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Integron Carriage among Carbapenem Resistant Enterobacteriaceae Isolated from a Tertiary Hospital in North India

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Authors' contributions

This work was carried out in collaboration between both authors. Authors JF and TB contributed to conception of study and managed the literature searches. Authors JF and TB wrote the protocol, managed data collection, analyzed and drafted the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Background: Carbapenem resistant enterobacteriaceae (CRE) constitute major public health threat. They account for significant morbidity and mortality and also present patients with difficult to treat infection. This study examines the integron carriage rate and gene content among CRE. **Methods:** Samples were collected from patients and enterobacteriaceae identified in accordance with standard practice. Antimicrobial susceptibility testing was performed in line with CLSI guidelines, and referenced criteria were used to screen for multidrug resistant enterobacteriaceae (MDRE) and carbapenem non susceptible isolates (CNSI). Further susceptibility test of the CNSI to carbapenems and third generation cephalosporin by agar dilution method as per CLSI guide lines was performed to screen for CRE, and *Int*1 and *Int*2 specific primers were used to amplify class 1 and 2 integron respectively. *Int*CS primer was used to amplify integrons variable region and a blast

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search on the amplicons' sequenced data was performed on NCBI site to identify the gene content.
Results: Of the 512 MDRE, 62.1% were CNSI and 56.0% of the CNSI were CRE. While *K. pneumoniae* and *E. coli* were the predominant isolates, 87.1% and 9.0% of the CRE isolates were positive for integron class 1 and 2 respectively. The amplified variable region did not reveal the presence of carbapenemase coding gene.
Conclusion: Although the variable region did not show presence of carbapenemase coding gene, high class 1 integron carriage rate underscore high potentials for dissemination of resistance determinants among isolates, thus the need to step up infection control measures in order to reduce spread of these pathogens.

Keywords: Integron; CRE; variable region; amplicon.

1. INTRODUCTION

Enterobacteriaceae is an important group of pathogen commonly found in humans, animals and the environment, and significantly responsible for both hospital and community acquired infections. The propensity of enterobacteriaceae to acquiring different mechanisms of resistance, depleting effective therapy and the lack of new antimicrobial agents, are major risk factor associated with this group of bacteria.

In recent time, an unprecedented increase in the evolution and spread of enterobacteriaceae with resistance to multiple classes of antibiotics has created a group of multidrug resistant enterobacteriaceae (MDRE), whose dissemination have been observed across the globe [1]. Equally troubling is the emergence of carbapenemases that confer on enterobacteriaceae the mechanism of resistance to carbapenem; a class of antibiotic that constitute last line effective therapy against MDRE. Carbapenem resistant enterobacteriaceae (CRE); defined as enterobacteriaceae resistant to third generation cephalosporin and non susceptible to any of the carbapenem, owe its resistance to carbapenemases; an enzyme known to degrade all known β-lactam antibiotics including carbapenems. In other words, the CRE are carbapenemase producers [2]. However, since resistance to carbapenem could also be mediated by other resistance mechanisms, enterobacteriaceae that are non susceptible to any of the carbapenems are referred to as carbapenem non susceptible isolates (CNSI) [3].

Mobile genetic elements including plasmids, transposons and integrons play an important role in acquisition and dissemination of resistance determinants in enterobacteriaceae. Additionally, studies have shown that multidrug-resistance in these bacteria is significantly associated with presence of integron and gene cassettes [4]. Integrons are sequences of conserved DNA that contain an integrase gene (*IntI*) encoding the *IntI* integrase and cause transmission and incorporation of gene cassettes via site-specific recombination mechanisms [5]. Eight classes of integron have been described and each class is distinguished by differences in the sequence of integrase genes, however, Integron class 1, 2 and 3 contain antibiotic resistance gene cassettes and the focus of numerous and wide spread studies. Class 1 integrons are the most prevalent among clinical isolates and significantly associated with resistance to aminoglycoside, sulfonamide, quinolones, quartenary ammonium and β-lactam agents [6]. Integron surveillance in bacteria has become a tool for clinical infection control and study of antibiotic resistance mechanisms. A study conducted by Xu et al. [7] reported integron gene cassette prevalence of 100% in *E. coli*, 100% in *P. aeruginosa*, 11% in *K. pneumoniae*, 18% in *A. baumannii* and 33.3% in *E. cloacae* from clinical isolates. Similarly, high association of integron and carbapenemase production, and its contribution to the prevalence of transferable extended spectrum cephalosporin resistance was observed in clinical isolates of enterobacteriaceae [8]. This study was performed to determine the prevalence of classes 1 and 2 integrons and cassettes within the integron variable region in clinical isolates of CRE from a tertiary care hospital.

