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Prevalence of Class I Integrons among Multidrug-resistant Gram-negative Bacterial Isolates from Tertiary Care Hospital, South India

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Abstract

Integrons are the special group of mobile genetic elements which can acquire, shuffle and spread antimicrobial resistance genes. They mediate drug resistance among *Enterobacteriaceae* and Non-fermenters. The present study investigation was undertaken to envisage the presence of Class I integrase gene among multidrug resistant Gram-negative bacteria. In this prospective study, 60 bacterial isolates from various clinical specimens were subjected to routine identification and susceptibility testing by conventional methods. Later the isolates were subjected for detection of *intI1* gene by conventional PCR. The overall prevalence of *intI1* gene among the clinical isolates was 60% (36/60) in our study. Class I integrase gene distribution among multidrug resistant bacteria was 80% (24/30) in comparison to non-multidrug resistant bacteria 43.34% (13/30). Antibiotics that were linked to Class I Integrons and shown to be statistically significant (p = 0.05) included ampicillin, aztreonam, ciprofloxacin, cefazolin, cefepime and tobramycin all showed high levels of resistance. Prevalence of *intI1* gene was high among *Enterobacteriaceae* than Non-fermenters. There is a significant association between *intI1* gene and multidrug resistance among these pathogens. *Klebsiella* species are highly multidrug resistant in comparison to other isolates and all of them harboured *intI1* gene. Integrons can be a platform for the discovery of certain new metabolic pathways which can bring revolution in the field of antibiotic drug resistance. The information on the Integrons will aid us in prompt utilization of antimicrobial agents for the treatment.

Keywords: Class I Integrons, *Enterobacteriaceae*, Gram-negative Bacteria, Multidrug-resistance, and Non-fermenters

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INTRODUCTION

Bacteria are present universally, infections caused by them have great impact on the public health. Bacterial infections are less severe in comparison to viral and parasitic infections. However, resistance to antimicrobial agents have made bacterial infections a leading cause of illness in the health care setup.[1]

Antimicrobial resistance has emerged to be the global threat of 21st century. It is estimated to cause 70,000 deaths worldwide and predicted to kill 10 million individuals by 2050.[2] Gram-negative Bacteria (GNB) develop multiple drug resistance, hence assume greater importance among high-risk groups. *Enterobacteriaceae* and Non-fermenting Gram-negative bacilli (NFGNB) are the two groups of organisms that comprise common pathogens causing hospital associated infection, which cause life-threatening infections.[3]

Bacteria develop resistance to antibiotics by various processes, which can be either by intrinsic or acquired in nature. Horizontal gene transfer techniques play a vital role in acquired drug resistance.[4] There are several methods involved in horizontal gene transferring such as transformation, transduction and conjugation.[4] Mobile genetic elements mediate the exchange of genetic material in the horizontal gene transfer process.[5] They promote the acquisition of genes and result in the spread of antibiotic resistance.[5,6] Integrons are a special group of mobile genetic elements which can acquire, shuffle and disseminate antimicrobial resistance genes.[7] To date five classes of integrons have been identified, among them Class I is most predominantly identified in clinical isolates. The organisms harbouring these genes are resistant to various classes of antimicrobial agents.[8] The rise of multidrug-resistance (MDR) in Gram-negative bacteria has dramatically raised in association with Class I integrons and has become a particularly serious problem for healthcare professionals.[9,10]

The significance of antibiotic resistance associated integrons in clinical setup has primarily been reflected in their global epidemiological observation, monitoring, prevalence, and evolution. Although there are studies demonstrating the significance of integrase gene carriage and multidrug-resistance among the GNB, there is a lacuna in the epidemiology of antibiotic resistance associated integrons among the bacterial isolates. The following study was undertaken with the objectives of firstly to identify the multidrug resistant isolates of *Enterobacteriaceae* and non-fermenter, secondly to assess the prevalence of Class I integrase gene (*intI1*) in these isolates and lastly to assess the distribution of Class I integrons among MDR and non-multidrug resistant isolates.

