ORIGINAL RESEARCH PAPER
Volume - 10 | Issue - 01 | January - 2021 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

SCREENING FOR 16S RIBOSOMAL RNA METHYLASES *(armA, RmtB & RmtC)* GENE IN CLINICAL ISOLATES OF ENTEROBACTERIAECEAE FROM A TERTIARY CARE HOSPITAL IN SOUTH INDIA

Microbiology

V.K.M.Banu Assistant Professor, KAPV Government Medical College, Trichy, India.

R.Beaula Lilly* Assistant Professor, KAPV Government Medical College, Trichy, India. *Corresponding Author

ABSTRACT

Gram-negative bacilli may commonly produce aminoglycoside modifying enzymes. However, any one of these enzymes alone cannot confer resistance to all commonly used aminoglycosides because of their narrower substrate specificities(3,13).But ,the mechanism of resistance mediated by 16S rRNA methylases that methylates residue G1405 is the very high level of resistance to all parenterally formulated aminoglycosides (MIC>128ug/ml) commonly used (16). Screening for 16S rRNA methylase producing organisms has become an essential measure to be taken for epidemiological as well as diagnostic purposes when nosocomial spread of such bacteriae is suspected. Only by early identification of these resistant determinants (armA, rmtB and rmtC) by molecular methods can help us to design appropriate antibiotic and infection-control policies which are necessary to limit the nosocomial spread of these resistance organisms (2).

KEYWORDS

16S rRNA methylases, high level pan aminoglycoside resistance, nosocomial spread, resistant determinants and continued surveillance

INTRODUCTION

Resistance to commonly used aminoglycoside antibiotics has been on the rise in clinical isolates of enterobacteriaceae (10). The most frequent use of aminoglycosides is empiric therapy for serious infections such as septicemia, intra- abdominal infections, complicated UTI, and also nosocomial respiratory tract infections caused by aerobic Gram-negative bacteria and organisms belonging to family Enterobacteriaceae (3). Apart from the many aminoglycosides modifying enzymes which causes resistance to this group of antibiotics, the emergence of high level pan-aminoglycoside resistance caused by the 16S rRNA methylase genes in clinical pathogens has been on the increase since its discovery in 2003 (13).

There are five types of plasmid-mediated 16s rRNA Methylases (*ArmA*, *RmtA*, *RmtB*, *RmtC*, and *RmtD*) so far identified in East Asia, Europe, South America and also in India (11, 21). People infected with resistant organisms are more likely to have longer, more expensive hospital stays and it also increases the mortality rate (4). In order to reduce the time to results in identifying pathogens and antibiotic resistance we can make use of molecular methods to overcome the limitations encountered in microbiological methods (5, 8). Hence the present study was designed to confirm the presence of 16srRNA methylase gene *armA* (also *rmtB* and *rmtC*) in clinical isolates of Enterobacteriaceae showing high level resistance to commonly used aminoglycosides both by phenotypic and genotypic methods.

MATERIALS AND METHODS

Study Design

A total of 200 clinical samples were collected and processed for isolates belonging to the family *Enterobacteriaceae* from patients attending the inpatient facility and septic wards of the Mahatma Gandhi Memorial Government Hospital, Trichy. The clinical samples included pus swabs from skin ulcers, ear discharges and surgical wound cases and also from urine samples collected between the period January 2017 to March 2019. A total of 198 isolates belonging to the family Enterobacteriaceae were taken up for the study and their susceptibility patterns to various antibiotics including aminoglycosides (Amikacin, Gentamicin netilmicin, Tobramycin) were determined using Kirby–Bauer Disc Diffusion Method. The minimal inhibitory concentrations of Amikacin and Gentamicin were determined to screen for low-level and high-level aminoglycoside resistance and was further evaluated for the presence of *armA* gene by PCR method among the isolates showing high level aminoglycoside resistance.

Disc diffusion method

Amikacin/ Netilmicin discs (30µg Hi Media Labs) were employed. Sensitivity testing was done according to the standard disc diffusion method (Bauer *et al.,* 1996). Zone of inhibition was measured and interpreted according to CLSI 2018 guidelines, ≤ 14mm resistant, ≥ 17mm or above, sensitive. *E.coli* ATCC 25923 was used as quality control. Similarly Gentamicin/Tobramycin discs (10µg Hi Media Labs) were employed. Zone of inhibition was measured and interpreted according to CLSI 2018 guidelines, < 12mm resistant, and > 15mm, sensitive.

