

Original Research Article

Integron carriage among carbapenem resistant enterobacteriaceae isolated from a tertiary hospital in North India

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. **Method:** Samples were collected from patients and enterobacteriaceae identified in accordance with standard practice. Disk susceptibility test was performed in line with CLSI guidelines, and referenced criteria were used to screen for MDRE and 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 the CRE, and *Int1* and *Int2* specific primers were used to amplify class 1 and 2 integron respectively. *IntCS* primer was used to amplify integrons variable region and a blast search on the amplicon sequenced data was performed on NCBI site to identify the gene content. **Result:** 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. However, 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 the resistance determinants among isolates, thus the need to implement precautionary measures in order to reduce spread of these pathogen.

Keywords: *Integron, CRE, variable region, amplicon*

1. Introduction

Enterobacteriaceae is an important group of pathogen commonly found in humans, animals and the environment, and responsible for both hospital and community acquired infections. The propensity with which this group of organisms acquires different mechanisms of resistance, depleting effective therapy and the lack of new antimicrobial agents, are major risk factor associated this group of bacteria. 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 [1].

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 [2]. 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, quaternary ammonium and β -lactam agents [3]. 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 [4] 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 [5]. This study was performed to determine the prevalence of classes 1 and 2 integrons and cassettes within the integron variable region of clinical isolates of Enterobacteriaceae 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, ascetic 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 [6].

2.2 Identification of carbapenem resistant enterobacteriaceae (CRE)

From previously characterized 152 (n= 761) clinical isolates of multidrug resistant enterobacteriaceae (MDRE) [7], carbapenem non susceptible isolates (CNSI) was determined [8] and carbapenemase resistant isolates was identified from the CNSI [8,9]. Briefly, susceptibility to carbapenems and cephalosporin; ceftazidime, ceftriazone and cefotaxime by minimum inhibitory concentration (MIC) breakpoints was determined by CLSI referenced agar dilution method on the CNSI and CRE, defined as isolates not susceptible to any of the carbapenems and resistant to all the third generation cephalosporins [8,9]. *Escherichia coli* 25922 was used as control.

2.3 DNA isolation

DNA was extracted by phenol chloroform (classical) methods. 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 10mg/ml. After incubation for 2hrs, 600µl of phenol: chloroform:isoamyl alcohol (P:C:I - 1:25:24) was added and mixed by gentle inversion and centrifuged at 10,000rpm 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°C. 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 [10]. DNA was extracted by boiling centrifugation method as earlier described. A volume of 25µl PCR reaction mixture containing 5µl of DNA template, 1µl of 10 pmol of each primer (Table 1), 2 µl 10mM dNTPs, 1 U *Taq* DNA polymerase and 3µl 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°C for 5min; 39 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1min and a final extension at 72°C for 7min. DNA fragment of the integrase gene amplified by PCR were identified by agarose gel electrophoresis. Briefly 5µl of amplified product from PCR was mixed with 2µl DNA loading dye and electrophoresed on 2% agarose gel with Tris Acetate EDTA (TAE) buffer and ethidium bromide (Genei, Bangalore, India), at constant 70 volts for 90 min.

Molecular marker of 100 bp DNA ladder, (Genei, Bangalore, India) was run concurrently. The gel was visualized under ultraviolet illumination in a gel documentation system. Presence of band of molecular weight, 160bp and 288bp indicates occurrence of class 1 and class 2 integron genes respectively.

Table 1: Oligonucleotides of integron gene primers

Primer	Primer oligonucleotide sequence (5'–3')	Size	Reference	Source
<i>Int 1</i>	F: 5'-CAG TGG ACA TAA GCC TGT TC-3' R: 5'-CCC GAG GCA TAG ACT GTA-3'	160kb	Koeleman <i>et al.</i> , 2001[10]	Hysel India
<i>Int 2</i>	F: 5'-TTG CGA GTA TCC ATA ACC TG-3' R: 5'-TTA CCT GCA CTG GAT TAA GC-3'	288kb	Koeleman <i>et al.</i> , 2001[10]	Hysel India
<i>Int CS</i>	F: 5'-GGG ATC CAA GCA GCA AG-3' R: 5'-AAG CAG ACT TGA CCT GA-3'		Mokracka <i>et al.</i> , 2011[11]	Hysel India

2.5 Amplification and sequencing of integron conserved sequence.

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 25µl reaction mix was amplified by PCR in thermal cycler (BioRad, USA) with the following reaction conditions; initial denaturation at 94° C for 5min; 39 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1min and a final extension at 72°C for 7min. The amplified DNA fragment of integrase conserved region containing the variable gene was visualized gel documentation unit as described in the earlier. The amplicons were sent for sequencing and blast search for identity of sequencing results was performed on NCBI site.