MATERIALS AND METHODS

The present study was undertaken in the Microbiology department of tertiary care hospital. Sixty bacterial isolates were included in this study, of which 30 were members of the family Enterobacterales (previously Enterobacteriaceae) and 30 were non-fermenters. These were isolated from various clinical specimens collected from patients, both inpatients and outpatients, belonging to all age groups attending our hospital. These isolates were collected between May 2022 to August 2022. Isolates were identified by conventional method followed by susceptibility testing and detection of *IntI1* gene by molecular technique.

Identification and susceptibility

The isolates thus obtained were subjected for conventional identification which includes culture and staining characteristics (Gram stain) and standard identification techniques by biochemical reactions. Susceptibility testing by Kirby Bauer Disc diffusion test as per CLSI M100 Ed 32.

The antimicrobial susceptibility testing was carried out by disk diffusion method (Kirby Bauer) according to CLSI guidelines for all the isolates.[11] The following antibiotics which were procured from HiMedia Labs Mumbai, India were used: amikacin (30 µg), aztreonam (30 µg), cefazolin (30 µg), cefepime (30 µg), cefuroxime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), tobramycin (10 µg), gentamicin (10 µg), ofloxacin (5 µg), imipenem (10 µg), meropenem (10 µg) and piperacillin/tazobactam (100 µg/10 µg). Standard culture suspension of the isolates were prepared and turbidity was matched with 0.5 Mc Farland standard. Culture inoculum

was spread on the surface of Mueller Hinton agar (MHA) using the sterile swab. After allowing the plates to dry for 5 minutes antibiotic discs were placed and incubated at 37°C. The sensitivity zone was measured comparing with standard zone size according to CLSI guidelines (2022). The quality control was set up using *P. aeruginosa* (ATCC 27853) and *E. coli* (ATCC 25922) strains. Microbroth dilution method was performed for determining the MIC for colistin (Hi-Media Labs Mumbai, India).

Molecular Method

DNA Extraction[12]

The extraction of DNA was done by boiling lysis method. Fresh culture of the organism was centrifuged for 10 minutes at 10,000 rpm. Later the supernatant was separated and 300 µl of nuclease free water was added. The culture was boiled for 10 minutes at 100°C. After boiling, the culture was immediately cooled to -20°C and was maintained at same temperature for 6 hours. The culture was then centrifuged for 10 min at 10,000 rpm. Later 2 µl supernatant was separated and stored at -20°C and was used as DNA template.

PCR Amplification

The PCR reaction mixture was 25 µl. It contained 200 µM of dNTPs, 1X PCR buffer Tris HCl [10 mM]; KCl [50 mM]; $MgCl_2$ [1.5 mM] and 0.1 Units Taq DNA polymerase, primers (10 pmol each) and 5 ng of template DNA. Amplification was done using Master cycler. The primers used were according to Goldstein *et al*., the primers sequence mentioned in the Table 1.[13]

Analysis of PCR products

PCR products was analysed by electrophoresis method with 2% agarose gel in 1X Tris acetic acid EDTA (TAE) buffer. The products of

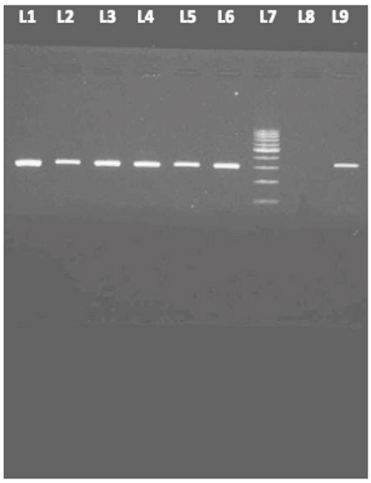

Figure. PCR amplification gel documentation picture

Table 2. Total number of clinical isolates positive for Class I Integron

Clinical isolates (N)	Class I Integron N (%)	
	Present	Absent
Bacterial Isolates (60)	36 (60)	24 (40)
Enterobacteriaceae (30)	24 (80)	6 (20)
Non-fermenters (30)	12 (40)	18 (60)
Multidrug-resistant isolates (30)	24 (80)	7 (23.34)
Non-multidrug resistant isolates (30)	13 (43.34)	17 (56.67)