Determination of low-level and high-level Aminoglycoside resistance by MIC

The MICs for Amikacin and Gentamicin (Hi Media Labs) was determined by agar dilution method. If the MIC value was ≤ 4µg/ml, isolates were reported to be sensitive, 8 - 64µg/ml to have low-level resistance, ≥ 128µg/ml to have high-level resistance. E.coli ATCC 25923 was used for quality control.

Molecular Detection of High-Level Aminoglycoside Resistance

Detection of high-level aminoglycoside resistance was done by performing *armA* gene PCR. PCR was carried out (50µl reaction) using the following components in the reaction mixture: 1x PCR Buffer /200 µM of each dNTP/0.2 µM - Forward Primer/ 0.2 µM - Reverse Primer /0.2 units - *Taq* Polymerase/1 µL (5-10ng) – DNA .
Primer sequence: Size of *armA* gene – 315 bp
Forward – 5'-ATT CTG CCT ATC CTA ATT -3'
Reverse – 5'-ACC TAT ACT TTA TCG TCG TC -3'

PCR Cycling Conditions

An initial denaturation step was carried out at 94ºC (2 min), followed by denaturation at 94ºC (1min), annealing at 53ºC (1min) and elongation at 72ºC (1 min) for 30 cycles and a final extension was carried out for 5 min at 72ºC. Agarose gel electrophoresis was then carried out with the final PCR product at 70V/100mA, with a 100bp ladder as marker.

RESULTS

Isolation and Identification

All 198 isolates were from inpatients of two tertiary care hospitals in and around Trichy. The predominant organism in all our enterobacterial isolates was found to be E.coli 64/198 isolates, the other isolates being K.*pneumoniae*, K.*oxytoca*, C.*freundii*, C.*koseri*, Enterobacter, P.*mirabilis* and P.*vulgaris* .The isolates showing resistance to two or more groups of antibiotics were take-up for this study. All of 198 isolates were screened for aminoglycoside resistance. 15/198 isolates were found to be resistant to all four amino glycosides screened.

Antibiotic susceptibility test of Enterobacterial isolates

The antibiotic susceptibility test was done for all 198 isolates. The antibiotic disks used were amikacin, gentamicin, netilmicin and tobramycin and also cefotaxime, piperacillin, piperacillin/tazobactam, ceftazidime, ceftazidime/ clavulanic acid, cotrimoxazole, imepenem, meropenem, cefipime, ciprofloxacin and ofloxacin.

- Among the aminoglycosides tested, isolates showed highest resistance to tobramycin 32/198 (16.2%), then gentamicin 24/198 (12.1%), amikacin 21/198 (10.6%) and netilmicin 16/198 (8.1%).

The orders of resistance for the other antibiotics tested were as follows: > piperacillin 56/198 (28.3%) > ciprofloxacin 49/198 (24.7%) cefotaxime 41/198 (20.7%) > ofloxacin 32/198 (16.2%) > cefipime 28/198 (14.1%) > cotrimoxazole 21/198(10.6%) > ceftazidime 20/198 (10.1%) > meropenem 18/198(9.1%) > piperacillin/tazobactam15/198 (7.6%) > ceftazidime/clavulanic acid 14/198 (7.1%) > imipenem 12/198(6.1%).