3. RESULT

Of the 512 MDRE screened from a total of 761 isolates of enterobacteriaceae, comprising predominantly of *E. coli* and *K. pneumoniae*, 318(62.1%) of the MDRE were CNSI and 178(56.0%) of the CNSI were CRE (Table 2)

Table 2: Proportion of MDRE that were CNSI and CRE

Bacterial isolates	n	Number and proportion (%) of enterobacteriaceae isolates that were*		
		MDRE	CNSI [†]	CRE ^{††}
<i>E. coli</i>	292	218(74.7)	142(65.1)	64(45.1)
<i>K. pneumoniae</i>	236	173(73.3)	113(65.3)	75(66.3)
<i>K. oxytoca</i>	39	25(64.1)	12(48.0)	8(66.7)
<i>C. freundii</i>	55	29(52.7)	7(24.1)	6(85.7)
<i>C. koseri</i>	51	36(70.6)	17(47.2)	10(58.8)
<i>E. aerogenes</i>	11	5(45.5)	5(100)	3(60.0)

<i>E. cloacae</i>	7	2(28.5)	2(100)	2(100)
<i>M. morganii</i>	16	2(12.5)	1(50.0)	0(0.0)
<i>P. mirabilis</i>	34	16(47.1)	13(81.3)	7(53.8)
<i>P. vulgaris</i>	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 CRE as n

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 (Figure 1, Table 3). Amplification of integron variable region by the integron conserved sequence PCR revealed variable length ranging from 800bp to 3000bp. Eight randomly selected amplicons were sent for sequencing and of these, 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.

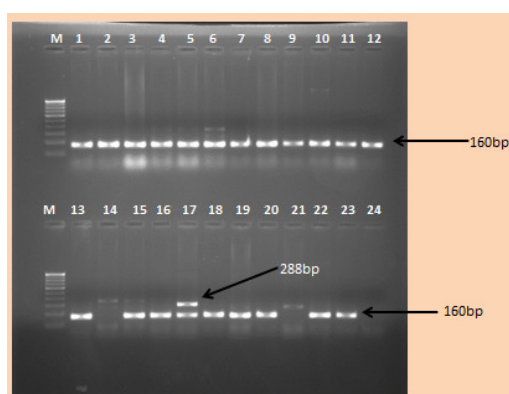


Figure 1 : PCR amplification of class 1 and class 2 integrase gene

Table 3 : Showing integron carriage rate and variable regions amplified.

Bacteria isolates	Number and proportion of isolates that are				
	CRE	<i>Int 1</i>	<i>Int 2</i>	<i>Int 1&2</i>	<i>Int CS</i>
<i>E. coli</i>	64	55(85.9)	5(7.8)	4(6.3)	1 (900bp)
<i>K. pneumoniae</i>	75	68(90.6)	9(12.0)	8(10.7)	1(2kb)
<i>K. oxytoca</i>	8	8(100)	2(25.0)	2(25.0)	-
<i>C. freundii</i>	6	5(83.3)	0(00.0)	0(00.0)	1(900bp)

<i>C. koseri</i>	10	10(100)	0(00.0)	0(00.0)	-
<i>E. aerogenes</i>	3	3(100)	0(00.0)	0(00.0)	-
<i>E. cloacae</i>	2	2(100)	0(00.0)	0(00.0)	1(1kb)
<i>M. morgani</i>	0	0(00.0)	0(00.0)	0(00.0)	-
<i>P. mirabilis</i>	7	4(57.1)	0(00.0)	0(00.0)	-
<i>P. vulgaris</i>	3	1(33.3)	0(00.0)	0(00.0)	-
Total	178	156(87.6)	16(9.0)	14(7.9)	

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 [12]. High frequency of integron class 1 carriage rate (87.6%) among CRE strains observed in this study underscore their 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 [13]. 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-bla_{OXA-10}-aadA1*, *aadA1*, *aar2-aacA4-dfrA1-orfC*, and *dfrA30*) reflected the collection excision and rearrangement of gene within the integron as have been reported [14]. The most abundant cassettes observed were those conferring resistance to aminoglycosides (*aadA1*, *aacA4*, *aadB* and *dfrA*). Previous studies have indicated dihydrofolate reductase and aminoglycoside resistance gene as probably the most abundant gene cassette found on integron variable region of enterobacteriaceae [14,15,16]. Although we could not observe presence of any carbapenemase resistance cassette or β -lactamase gene except *bla_{OXA-10}* in *E. cloacae*, carbapenemase resistance gene maybe be located outside the integron [14], however this does not preclude the possibility that integron from other isolates could harbour more cassettes combination containing these genes, in view of our limitation of amplifying and sequencing variable region for only very few isolates. A study on integron by [14] reported that although ESBL gene was not found on the integron conserved sequence, ESBL production was significantly higher in isolates positive for integron class 1 than integron negative isolates. Nonetheless, 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 [14,15,16,17].

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. This calls for caution particularly in the

area of antibiotic prescription policy and hospital hygiene in order to reduce the spread of these pathogens within the hospital setting.

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