Table 1. List of primers used for the detection of Class I integron gene

Target genes	Primer sequences 5' to 3 '		Expected Amplicon Size	
Class I integron gene (*intI1*)	F: CCTCCCGCACGATGAT R: TCCACGCATCGTCAGGC		280 bp	
		PCR Conditions		
1 Cycle		30 cycles		1 Cycle
Initial denaturation	Denaturation	Annealing	Extension	Final extension
94°C for 5 minutes	94°C for 45 seconds	52°C for 45 seconds Hold at 4°C	72°C for 1 minute	72°C for 7 minute

Table 3. Shows distribution of Class I integron among various clinical isolates

No.	Clinical isolate	No of isolates (N)	Class I Integron (N)	Percentage (%)
1.	*Acinetobacter* species	14	4	28.5
2.	*Citrobacter freundii*	01	0	0.0
3.	*E. coli*	16	11	68.8
4.	*Klebsiella* species	13	13	100.0
5.	*Pseudomonas* species	16	8	50.0

Table 4. Comparison of Antibiotic Resistance pattern among the Class I Integron positive and negative isolates

Antibiotics	Total (N = 60)			Class I Integron positive (N = 36)			Class I Integron negative (N = 24)			p value
	R	I	S	R	I	S	R	I	S	
AK	30	2	26	23	1	12	7	1	14	0.06
AM	44	0	0	28	0	0	16	0	0	-
CZ	44	0	0	28	0	0	16	0	0	-
CPM	46	1	10	31	0	4	15	1	6	0.12
CTX	38	2	5	26	1	2	12	1	3	0.41
CXM	42	0	2	27	0	1	15	0	1	0.68
CIP	43	0	16	29	0	6	14	0	10	0.03
CL	0	30	0	0	23	0	0	7	0	-
FOS	1	0	12	0	0	9	1	0	3	0.11
GN	34	0	26	26	0	10	8	0	16	0.003
IMI	31	0	29	23	0	13	8	0	16	0.02
MEM	30	1	29	23	0	13	7	1	16	0.02
NIT	4	0	11	2	0	8	2	0	3	0.40
PTZ	33	0	25	24	0	12	9	0	13	0.05
COT	30	0	12	24	0	2	6	0	10	0.000
TB	25	1	19	20	0	6	5	1	13	0.003
AZT	6	1	9	5	0	3	1	1	6	0.09
CAZ	15	0	0	8	0	0	7	0	0	-
OF	8	0	7	7	0	1	1	0	6	0.005

Abbreviations: R, resistant; I, intermediate resistant; S, Susceptible; AK, Amikacin; AM, Ampicillin; CZ, Cefazolin; CPM, Cefepime; CTX, Cefotaxime; CXM, Cefuroxime; CIP, Ciprofloxacin; CL, Colistin; FOS, Fosfomycin; GN, Gentamicin; IMI, Imipenem; MEM, Meropenem; NIT, Nitrofurantoin; PTZ, Piperacillin-Tazobactam; COT, Cotrimoxazole; TB, Tobramycin; AZT, Aztreonam; CAZ, Ceftazidime; OF, Ofloxacin

PCR reaction was visualised by gel electrophoresis after the gel was stained with ethidium bromide (EtBr) (Figure).

Statistical analysis

Data were entered in Microsoft Excel and analyzed using SPSS (version 27.0; SPSS Inc., Chicago, IL, USA). The categorical variables were expressed in proportion and numbers. The statistical significance was tested using Chi^2 test for the categorical variables. The p-value less than 0.05 was considered to be significant statistically.

RESULTS

In the following study, the extracted Deoxyribonucleic acid (DNA) from 60 bacterial isolates were subjected to detection of *intI1* gene by polymerase chain reaction (PCR) using the specific primers (Table 1). Of total 60 bacterial isolates,

Table 5. The comparison of antibiotic resistance among the MDR and Non-MDR Isolates

Antibiotics	MDR Isolates (N = 30)			Non-MDR Isolates (N = 30)			p value
	R	I	S	R	I	S	
AK	28	0	1	2	1	26	0.000
AM	23	0	0	21	0	0	-
CZ	23	0	0	21	0	0	-
CPM	30	0	0	16	1	10	0.001
CTX	23	0	0	16	2	5	0.01
CXM	23	0	0	19	0	2	0.13
CIP	30	0	0	12	0	16	0.000
CL	0	30	0	0	0	-	-
FOS	0	0	1	1	0	11	0.76
GN	28	0	2	6	0	24	0.000
IMI	30	0	0	1	0	29	0.000
MEM	30	0	0	0	1	29	0.000
NIT	0	0	1	4	0	10	0.53
PTZ	30	0	0	3	0	25	0.000
COT	21	0	1	9	0	10	0.000
TB	24	0	1	1	1	18	0.000
AZT	6	0	1	0	1	8	0.002
CAZ	7	0	0	8	0	0	-
OF	7	0	0	1	0	7	0.001