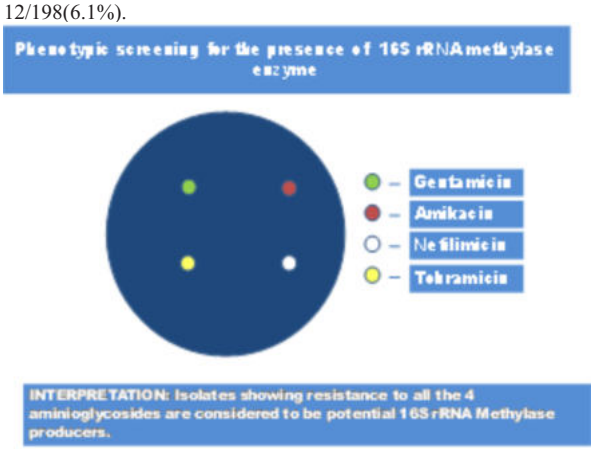

Figure 1: Phenotypic screening for the presence of 16s rRNA methylase enzyme

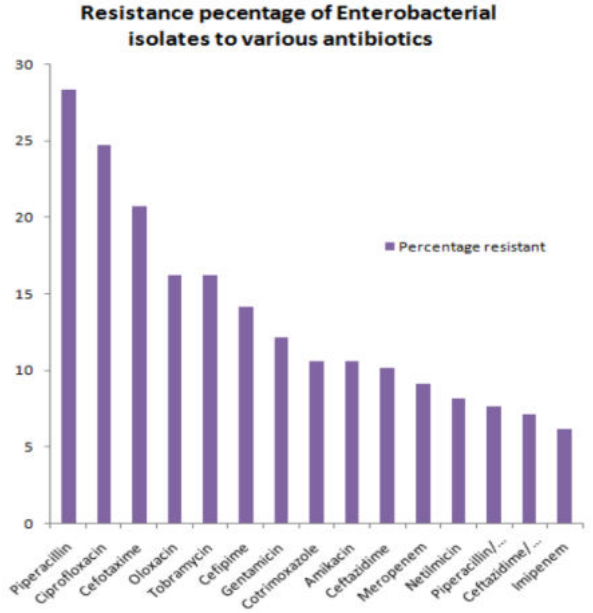

Figure 2. Resistance Percentage of Enterobacterial isolates to various antibiotics

15/198 (7.6%) isolates were found to be resistant to all the four aminoglycosides tested. 42/198 isolates were found to be resistant to two aminoglycosides tested. 24/74 isolates were found to be resistant to one aminoglycoside tested.

- 8/15 isolates were also ESBL producers, by phenotypic methods.
- Highest sensitivity was seen for imipenem 186/198 (94.1%) with a sensitivity index higher than all other antibiotics.

MIC determination of the isolates

The MIC of the isolates for gentamicin and amikacin was determined.

- MIC_{50} and MIC_{90} for gentamicin was 16 µg/ml and 128µg/ml respectively.
- MIC_{50} and MIC_{90} for amikacin was 16µg/ml and >128µg/ml respectively.

TABLE - 1 CORRELATION OF MIC TO THE DETECTION OF 16S rRNA METHYLASES

Serial No	Strain No	Positive for gene	Amikacin MIC
1	16	*armA*	>128
2	17	*armA*	>128
3	18	*armA*	>128
4	19	*rmtC*	128
5	26	*armA*	128
6	50	*armA*	>128
7	51	*armA*	>128
8	52	*rmtB*	128
9	53	*rmtC*	>128
10	66	*rmtB*	128
11	67	*armA*	128
12	71	*rmtB*	>128
13	73	*rmtC*	>128
14	82	*armA*	>128
15	93	*rmtC*	128

- The MIC amikacin and gentamicin of all the 15 isolates positive for 16S rRNA methylases were in the range of >128 and 128 µg/ml confirming the presence of high level aminoglycoside resistance in these isolates.

Genotypic detection of high level aminoglycoside resistance using *armA*, *rmtB* and *rmtC* gene PCR

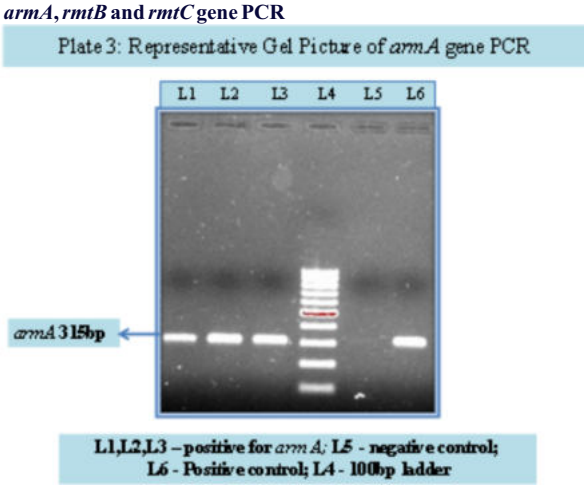

Figure 3. Gel doc picture of the isolate indicating the presence of *armA* gene

All the 198 isolates were tested for the presence of *armA, rmtB* and *rmtC* genes by PCR. Of these, 8 isolates with amikacin MIC >128µg/ml were found to be positive for *armA* gene, 3 for *rmtB* gene and 4 for *rmtC* gene , thereby confirming high-level aminoglycoside resistance in these isolates. Among the 8 *armA* positive isolates, 6 were ESBL producers.

