-PRODUCING MULTIDRUG-RESISTANT GRAM-NEGATIVE PATHOGENS

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In the recent years, it has been noted that microorganisms with acquired resistance to almost all available potent antibiotics are increasing worldwide. Hence, the use of antibiotics in every clinical setup has to be organized to avoid irrational use of antibiotics. This study was aimed to establish the pattern of antibiotic sensitivity and relevance of antimicrobial resistance in aerobic Gram-negative bacilli. A total of 103 aerobic Gram-negative bacteria namely *Escherichia coli, Klebsiella pneumoniae, Enterobacter* spp., *Citrobacter koserii, Proteus* spp., and *Pseudomonas aeruginosa* were collected from tertiary care centers around Chennai. Kirby–Bauer Disk Diffusion test and study for genes of cephalosporin, carbapenem, and aminoglycoside resistance were done. A descriptive analysis of the data on altogether 103 clinical urine isolates was performed. All strains showed susceptibility to colistin. The frequency of genes encoding 16S rRNA methylases *armA* and *rmtB* were 7.8% and 6.8%, respectively. Among metallo-β-lactamases, bla_{VIM}, bla_{IMP}, and $bla_{\text{NDM-1}}$ were detected in 6.8%, 3.8%, and 3.8%, respectively. One *E. coli* strain harbored $bla_{\text{SIM-1}}$ gene. Cumulative analysis of data suggested that 30% of the strains carried more than one resistance gene. The current research evidenced the increasing frequency of resistance mechanisms in India. Combined approach of antibiotic restriction, effective surveillance, and good infection control practices are essential to overcome antibiotic resistance.

Keywords: multidrug resistance, ESBL, MBL, *armA*, *rmtB*

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Introduction

Enterobacteriaceae and non-fermentative Gram-negative *Pseudomonas* and *Acinetobacter* species are responsible for majority of hospital- and community-acquired infections globally due to their accelerated antibiotic resistance and difficulty in treatment by conventional antibiotics. Colistin and polymyxin B have become the only drugs of choice for serious infections caused by the beta-lactamases-producing aerobic Gram-negative bacilli. The incidence of infections caused by these organisms has increased in recent years [1]. To minimize the spread of these bacteria in the community, there is an increasing need for clinical laboratory to identify and characterize the strains up to the molecular level especially for drug resistance. Moreover, the inappropriate use of antibiotics and acquisition of mobile genetic elements by horizontal transfer may result in a "superbug," with resistance to all licensed antibiotics. Deliberate and perpetual surveillance studies are warranted since the epidemiology of beta-lactamase-producing bacteria have become complex. In India, many studies have identified disseminated multidrug resistance among Gram-negative bacilli due to extended-spectrum beta-lactamases (ESBL) and carbapenamases [1].

Owing to the dramatic increase in bacterial resistance to β-lactam antibiotics, aminoglycosides have become the substitutes for the therapeutics due to their broad antimicrobial spectrum and also their synergistic effect in association with other antibiotics. Increased prescription of aminoglycosides for therapy accounts for the growing resistance to aminoglycoside antibiotics. Three groups of enzymes produced by bacteria confer aminoglycoside resistance namely, acetyltransferases, nucleotidyl transferases, and phosphotransferases, which inactivate gentamicin (GEN) and tobramycin (TOB). However, recently plasmid-mediated 16S rRNA methylases have emerged which confer remarkably high-level resistance to arbekacin, amikacin (AK), TOB, and GEN (3) through post-transcriptional methylation of the aminoglycoside-binding site leading to the loss of affinity [2].

Until now, 10 plasmid-mediated 16S rRNA methylases (*armA, rmtA, rmtB, rmtC, rmtD, rmtE, rmtF, rmtG, rmtH*, and *npmA*) have been identified in at least 30 countries worldwide [2–11]. Since the 16S rRNA methylase genes are frequently located on plasmids, there is an increased probability of other resistance mechanisms to interplay, thus leading to multidrug resistance. In particular, very recently, this coexistence is seen with genes encoding ESBL and metallo-β-lactamases (MBL). As a consequence, those strains remain resistant to aminoglycosides, beta-lactams, and carbapenems.

Limited studies that are available in India show the percentage of 16S rRNA methylases-mediated aminoglycoside resistance in Gram-negative isolates.