Abbreviations: R, resistant; I, intermediate resistant; S, Susceptible; AK, Amikacin; AM, Ampicillin; CZ, Cefazolin; CPM, Cefepime; CTX, Cefotaxime; CXM, Cefuroxime; CIP, Ciprofloxacin; CL, Colistin; FOS, Fosfomycin; GN, Gentamicin; IMI, Imipenem; MEM, Meropenem; NF, Nitrofurantoin; PTZ, Piperacillin-Tazobactam; COT, Cotrimoxazole; TB, Tobramycin; AZT, Aztreonam; CAZ, Ceftazidime; OF, Ofloxacin

36 were identified as being positive for *intI1* gene. All the products obtained showed the same melting point as that of positive control in each assay run. The prevalence of *intI1* gene among the isolates was 60% in our study. These 60 isolates were further evaluated on the basis of antimicrobial sensitivity. The presence of *intI1* gene among *Enterobacteriaceae* and Non-fermenters were 80% (24/30) and 40% (12/30) respectively. The presence of *intI1* gene among MDR pathogens was 80% (24/30) in comparison to non-multidrug resistant pathogens 43.34% (13/30) (Table 2).

Highest distribution of *intI1* gene was observed among *Klebsiella* species 100% (13/13), followed by *Escherichia coli* 68.75% and Non-fermenters. Table 3 Shows distribution of *intI1* gene among various clinical isolates. The distribution of *intI1* gene among exudate samples were 61% (n = 16/26), urine samples were 62.5% (n = 15/24) and respiratory isolates were 50% (n = 5/10).

The cases were distributed between the two to 82 years of age group. The highest incidence was seen among the age group above 61 years, comprising 28.3% of cases, followed by others. It was observed that among the 60 cases, there were 38 males and 22 females. The incidence was high among males with male to female ratio 1.7:1.

The sensitivity pattern was observed for 19 antibiotics. The resistance was high among the *intI1* gene positive bacteria. It was statistically significant with ciprofloxacin, gentamicin, imipenem, meropenem, cotrimoxazole, tobramycin, and ofloxacin ($p < 0.05$) (Table 4).

The antibiotic resistance pattern of the MDR and Non-MDR isolates were compared for all 19 antibiotics. The resistance was statistically significant with amikacin, cefepime, cefotaxime, ciprofloxacin, gentamicin, imipenem, meropenem,

piperacillin-tazobactam, cotrimoxazole, tobramycin, aztreonam and ofloxacin ($p < 0.05$) (Table 5).

DISCUSSION

Integrons are the mobile genetic elements which are usually located on transposons and have the ability to incorporate and disseminate antimicrobial resistance gene cassettes amongst the microorganisms.[14] The general structure of integron comprises of a conserved sequence with variable region which contains inserted genes cassettes. The Class I integron comprises of three important components firstly an *intI* gene encodes an enzyme integrase which mediates insertion, excision and shuffling of gene cassettes; secondly an *attI* recombination site for gene cassette insertion and thirdly a Pc promoter region responsible for gene cassette expression.[15] They play a vital role in disseminating MDR genes among Gram-negative bacteria.[16] In developing countries including India, Gram-negative organisms are the predominant pathogens causing infectious diseases and are responsible for high mortality and morbidity among them.[17] There are rising concerns about the integrons associated MDR clinical isolates.