2. METHODOLOGY

2.1 Bacterial Isolates

Non duplicate isolates of enterobacteriaceae were recovered from various clinical specimens such as urine, blood, sputum, endotracheal tube, pus aspirates, intravascular catheter tip, ascitic fluid and wound swab received in microbiology laboratory for routine analysis, from patients

attending the various outpatient and inpatient departments of the University hospital. Isolation was done by plating on cysteine lactose electrolyte deficient (CLED) agar or blood and MacConkey agar as per nature of the specimen. Standard bacteriological methods were followed for identification of enterobacteriaceae isolates [9].

2.2 Identification of Carbapenem Resistant Enterobacteriaceae (CRE)

From previously characterized 152 (n= 761) clinical isolates of MDRE [10], carbapenem non susceptible isolates (CNSI) was determined [3] and carbapenemase resistant isolates were identified from the CNSI [2,3]. Briefly, susceptibility to carbapenems and third generation cephalosporins; ceftazidime, ceftriazone and cefotaxime by minimum inhibitory concentration (MIC) breakpoints was determined by CLSI referenced agar dilution method on the CNSI, and CRE was defined as isolates not susceptible to any of the carbapenems and resistant to all the third generation cephalosporins [2,3]. *Escherichia coli* 25922 was used as control.

2.3 DNA Isolation

DNA was extracted by phenol-chloroform (classical) methods [11]. Briefly a suspension of five colonies of cell in 400 µl of sterile distilled water in eppendorf tube was pelleted by centrifugation at 10,000rpm for 10 minutes. The pellet was dissolved in 564 µl Tris EDTA (TE buffer) and vortexed followed by addition of 10% SDS and 6µl proteinase K of concentration 10 mg/ml. After incubation for 2 hrs, 600µl of phenol**:**chloroform:isoamyl alcohol (P:C:I - 25:24:1) was added and mixed by gentle inversion and centrifuged at 10,000 rpm for 10 minutes. The aqueous phase was decanted into a new tube and 1/10th volume of chilled sodium acetate and equal volume of isopropanol was added and the mixture incubated overnight at 4^0C. DNA was centrifuged at 10,000 rpm for 10 minutes and washed in 70% ethanol. The supernatant was removed and the resulting DNA pellet dried at room temperature, and then resuspended in TE buffer.

2.4 Identification of Integron by Integrase Gene PCR

CRE isolates were screened for presence of integrons by PCR for amplification of integrase class 1 and 2 gene by targeting 160 bp (for integron class 1) and 288 bp (for integron class 2) fragments [12]. DNA was extracted by phenol-chloroform method as earlier described. A volume of 25 µl PCR reaction mixture containing 2 µl of DNA template, 1µl of 10 pmol of each primer (Table 1), 2 µl 10 mM dNTPs, 1U *Taq* DNA, polymerase and 3 µl of 10x *Taq* buffer (Genei, Bangalore, India) was prepared. Amplification reaction mixture containing DNA template was performed using thermal cycler (BioRad, USA) with the following reaction conditions; initial denaturation at 94℃ for 5min; 39 cycles of 94℃ for 1 min, 55℃ for 1min and 72℃ for 1 min and a final extension at 72℃ for 7 min. DNA fragment of the integrase gene amplified by PCR were identified by agarose gel electrophoresis. Briefly 5 µl of amplified product from PCR was mixed with 2 µl DNA loading dye and electrophoresed on 2% agarose gel with Tris Acetate EDTA (TAE) buffer, to which ethidium bromide (Genei, Bangalore, India) was added, at constant 70 volts for 90 min [8]. Molecular marker of 100 bp DNA ladder, (Genei, Bangalore, India) was electrophoresed concurrently with test samples. The gel was visualized under ultraviolet illumination in a gel documentation system. Presence of band of molecular weight, 160 bp and 288 bp indicates occurrence of class 1 and class 2 integron genes respectively.