Nevertheless, surveillance in Southern and Eastern parts of Europe reported approximately 10%–50% aminoglycoside resistance in invasive strains with consistent increase every year. Moreover, *armA* and *rmtB* are often identified genes among the methylases in Gram-negative bacilli from East Asia and South America [3]. Hence, we intended to investigate the prevalence of *armA* and *rmtB* genes among the ESBL- and/or MBL-producing *Enterobacteriaceae* and non-fermenting Gram-negative bacilli prospectively recovered from patients at multiple centers in Chennai.

Materials and Methods

Clinical strains

About 103 aerobic Gram-negative urinary isolates resistant to at least one antibiotic were collected from multicenters, in and around Chennai, Tamil Nadu. Identification of bacterial isolates was done to the species level by cultural characteristics and biochemical reactions by conventional methods.

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was carried out on all strains by Kirby–Bauer Disk Diffusion Agar method using commercial disks (HiMedia) according to the criteria recommended by Clinical and Laboratory Standards Institute (CLSI) [12]. The antibiotics tested were ampicillin (AMP), ciprofloxacin (CIP), ceftazidime (CAZ), CAZ/clavulanic acid (CAC), piperacillin (PIP), AK, GEN, TOB, imipenem (IPM), meropenem (MEM), cefoxitin (CX), aztreonam (AZT), cefepime (CPM), and PIP/tazobactam (PTZ). All isolates were tested for colistin susceptibility by E-test colistin strip (ranging from 0.016 to 256 mg/ml) according to the manufacturer's guidelines (Liofilchem, Italy). The minimum inhibitory concentration (MIC) was read at the point of complete inhibition of all growth, including hazes. The interpretive criteria used were those established in CLSI. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains for antibiotic susceptibility testing and E-test.

Phenotypic screening for ESBLs and MBLs

ESBL production was detected by double-disk synergy test as recommended by the CLSI guidelines (CLSI, 2010). The strains showing any synergy between CAZ/CAC disk and CPM/CAZ disk were considered to be ESBL producer [13–17].

Combined disk test with IPM/MEM–EDTA-impregnated disk was performed for MBL screening as previously described by Arunagiri et al. [18].

Isolation of genomic DNA

A single colony was inoculated into 5-ml Luria–Bertani broth and incubated with shaking at 37 °C for 20 h. Overnight culture was harvested by centrifugation for 5 min and the pellet was resuspended in 500 µl distilled water. The cells were lysed by heating at 95 °C for 5 min and centrifuged for 1 min. After centrifugation, 5 µl of supernatant was used for PCR amplification.

Genotypic ESBL and MBL characterization

Amplification of DNA was performed in a thermal cycler for the detection of bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ ESBLs and bla_{VIM}, bla_{IMP}, $bla_{SIM\text{-}1}$, $bla_{GIM\text{-}1}$, $bla_{SPM\text{-}1}$, and $bla_{NDM\text{-}1}$ MBLs. Primers, PCR amplicon size, and annealing temperature were indicated in Table I. PCR-amplified products were subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light to observe the anticipated PCR product.

Detection of armA and rmtB genes

The *armA* and *rmtB* genes were detected by PCR as described by Doi and Arakawa [3]. Primers for *armA* and *rmtB* are described in Table I. Primers for *armA* detected a region of 315 bp and *rmtB* detected a region of 173 bp. PCR amplification was done; the products were electrophoresed in 1.5% agarose gels, and visualized under UV light. The cycling conditions were initial DNA denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing as given in Table I, extension at 72 °C for 1 min, and a final elongation at 72 °C for 5 min.

Results

During the study period, 103 clinical isolates resistant to at least one antibiotic were collected from clinical urine samples from multicenters, in and around Chennai, Tamil Nadu. Samples included 66 (64.1%) females and 37 (35.9%) males whose age group ranged from 1 to 68 years with mean age of 38.4 ± 19.1 years. Isolates were distributed as *E. coli* (73.7%), *Klebsiella pneumoniae* (10.6%), *P. aeruginosa* (9.7%), *Enterobacter* (2.9%), *Citrobacter koseri* (1.9%), and *Proteus* spp. (0.9%).

