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Diversity of Class 1 Integrons and Carriage of Trimethoprim Resistance in Clinical Isolates of Enterobacteriaceae from India

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Abstract

Class 1 integron continues to be one of the major factors responsible for multidrug resistance in *Enterobacteriaceae* family. The diversity of integron cassette array with special reference to trimethoprim and sulfomethoxazole resistance determinants often compromise treatment regime. The present works investigate class 1 integron and carriage of trimethoprim resistant marker as gene cassette. Out of 268 consecutive, non-duplicate clinical isolates of *Enterobacteriaceae* resistance pattern against 5 antimicrobial agents assessed by disk diffusion and minimum inhibition concentration. The presence of integron was done by PCR targeting integrase gene (*intI1* and *intI2*). Whole gene mapping was done by amplifying 5´-CS and 3´-CS region of class I integron. The amplified gene cassette was cloned and antibiogram of clone was assessed.The isolates showed significantly high amount of resistant against co-trimoxazole (89%). The carriage of class I integron was found detected in 187 isolates. Five different cassette arrangements were detected: (*dfrA*17-*aadA*5), (*dfrA*30-*aadA*5), (*dfrA*12-*orf*F-*aadA*2), (*dfrA*1-*aadA*1) and (*aacA*7-*aadA*6-*qacEΔ*1-*sul*-*orf*5) with complete 3´-Conserved segments. The most prevalent cassette combination was (*dfrA*17-*aadA*5). This study was identified a set of gene cassette carrying *dfrA*30 gene with *aadA*5 aminoglycoside resistance gene. Further investigation is required to determine the prevalence of different *dfr* variants among *Enterobacteriaceae* and their contribution in hospital environment.

Keywords: Integron; Dihyrofolate reductase (dfr); Multidrug resistance (MDR); Gene cassette; Enterobacteriaceae

Introduction

Integrons are mobile genetic elements which play a vital role in rapid dissemination of multidrug resistance gene (MDR) among *Enterobacteriaceae* [1]. The selection processes suggest their accumulation may not involve a single mechanism, but augmented by transposon and plasmids [2]. Integrons carry more than one resistance genes in the form of tandem gene cassettes, mainly based on three essential components in their 5´Conserved Segment (CS): *intI* gene for encoding enzyme integrase, *attI* site for site specific recombination and transcription is initiated by a strong promoter *Pc* upstream of the gene cassette and the 3´CS confers the presence of sulphonamide resistance gene (*sul1*) and *qacEΔ*1 genes encoding resistance to quaternary ammonium compounds [3,4]. Based on integrase genes *(intI*) integrons have been grouped into four classes (Class 1,2,3 and 4) [4] and the genetic arrangement of Class I integrons are strongly associated with multidrug resistance in clinical environment [5]. Acquisition of trimethoprim resistance dihydrofolate reductase (dfr) gene in *Enetrobacteriaceae* is most other encoded by Integron system [6]. Previously 17 types of trimethoprim resistance dfr genes have been reported [6]. Two more *dfr* gene was reported recently; found associated with common region (CR1) an IS91-related element in the downstream of 3´CS in integron viz: *dfrA*24

and *dfrA*26 [7]. Carriage of other resistant genes as cassette within class I integron, forms a multidrug resistant phenotype, which severely restricts treatment alternatives in clinical settings [7]. So, the current study aim to find out the presence of integron harbouring–positive isolates as well as mapping of whole class 1 integron and to explore the prevalence of integron mediated dfr variants, with special reference with trimethoprim-sulphomethoxazole resistant isolates in hospital environment.

Methods

Collection of samples

A total of 268 consecutive non-duplicate clinical isolates of Enterobacteriaceae were taken from the patients admitted to various wards or attended clinics of Silchar medical college and Hospital, Silchar, India from March 2013 to February 2014 **(Table 1).** Bacterial isolation and identification were according to microscopical investigation, cultural characteristics and biochemical typing [8]. Antibiotic susceptibility was performed against ampicillin (10 µg), co-trimoxazole (1.25/23.75 µg), ciprofloxacin (5 µg), gentamicin (10 µg) and cefepime (30 µg) [Hi-Media, Mumbai, India], by Kirby Bauer disc diffusion method and results were interpreted according to CLSI criteria [9] **(Table 2).** Escherichia coli ATCC 25922 was taken as negative control.