The overall distribution of *intI1* gene among *Enterobacteriaceae* and Non-fermenters in our study was 60% and the prevalence of *intI1* gene among MDR was 80% (24/30) in comparison to Non-MDR strains 43.34% (13/30). There is an high prevalence of *intI1* gene in our study which is supported by other studies. An Indian study by Kaushik *et al*. in the year 2012 showed Class I and Class II integron to be associated with 66.10% (39/59) of Gram-negative bacteria and 32 among them were MDR and *E. coli* was the predominant isolate.[18] Lavanya *et al*. in their study on *Pseudomonas* observed that all the carbapenem resistant isolates were harbouring *intI1* gene.[19]

Kargar *et al*. in his work on *E. coli* observed 42% of isolates to be MDR and 78.26% were associated with Class I integrons.[20] Another study by Mohadeseh zarei-yazdeli on various clinical isolates showed 82.6% of isolates were associated with *intI1* gene, among them 59.7% were MDR and 22.9% were sensitive and intermediate strains.[21] Kor *et al*. investigated 147 multidrug-resistant *Enterobacteriaceae* and *Pseudomonas* isolates from patients admitted to Malaysian hospitals and revealed prevalence of *intI1* gene to be 45.6%.[22] Maurine *et al*. in his study also showed high connection between MDR and the integron gene in *Enterobacteriaceae*.[10] Nikibakhsh *et al*. in their work, all the *Acinetobacter* strains were MDR and 58.8% of them were associated with *intI1* gene.[23] In our study gentamicin, imipenem, meropenem, ciprofloxacin, cotrimoxazole, ofloxacin and tobramycin are the drugs predominantly associated with *intI1* gene and are statistically significant. MDR isolates were resistant to the antimicrobials such as cotrimoxazole, gentamicin, imipenem, meropenem, tobramycin, amikacin, cefepime, ciprofloxacin, ampicillin, piperacillin/tazobactam, aztreonam, ofloxacin and cefuroxime. Maurine *et al*. in a study also showed MDR to be associated with resistance to the antimicrobials such as Cotrimoxazole, gentamicin, tobramycin, amikacin, ciprofloxacin, ampicillin, piperacillin/tazobactam, cefuroxime.[10] Mohadeseh *et al*. observed highest resistance for piperacillin (90.03%), ciprofloxacin (87.54%) and cotrimoxazole (81.13%).[21] All these studies support the fact that the resistance rate is high among the isolates which were associated with *intI1* gene. In our study, the prevalence of Class I integrons and MDR were high among *Enterobacteriaceae* in comparison to Non-fermenters.

The predominant isolates in our study were *Klebsiella* species, *E. coli* and *Pseudomonas*: among them, the distribution of *intI1* gene was 100%, 68.8% and 50% respectively. Farzaneh *et al*. also observed all the *Klebsiella* species (100%) harbour *intI1* gene in his research.[24] But in a study by Kuihai *et al*. in China, *Acinetobacter* spp. was the predominant isolate to harbour *intI1* gene which was followed by *K. pneumoniae* (72.5%) and *E. coli* are other isolates.[25]

We had clinical isolates from all the age group ranging from two years to 82 years. However, we did not observe age playing any role in the prevalence of MDR of the isolates. Male predominance was observed in our study. With respect to *intI1* gene or MDR we did not observe any preference for a particular gender. Wound

swab/pus samples as predominant clinical samples followed by urine and respiratory. The ability of the integrons to spread the drug resistance, pathogenicity and virulence has a great impact on the healthcare system. Integrons can be a platform for the discovery of certain new metabolic pathways which can bring revolution in the field of antibiotic drug resistance. The information on the integrons will aid us in the prompt utilization of antimicrobial agents for the treatment.

CONCLUSION

Antimicrobial resistance is of major health concern. Integrons are unique mobile genetic elements which mediate resistance among bacteria, especially gram-negative bacteria**.** We analysed the distribution of *intI1* gene among Gram-negative bacteria in the various clinical samples and their association with multidrug resistance. A significant correlation between the *intI1* gene and multidrug-resistance was observed. Detection of *intI1* gene was high among *Enterobacteriaceae* in comparison to non-fermenters. The majority of *Klebsiella* species were multidrug-resistant, and all of them carried the *intI1* gene. The Knowledge on the integron mediated antimicrobial resistance will aid us in prompt utilization of antimicrobial agents for the treatment.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

KP and PS designed the study. KP, PR and LM performed data collection. KP, PR, PS and LM performed experiments. KP, PR, PS and LM performed result analysis. KP, PR, PS and LM wrote, reviewed, edited and approved the manuscript for publication.

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None.

DATA AVAILABILITY

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

ETHICS STATEMENT

This study was approved by the Institutional Ethical Committee, with reference number is IHEC-II/0012/21.

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