Table I. Oligonucleotide primers used for gene detection by PCR amplification (nucleotide sequence)

Target gene		Primer sequence (5′→3′)	Product size (bp)	Annealing temperature	Ref.
bla_{TEM}	TEM-F	GAAGACGAAAGGGCCTCGTG	1,074	55 °C for 1 min	[18]
	TEM-R	GGTCTGACAGTTACCAATGC			
bla_{SHV}	SHV-F	CGCCGGGTTATTCTTATTTGTCGC	1,016	68 °C for 1 min	[18]
	SHV-R	TCTTTCCGATGCCGCCGCCAGTCA			
bla_{CTX-M}	CTX-M-F	TTTGCGATGTGCAGTACCAGTAA	544	51 °C for 1 min	[19]
	CTX-M-R	CGATATCGTTGGTGGTGCCATA			
bla_{VIM}	VIM-F	TTTGGTCGCATATCGCAACG	500	46°C for 1min	[20]
	VIM-R	CCATTCAGCCAGATCGGCAT			
bla_{IMP}	IMP-F	GTTTATGTTCATACWTCG	432	45 °C for 1 min	[20]
	IMP-R	GGTTTAAYAAAACAACCAC			
bla_{GIM-1}	GIM-F	TCGACACACCTTGGTCTGAA	477	52 °C for 40 s	[21]
	GIM-R	AACTTCCAACTTTGCCATGC			
bla_{SIM-1}	SIM-F	TACAAGGGATTCGGCATCG	570	52 °C for 40 s	[21]
	SIM-R	TAATGGCCTGTTCCCATGTG			
bla_{SPM-1}	SPM-F	AAAATCTGGGTACGCAAACG	271	52 °C for 40 s	[21]
	SPM-R	ACATTATCCGCTGGAACAGG			
bla_{NDM-1}	NDM-F	GGTTTGGCGATCTGGTTTTC	475	55 °C for 1 min	[22]
	NDM-R	CGGAATGGCTCATCACGATC			
armA	armA-F	ATTCTGCCTATCCTAATTGG	315	51 °C for 1 min	[3]
	armA-R	ACCTATACTTTATCGTCGTC			
rmtB	rmtB-F	GCTTTCTGCGGGCGATGTAA	173	58 °C for 1 min	[3]
	rmtB-R	ATGCAATGCCGCGCTCGTAT			

Antimicrobial susceptibility testing

All the 103 isolates were sensitive to colistin by E-test method with MIC ≤ 2 μg/ml and were found resistant to AMP. The resistant trend decreased with respect to MEM, CIP, PIP, AZT, CPM, CAZ, and GEN substantially. More than 50% were resistant to five antibiotics, such as AMP, MEM, CIP, PIP, AZT, and CPM, and more than 50% were sensitive to GEN, CX, AK, CAC, TOB, PTZ, and IPM.

β-lactamase characterization

Of the 103 isolates, 57 (55.3%) were positive for ESBL screening as it showed synergy between CAZ/CAC and CPM/CAZ disk and 14 (13.5%) strains were positive for MBL screening since they exhibited significant zone size enhancement with the ethylenediaminetetraacetic acid-impregnated disk when compared with the IPM/MEM disk. Ten isolates showed positive for both ESBL and MBL screening. The most prevalent gene was $bla_{\text{CTX-M}}$ as it appeared among the clinical isolates as single resistance gene (16.5%) or in combination with other resistance determinants (23.3%), such as bla_{TEM}, bla_{VIM}, bla_{SHV}, bla_{IMP}, and $bla_{\text{NDM-1}}$. The second most prevalent genes were TEM and SHV present alone in 6.7% and 3.8%, respectively. Genes bla_{VIM}, bla_{IMP}, $bla_{\text{SIM-1}}$, and $bla_{\text{NDM-1}}$ were found in 5.8%, 2.9%, 0.9%, and 3.8% of strains, respectively, alone or in combination with other resistance genes. Altogether, 38/103 (36.8%) were found to be negative for any gene, which constitutes of 35 *E. coli* and 3 *K. pneumoniae*.

Prevalence of methylase genes

Out of 103 isolates, only 12 (11.6%) were harboring methylase gene *armA* and *rmtB* and only four of them were presented as individual gene. The remaining eight isolates were positive for these genes together with other beta-lactamase resistance genes.

Discussion

Beta-lactamase production has been identified as one of the most important mechanism of resistance among Gram-negative strains leading to global threat. Recently, the high prevalence of aminoglycoside resistance mechanisms among these strains is alarming. In this study, resistance rates of the Gram-negative isolates to GEN were more than 50.0%, but resistance to AK and TOB were

relatively low. Yu et al. [23] reported similar resistance to GEN among the *E. coli* isolates but showed higher resistance to TOB.