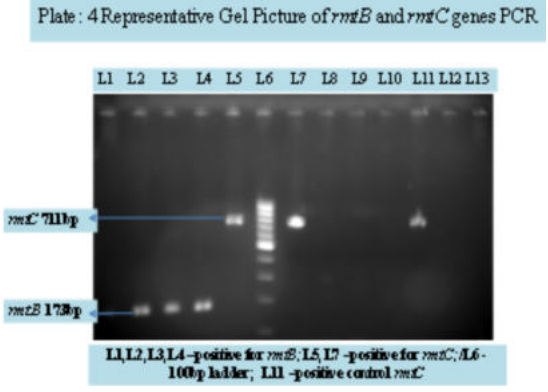

Figure 4. Gel doc picture of isolates indicating the presence of *rmtB* and *rmtC* genes

DISCUSSION

Production of 16S rRNA methylase is suspected when we observe little or no inhibitory zone with any of the four aminoglycoside disks used namely amikacin, gentamicin, tobramycin and netilmicin (6, 22). When considering the MICs of these aminoglycosides used, a cutoff value of 256µg/ml provides an excellent positive predictive value (5, 8). In the present study, the MIC amikacin and MIC gentamicin of isolates having high level panaminoglycosideresistace have been reported to range from 128µg/ml - >128µg/ml, which is close to the positive predictive value(23). The 16S rRNA methylase genes (*armA,*

rmtB, rmtC) have been detected by PCR in all these HLR isolates. PCR seems to be a reliable tool in the detection and confirmation of this resistance mechanism which confers resistance to all the four aminoglycosides commonly used (1, 15). The post-transcriptional methylation of rRNA is the mechanism involved in this high level aminoglycoside resistance caused by the 16SrRNA methylases and it seems to be efficient even when the corresponding gene is present in a low copy number (12).

The present study shows that the overall prevalence of 16S rRNA methylase genes in clinical isolates of *Enterobacteriaceae* was 7.6 % which is a little higher than those previously reported of 5.4% in China (10). *ArmA*, *rmtB* and *rmtC* were detected in 8, 3 and 4 isolates respectively. Among the 8 *armA*positive isolates, 3 were found in *Klebsiella pneumoniae*, 4 from *Escherichia coli* and 1 from *Klebsiella oxytoca*. The 3 *rmtB* genes were found in 3 *E.coli* isolates. The *rmtC* genes were found 1 in K.pneumoniae, 2 in *Enterobacter sps* and 1 in *Proteus mirabilis* isolate.

The most prevalent 16S rRNA methylase gene present in our isolates was the *armA* gene, which was consistent with findings in other studies (23). In the 8 isolates producing *armA* gene 6 were ESBL producers by phenotypic methods, 4 were resistant to both ciprofloxacin and cotrimoxazole, which was consistent with previous reports indicating the association *rmtB* and *armA* genes to CTX-M3 and different *qnr* genes with CTX-M-1,15,9 (18,24). Our isolates were resistant to multiple antibiotics and there was an apparently higher prevalence of *armA* gene compared with that of *rmtB* and *rmtC* gene (17). This was due to its association with the same conjugative plasmid which harbours the gene for CTX-M-3 encoding ESBLs and its location on functional transposon Tn1548 (16, 20). *ArmA* and *rmtB* may also spread by horizontal transfer as well as by clonal spread of some resistant strains (10). The 16S rRNA methylase gene *armA* was previously reported in India in Acinetobacter isolates from Chennai producing metallo-beta lactamases (7, 9 and 14). Among the aminoglycosides tested highest level of resistance was observed or tobramycin (16.2%), followed by gentamicin (12.1%), amikacin (10.6%), and netilmicin (8.1%).