Determination of trimethoprim resistance

All the co-trimoxazole resistant isolates were further tested for susceptibility towards trimethoprim (10 µg) (Hi-Media, Mumbai, India). For further confirmation, MICs of trimethoprim were detected by Hi-Comb MICs Strip (Hi-Media, Mumbai, India).

Screening of class I integron

DNA templates were prepared by using boiling centrifugation method. Overnight culture was suspended in 200 µl sterile distilled water and heated to 80˚C for 10 min. Cell suspensions were centrifuged and the supernatant was used for PCR. The presence of class I integron was screened by multiplex PCR using primer *int1* and *int2* [10] **(Table 3).** Each single reaction mixture (25 µl) contained 1µl of DNA suspension, 10 pmol of each primer, 2x GoTaq Green Master Mixture [Promega, Madison, USA]. The PCR conditions were as follows; 94°C for 3 minutes, followed by 32 cycles at 94°C for 20 seconds, 54°C for 20 seconds, 72°C for 1 minute and final extension at 72°C for 5 minutes.

Mapping of class I integron

PCR was performed with presumptively class I integrase positive isolates using primers 5´– CS GGC ATC CAA GCA GCA AG and 3´– CS AAG CAG ACT TGA CCT GA [11] **(Table 3)** to amplify the variable region of integron. Each single reaction mixture (30*µl*) contained 1 µl of DNA suspension, 10 pmol of each primer, 2 x GoTaq Green Master mixtures [Promega, Madison, USA]. The PCR conditions were as follows 95°C for 2 minutes, followed by 35 cycles at 95°C for 20 seconds, 50°C for 45 seconds, 72°C for 2 minute and final extension at 72°C for 7 minutes.

DNA sequence analysis

A number of PCR product that appeared as a unique size of band on the gel was processed with the QIA quick PCR purification Kit (QIAGEN, Germany) and used for direct sequencing. Sequences were compared using BLAST search (**http://www.ncbi.nlm.nih.gov/BLAST**).

Cloning of class I integron

In order to determine the genetic array of each gene cassettes within the variable region of class I integron amplified products were cloned using pGEM-T vector [Promega, Madison, USA] and transformed into *E.coli,* JM107. The transformants were subjected to antimicrobial susceptibility testing.

Table 1 List of clinical samples showing their antibiotic susceptibility pattern.

Isolates	Clinical Samples											
	Urine	Pus	Stool	Ear swab	Throat swab	Oral swab	Sputum	Drain tip	Blood	Pseudo pancreatic fluid	Wound aspirate	Nasal secretion
Escherichia.coli n= 174	104	40	11	2	6	4	2	1	0	1	2	1
Klebsiella Pneumonia n= 71	42	18	0	2	4	0	3	1	0	0	0	1
Proteus mirabilis n=23	3	12	2	1	1	3	0	0	1	0	0	0

Table 2 Antibiotic susceptibility pattern.

Isolates	Antibiotics									
	Co-trimoxazole		Ampicillin		Ciprofloxacin		Gentamicin		Cefepime	
Escherichia.coli n= 174	167	96%	142	82%	125	72%	88	51%	83	48%
Klebsiella Pneumonia n= 71	49	69%	44	62%	35	49%	29	41%	36	47%
Proteus mirabilis n=23	2	96%	21	91.3%	15	65%	15	65%	14	61%

Table 3 List of Primers.