The *armA* gene was initially sequenced from a *Citrobacter freundii* strain in Poland but first characterized from *K. pneumoniae* BM4536 in France in 2000, and the *rmtB* gene was first identified in *Serratia marcescens* S-95 isolated from a Japanese patient in 2002. Since then these two genes have been found in *Enterobacteriaceae, P. aeruginosa*, and *Acinetobacter baumannii* in Asia-Pacific region [23–26]. In this study, the overall prevalence rate of 16S rRNA methylase genes *armA* and *rmtB* alone/in combination with beta-lactamase genes was 11.6%. This is higher than the percentage of *armA* and *rmtB* previously reported in a Chinese study (5.4%), a Taiwanese study (0.4%), and a study from Shanghai, China (3.4%) [24, 26]. Very few studies have reported *armA* and *rmtB* genes from India [26]. Unlike other reported studies, the data from this study showed *armA* to be more prevalent than *rmtB* gene [24, 28, 29].

In this study, 16S rRNA methylase genes have been identified to be linked with other beta-lactamase resistance determinants bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{VIM}, bla_{IMP}, and $bla_{\text{NDM-1}}$, and cotransferred with other resistance determinants on self-transferable plasmids to recipients by conjugation as reported previously [23–25, 27], thus, conferring resistance to multiple antibiotics. The *armA* gene was detected as a single gene in one (0.9%) *K. pneumoniae* and two (1.9%) *P. aeruginosa* strains. However, in various combinations with other beta-lactamase genes, it was seen in four (3.9%) *E. coli* strains and one (0.9%) *K. pneumoniae* strain. Similarly, *rmtB* gene was detected as a single gene in one (0.9%) *P. aeruginosa* strain but four (3.9%) *E. coli* and one (0.9%) *K. pneumoniae* strains harbored this gene in combination with other beta-lactamase gene. Overall, out of the 14 isolates carrying 16S rRNA methylases, one (0.9%) strain harbored $bla_{\text{CTX-M}}$, bla_{TEM}, $bla_{\text{NDM-1}}$, *armA*, and *rmtB* genes and was highly resistant to all the tested antibiotics except colistin. *Enterobacter, C. koseri*, and *Proteus* spp. did not carry any 16S rRNA methylase gene either alone or in combination.

Numerous previous studies have reported ESBL production ranging from 68% to 72% [30, 31] and MBL production 28% to 42% [32, 33] among the Gram-negative strains. In this study, genotypic screening for ESBLs and MBLs identified 55.3% strains carried at least one ESBL gene and 13.5% strains carried at least one of the MBL gene by PCR. The presence and diversity of ESBLs and MBLs in different isolates as a single gene and in association with other resistance genes are shown in Table II. Among the ESBLs, CTX-M was the most common gene found (16.5%) both individually and in combination (22.3%), followed by TEM-type ESBLs (9.7%) individually and (15.5%) in association and SHV-type ESBLs (4.8%) individually and (1.9%) in association. PCR for MBLs showed $bla_{\text{NDM-1}}$ and bla_{VIM} (6.7%) to be the most dominant type found followed by bla_{IMP} (3.8%)