Although there are many alternative treatment options for MRSA and VRE there are not much alternatives to treat infections caused by pan-aminoglycoside resistant gram negative bacteria. The clinical outcome for patients infected with these organisms is still unclear. Changes in the prevalence of genes encoding 16S rRNA methylases could be caused only by changes in antibiotic policies and effective infection control methods (19). Hence, continued surveillance by both the rapid genotypic *pcr* method and also by phenotypic methods is necessary to understand and optimize antimicrobial therapy and infection-control methods which in turn could bring down the prevalence of these resistant determinants (2).

CONCLUSION

Molecular methods can overcome the limitations of microbiological methods, by reducing the time to results in identifying pathogens and antibiotic resistance. This enables evidenced based antibiotic use in critical care settings rather than empirical antibiotic treatment with broad–spectrum, high-level aminoglycoside antibiotics.

REFERENCES:

1. Ad, C. Fluit Maarten, R., Visser, Franz, and Josef Schmitz. (2001), "Molecular Detection of Antimicrobial Resistance." Clin Microbiol Rev., 14(4), 836–871.
2. Aparajita Singh ,Richard, V., Goering Shabbir Simjee, Steven, L., Foley, Marcus, J., and Zervos. "Application of Molecular Techniques to the Study of Hospital Infection."
3. Boehr, D. D., I. F. Moore., and G. D., Wright. (2005), "Aminoglycoside resistance mechanisms. In Frontiers in antimicrobial resistance: a tribute to Stuart B. Levy." ASM Press, Washington, DC, 85–100.
4. Boucher, H. W., G. H., Talbot, J. S., Bradley, J. E., Edwards, D. Gilbert, L. B., Rice, M. Scheld, B. Spellberg, and J. Bartlett. (2009), "Bad bugs, no drugs: no ESCAPE! An update from the Infectious Diseases Society of America." Clin. Infect. Dis., 48(1), 12.
5. Bruno González-Zorn, Tirushet Teshager, María Casas, María C. Porrero, Miguel A. Moreno, Patrice Courvalin, and Lucas Domínguez. (2005), "*ArmA* and Aminoglycoside Resistance in *Escherichia coli*." Emerg. Infect. Dis., 11(6), 954–956.
6. Davis, MA., Baker KN., Orfe, LH., Shah, DH., Besser, TE., and Call, DR. (2010), "Discovery of a gene conferring multiple-aminoglycoside resistance in *Escherichia coli*." Antimicrobial Agents Chemotherapy, 54(6), 2666-2669.
7. Doi, Y., A. C., Ghilardi, J. Adams, D. de Oliveira Garcia, and D. L., Paterson. (2007), "High prevalence of metallo-lactamase and 16S rRNA methylase coproduction among imipenem-resistant *Pseudomonas aeruginosa* isolates in Brazil." Antimicrob. Agents Chemother, 51, 3388–3390.
8. Doi, Y., and Y. Arakawa. (2007), "16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides." Clin. Infect. Dis., 45, 88–94.
9. Doi, Y., D. de Oliveira Garcia, J. Adams, and D. L., Paterson. (2007) "Coproduction of novel 16S rRNA methylase *rmtD* and metallo-lactamase SPM-1 in a pan resistant *Pseudomonas aeruginosa* isolate from Brazil." Antimicrob. Agents Chemother, 51, 852–856.
10. Fang-you, Yu, Dan Yao, Jing-ye Pan, Chong Chen, Zhi-qiang Qin, Chris Parsons, Le- he Yang, Qiao-qiao Li, Xue-qing Zhang, Di Qu1 and Liang-xing Wang. (2010), "High prevalence of plasmid-mediated 16S rRNA methylase gene *rmtB* among *Escherichia coli* clinical isolates from a Chinese teaching hospital." BMC Infectious Diseases.
11. Fritsche, TR., Castanheira, M., Miller, GH., and Jones, RN. (2008), "Armstrong ES. Detection of methyl transferases conferring high-level resistance to aminoglycosides in enterobacteriaceae from Europe, North America, and Latin America." Antimicrob. Agents Chemother, 52(5), 1843-5.