Table 1. Oligonucleotides of integron gene primers

Primer	Primer oligonucleotide sequence (5^1–3^1)	Size	Reference	Source
Int 1	F: 5^l-CAGTGGACATAA GCCTGTTC-3^l R: 5^l-CCCGAGGCATAGACTGTA-3^l	160 kb	Koeleman et al. [12]	Hysel India
Int 2	F: 5^l-TTGCGAGTA TCCATA ACCTG-3^l R: 5^l-TTACCTGCACTGGATTAAGC-3^l	288 kb	Koeleman et al. [12]	Hysel India
Int CS	F: 5^1-GGGATCCAAGCAGCA AG-3^1 R: 5^1-AAGCAGACTTGACCT GA-3^1	Variable	Mokracka et al. [13]	Hysel India

2.5 Amplification and Sequencing of Integron Class 1 Variable Region

Twenty Isolates positive for integrase class 1 were randomly selected and subjected to PCR for amplification of variable region with integron conserved sequence primers (*Int CS)* (Table 1) [10,11]. DNA template in 25 µl reaction mix was amplified by PCR in thermal cycler (BioRad, USA) with the following reaction conditions; initial denaturation at 94℃ for 5min; 39 cycles of 94℃ for 1 min, 55℃ for 1min and 72℃ for 1 min and a final extension at 72℃ for 7 min. The amplified DNA fragments of integrase conserved region containing the variable genes were visualized in gel documentation unit as described earlier. The amplicons were sent for sequencing and blast search for identity of the sequenced data was performed on NCBI site.

3. RESULTS

Escherichia coli and *K. pneumoniae* constitute the predominantly isolates of enterobacteriaceae recovered in this study. Of the 512 MDRE screened, 318(62.1%) were CNSI and 178(56.0%) of the CNSI were CRE (Table 2)

Of 178 CRE isolates screened for integron class 1 and 2, 87.1% and 9.0% carried integron class 1 and class 2 respectively and 7.9%, predominantly *K. pneumoniae* showed the presence of both class 1 and class 2 (Fig. 1, Table 3). Amplification of integron variable region by the integron conserved sequence PCR revealed variable length ranging from 800bp to 3000 bp in 12 isolates that produced visible bands. Amplicons of eight isolates whose band were most visible on gel were selected and sent for sequencing, of which five were successfully sequenced and three had mix sequence data. Performing blast search on NCBI, for the five successfully sequenced amplicons, revealed integron with different gene cassettes array which include; *dfrA5-aacA4-nit1-nit2-catB*, *aadB-blaOXA-10-aadA1*, *aadA1, aar2-aacA4-afrA1-orfC*, and *dfrA30*. However, no cassette for gene encoding carbapenemases was observed.

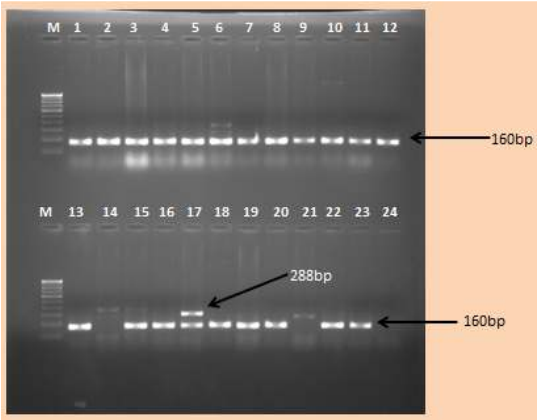

Fig. 1. PCR amplification of class 1 and class 2 integrase gene

M-100 bp DNA ladder, Class 1 integron-160 bp, Class 2 integron-288 bp

Table 2. Proportion of MDRE that were CNSI and CRE

Bacterial isolates	n	Number and proportion (%) of enterobacteriaceae isolates that were*		
		MDRE	CNSI†	CRE††
E. coli	292	218(74.7)	142(65.1)	64(45.1)
K. pneumoniae	236	173(73.3)	113(65.3)	75(66.3)
K. oxytoca	39	25(64.1)	12(48.0)	8(66.7)
C. freundii	55	29(52.7)	7(24.1)	6(85.7)
C. koseri	51	36(70.6)	17(47.2)	10(58.8)
E. aerogenes	11	5(45.5)	5(100)	3(60.0)
E. cloacae	7	2(28.5)	2(100)	2(100)
M. morganii	16	2(12.5)	1(50.0)	0(0.0)
P. mirabilis	34	16(47.1)	13(81.3)	7(53.8)
P. vulgaris	20	6(30)	6(100)	3(50.0)
Total	761	512(67.3)	318(62.1)	178(56.0)

**MDRE-Multidrug resistant Enterobacteriaceae, CNSI-Carbapenem resistant Enterobacteriaceae, CRE- Carbapenem resistant Enterobacteriaceae*