Table II. Prevalence of resistance determinants in clinical isolates

Type	Resistance genes		Organism producing	Total 103
Existence of a single genotype	Extended-spectrum beta-lactamase genes	$bla_{\text{CTX-M}}$	*E. coli* (11), *P. aeruginosa* (1), *K. pneumoniae* (1), and *Enterobacter* (1)	14
		bla_{TEM}	*E. coli* (7), *P. aeruginosa* (2), and *Proteus* spp. (1)	10
		bla_{SHV}	*E. coli* (4) and *P. aeruginosa* (1)	5
	Metallo-β-lactamase genes	bla_{VIM}	*K. pneumoniae* (1)	1
	16S rRNA methylase genes	*armA*	*P. aeruginosa* (2) and *K. pneumoniae* (1)	3
		rmtB	*P. aeruginosa* (1)	1
	Association of ESBL and MBL genes	$bla_{\text{CTX-M}} + bla_{\text{TEM}}$	*E. coli* (8) and *P. aeruginosa* (1)	9
Existence of two or more than two genotypes		$bla_{\text{CTX-M}} + bla_{\text{TEM}} + bla_{\text{SHV}}$	*K. pneumoniae* (1) and *C. koseri* (1)	2
		$bla_{\text{CTX-M}} + bla_{\text{VIM}} + bla_{\text{IMP}} + bla_{\text{NDM-1}}$	*K. pneumoniae* (1)	1
		$bla_{\text{CTX-M}} + bla_{\text{VIM-1}}$	*E. coli* (2) and *K. pneumoniae* (1)	3
		$bla_{\text{CTX-M}} + bla_{\text{TEM}} + bla_{\text{NDM-1}}$	*E. coli* (1)	1
		$bla_{\text{CTX-M}} + bla_{\text{IMP}}$	*E. coli* (1)	1
		$bla_{\text{IMP}} + bla_{\text{NDM-1}}$	*P. aeruginosa* (1)	1
		$bla_{\text{SIM-1}}$	*E. coli* (1)	1
		$bla_{\text{TEM}} + bla_{\text{VIM}}$	*E. coli* (1)	1
	Combination of 16S rRNA methylase genes with other genes	$bla_{\text{CTX-M}} + armA$	*E. coli* (1)	1
		$bla_{\text{CTX-M}} + rmtB$	*E. coli* (3)	3
		$bla_{\text{CTX-M}} + bla_{\text{TEM}} + bla_{\text{NDM-1}} + armA + rmtB$	*E. coli* (1)	1
		$bla_{\text{CTX-M}} + bla_{\text{TEM}} + armA$	*E. coli* (2)	2
		$bla_{\text{CTX-M}} + bla_{\text{SHV}} + rmtB$	*E. coli* (1)	1
		$bla_{\text{IMP}} + armA$	*K. pneumoniae* (1)	1
		$bla_{\text{SHV}} + bla_{\text{VIM}} + rmtB$	*K. pneumoniae* (1)	2
	Negative			38

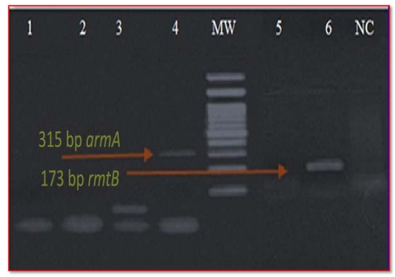

Figure 1. PCR amplification of 16S rRNA methylase genes

and $bla_{\text{SIM-1}}$ (0.9%) gene similar to a study by Somily et al. [34]. One *E. coli* strain was found to harbor SIM-1 gene and another *E. coli* strain, which was identified with high resistance to all the antibiotics except colistin was positive for $bla_{\text{CTX-M}}$, bla_{TEM}, $bla_{\text{NDM-1}}$, *armA*, and *rmtB* genes. Similarly, the emergence of the multidrug-resistant *E. coli* and *K. pneumoniae* strains producing both ESBLs and 16S rRNA methylases was identified in a previous study in Taiwan. Thus, the antibiotic resistance in Gram-negative strains lead to increased morbidity, mortality at hospital settings as revealed by surveillance studies from Europe, Asia-Pacific region, Latin America, and North America over the last 3–5 years [35].

The antibiotic resistogram showed an increasing resistance to various antibiotics. However, in contrast to the other reported studies, colistin was the most active drug with 100% sensitivity followed by IMP and PIP/PTZ [36]. Hence, colistin is the only choice available for the treatment of these multidrug-resistant strains, but it should be used in accordance with the antimicrobial consumption policy to prevent the dissemination of drug-resistant clones.

AMP showed highest percentage of resistance followed by CIP and MEM (Figure 1). Several published reports have documented reduced susceptibility to MEM among Gram-negative strains. Kaul et al. [37] reported increased carbapenem resistance in Gram-negative bacilli. A study by Srinivasa Rao et al. [38] has reported high-level resistance (>75%) to both carbapenem and other antibiotics routinely used for the treatment of Gram-negative bacilli.

Conclusions

Comparatively, high prevalence of plasmid-mediated *armA* and *rmtB* genes was described in this study among clinical aerobic Gram-negative isolates from urine samples from multicenter in Chennai. The majority of 16S rRNA methylase gene-positive isolates coproduced CTX-M, TEM, SHV-type ESBLs and IMP, VIM, NDM-1-type MBLs. Thus, both horizontal gene transfer and clonal spread

may occur leading to widespread dissemination of the *armA* and *rmtB* genes. A very rare gene is bla_{SIM} and it is usually found in *Acinetobacter* spp., but in this study, we have identified the presence of bla_{SIM-1} in *E. coli*, which may indicate the dissemination of the bla_{SIM-1} gene.

Conflict of Interest

The authors declare no conflict of interest.

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