12. Galimand, M., Sabtcheva, S., Courvalin, P., and Lambert, T. (2005), "Worldwide disseminated *armA* aminoglycoside resistance methylase gene is borne by composite transposon Tn1548." Antimicrob. Agents. Chemother, 49(7), 2949-2953.
13. Galimand, M. P., Courvalin, T., and Lambert. (2003), "Plasmid-mediated high-level resistance to aminoglycosides in Enterobacteriaceae due to 16S rRNA methylation," Antimicrob. Agents Chemother, 47, 2565–2571.
14. Kumarasamy Karthikeyan M. A., Thirunarayan, and Padma Krishnan. (2010), Coexistence of bla_{OXA-23} with bla_{NDM-1} and *armA* in clinical isolates of Acinetobacter baumannii, India." Journal of Antimicrobial Chemotherapy, 65, 10, 2253–2254.
15. Liou, G. F., S. Yoshizawa, P. Courvalin, and M. Galimand. (2006), "Aminoglycoside resistance by *armA*-mediated ribosomal 16S methylation in human bacterial pathogens." J. Mol. Biol., 359, 358– 364.
16. Marc Galimand, Thierry Lambert, and Patrice Courvalin. (2005), "Emergence and dissemination of a new mechanism of resistance to aminoglycosides in Gram-negative bacteria: 16S rRNA methylation." Eurosurveillance, 10(4), 27.
17. Pierre Bogaerts, Marc Galimand, Caroline Bauraing, Ariane Deplano, Raymond Vanhoof Ricardo De Mendonca, Hector Rodriguez-Villalobos, Marc Struelens, and Youri Glupczynski. (2007), "Emergence of *ArmA* and *RmtB* aminoglycoside resistance 16S rRNA methylases in Belgium." Journal of Antimicro. Chemotherapy, 59, 459–464.
18. Cantón, R., Novais, A., Valverde, A., Machado, E., Peixe, R., Baquero, F., and Coque, T.M., (2008), "Prevalence and spread of extended-spectrum beta-lactamase-producing Enterobacteriaceae in Europe." Clin Microbiol Infect., 14(1), 144-53.
19. Richard, J. Fair, Yitzhak Tor. (2006), "Antibiotics and Bacterial Resistance in the 21st Century." Clin Microbiol Rev., 19(3), 512–530.
20. Sabtcheva, S., Saga, T., Kantardjiev, T., Ivanova, M., Ishii, Y., Kaku, M. (2008), "Nosocomial spread of *armA*-mediated high-level aminoglycoside resistance in Enterobacteriaceae isolates producing CTX-M-3 beta-lactamase in a cancer hospital in Bulgaria." J. Chemother, 20 (5), 593-599.
21. Thomas, R., Fritsche, Mariana Castanheira, George, H. Miller, Ronald, N. Jones, and Eliana, S. Armstrong. (2008), "Detection of Methyltransferases Conferring High-Level Resistance to Aminoglycosides in Enterobacteriaceae from Europe, North America, and Latin America." Antimicrob. Agents Chemother, 52(5), 1843–1845.
22. Wachino, J., K. Yamane, K. Shibayama, H. Kurokawa, N. Shibata, S. Suzuki, Y. Doi, K. Kimura, Y. Ike, Y. Arakawa. (2006), "Novel plasmid-mediated 16S rRNA methylase, *RmtC*, found in a *Proteus mirabilis* isolate demonstrating extraordinary high-level resistance against various aminoglycosides." Antimicrob. Agents Chemother, 50, 178–184.
23. Yan, J. J., J. J. Wu, W. C. Ko, S. H. Tsai, C. L. Chuang, H. M. Wu, Y. J. Lu, and J. D. Li. (2004), "Plasmid-mediated 16S rRNA methylases conferring high-level aminoglycoside resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates from two Taiwanese hospitals." J. Antimicrob. Chemother, 54, 1007-1012.
24. Ying Zhang, Hua Zhou, Xiao-qiang Shen, Ping Shen, Yun-song Yu, and Lan-juan L. (2008), "Plasmid-borne *armA* methylase gene, together with blaCTX-M-15 and blaTEM-1, in a *Klebsiella oxytoca*isolate from China." Journal of Medical Microbiology, 57, 1273–1276.