† Proportion calculated with MDRE as n

†† Proportion calculated with CNSI as n

Table 3. Showing integron carriage rate and variable regions amplified

Bacteria isolates	Number and proportion of isolates that are				
	CRE	***Int* 1**	***Int* 2**	***Int* 1&2**	***Int* CS***
E. coli	64	55(85.9)	5(7.8)	4(6.3)	1 (900 bp)
K. pneumoniae	75	68(90.6)	9(12.0)	8(10.7)	1(2 kb), 1(800 bp)
K. oxytoca	8	8(100)	2(25.0)	2(25.0)	-
C. freundii	6	5(83.3)	0(00.0)	0(00.0)	1(900 bp)
C. koseri	10	10(100)	0(00.0)	0(00.0)	-
E. aerogenes	3	3(100)	0(00.0)	0(00.0)	-
E. cloacae	2	2(100)	0(00.0)	0(00.0)	1(1 kb)
M. morganii	0	0(00.0)	0(00.0)	0(00.0)	-
P. mirabilis	7	4(57.1)	0(00.0)	0(00.0)	-
P. vulgaris	3	1(33.3)	0(00.0)	0(00.0)	-
Total	178	156(87.6)	16(9.0)	14(7.9)	

**Sizes of successfully sequenced PCR amplicons of variable region*

4. DISCUSSION

Integron plays a vital role in the dissemination of antimicrobial resistance determinants through horizontal transmission and their contribution to increased prevalence of transferable resistance has been documented [14]. High frequency of integron class 1 carriage rate (87.6%) among CRE strains observed in this study underscores its contribution to development of resistance. Previous studies reported the association of integron and resistance in enterobacteriaceae and that strains with integron class 1 are 100% resistant to more than one antibiotic [15].

In this study, integron conserved sequence PCR was performed and the amplicons generated were sent for sequencing. A blast search on NCBI site was performed on the sequence data, and results obtained showed variation and similarity in cassettes on the sequenced fragments (*dfrA5-aacA4-nit1-nit2-catB*, *aadB-blaOXA-10-aadA1*, *aadA1, aar2-aacA4-dfrA1-orfC*, and *dfrA30*) reflecting the collection, excision and rearrangement of gene within the integron as have been reported [16]. The most abundant cassettes observed were those conferring resistance to aminoglycosides (*aadA1, aacA4, aadB*) and trimethoprim-sulfamethoxazole (*dfrA*). Previous studies have indicated dihydrofolate reductase and aminoglycoside resistance gene as probably the most abundant gene cassettes found on integron variable region of enterobacteriaceae [16,17,18].

Although we could not observe presence of any carbapenemase resistance cassette or β-lactamase gene except bla_{OXA-10} in *E. cloacae*, carbapenemase resistance genes maybe be located outside the integron [16]. A similar study on variable region of multidrug resistant *E. coli* did not identify any β-lactam antibiotic associated resistance cassette [19]. The result of Canal et al corroborated the finding obtained in this study and thus implies that resistance pattern must not always be associated to only integrons carrying antibiotic resistance cassettes, since other mobile genetic elements such as plasmid and transposons may carry resistance genes. Studies have indicated that plasmids carrying ESβL genes are frequently conjugative and could carry determinants of resistance to non β-lactams and carbapenemases [16,19]. However this does not preclude the possibility that integron from other isolates in this study could harbor more cassettes combination containing carbapenemase genes; in view of our limitation of amplifying and sequencing variable region for only very few isolates, or strains may have carried larger integron which were not amplified by PCR conditions used [19].

Nevertheless, a study has reported that while ESβL gene was not found on the integron conserved sequence, ESβL production was significantly higher in isolates positive for integron class 1 than integron negative isolates [16]. Furthermore, it has been established that class 1 integron plays a major role in the dissemination of drug resistance in clinical bacterial isolates and the use of broad spectrum antimicrobial agents has a profound effect on promoting this process [16,17,18,20].

5. CONCLUSION

Although, amplification and sequencing of integron variable region did not reveal presence of any carbapenemase coding gene, high class 1 integron carriage rate observed in this study is an

indication of high potential for resistance dissemination among the isolates. While high class 1 integron carriage rates have been demonstrated in this, and other studies, there is need to further study carriage of carbapenemase resistance genes in the variable region of class 1 integron. However, caution particularly in the area of antibiotic prescription policy and hospital hygiene are needed in order to control the spread of these pathogens within the hospital setting.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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