Primer	Neucleotide Sequence (5' to 3')	Product size (bp)	
Int 1 F	CAG TGG ACA TAA GCC TGT TC	160	[10]
Int 1 R	CAG TGG ACA TAA GCC TGT TC		
Int 2 F	TTG CGA GTA TCC ATA ACC TG	288	[10]
Int 2 R	TTA CCT GCA CTG GAT TAA GC		
5-CS	GGC ATC CAA GCA GCA AG	[11]	
3-CS	AAG CAG ACT TGA CCT GA		

Results

Antibiotic susceptibility pattern

Isolated bacteria (n=268), were identified as *Escherichia coli* 174 (64.9%), *klebsiella pneumoniae* 71 (26.5%) and *Proteus mirabilis* 23 (8.6**%) (Table 2).** Majority of the bacterial isolates were obtained from urine, followed by pus, stool and other clinical specimens **(Table 1).** Results of susceptibility profiling showed that, high levels of resistance were recorded towards co-trimoxazole (89%), ampicillin (77%), ciprofloxacin (65%), cefepime (50%) and gentamicin (49%) **(Table 2).**

PCR screening for integrons

A total of 187 isolates had integron, among which 149 isolates had only class 1 integron, 32 isolates had class 1 integron and class 2 integron, while 11 isolates had only class II integron (**Table 4**, **Figure 1).**

Mapping of class 1 integron

In amplifying the whole class I integron gene cassette for PCR, 95 isolates revealed visible amplification. Variable sizes of class I integrons was between 800 bp-2.5 kb (**Figure 2)** Sequencing results revealed five different arrays of variable regions and four different types of trimethoprim resistance determinants in gene cassette i.e; *dfrA1, dfrA12, dfrA17, dfrA30* **(Figure 3).** In arrangement I associated with *dfrA17* and *aadA5* cassette array, and found to be the most prevalent types of gene cassette array (n=44). Whereas, in arrangement II *dfrA30, aadA5* in the downstream of the integron I in one isolates. The arrangement III showed the presence of *orfF* in between of two resistant variants *dfrA12* and *aadA2* and as well. In arrangement IV, association of *dfrA1* and *aadA1* gene was observed and this association could be observed, which was observed only in one isolate. The V arrangement showed presence of *aacA*7 and *aadA*6 with two chronological 3´-conserved sequences which indicates the association of more than one transposable mechanism.

When tested for susceptibility of each arrangement that was cloned in *E.coli DH5α*, resistance towards co-trimoxazole was observed but found to be susceptible against aminoglycoside group of antibiotics.

Discussion

This study given a representative structure of the prevalence of integron mediated *dfr* gene among clinical isolates of *Enterobacteriaceae* in a hospital setting of Silchar, India. The existence of integron was confirmed in 73.1% of identified multiple antibiotic resistant *Enterobacteriaceae* strains, which possibly indicates maintenance and propagation of resistance determinants in clinical environment [12,13]. The presence of class I integron were predominant in the *Enterobacteriaceae* isolates (79.7%). Co-trimoxazole is prescribed for the treatment of urinary tract infection but recently nitrofurantoin, fluoroquinolones and cephalosporins are also being advised, suggesting that intake of these drugs might be sufficient to maintain the antibiotic stress in the clinical settings. Few isolates failed to amplify with 5´-CS and 3´-CS primer, these phenomena could be due to alteration, which occurs in the primer target sites or which are too large to amplify [13]. All the amplification negative isolates were found to confer high level of resistance in one or more antibiotics phenotypically, therefore it could be assumed that the resistance gene might be co-selected with the integrons in another mechanism. It is reported that in rare occasion 3´-CS of class I integron mobilized without any trace of a resistance gene cassette in its surrounding environment [12]. Sequence analysis identified different *dfr (dfrA17, dfrA30, dfrA12, dfrA1)*, four variants of *aadA(aadA6, aadA5, aadA2, aadA1*) and one *aacA7*gene (confers resistance to modern aminoglycosides such as amikacin and netilmicin) in gene cassette [13,14]. However, five different arrangements which confer antibiotics resistant to trimethoprim, spectinomycin, streptomycin and one isolates carrying two intact 3´-CS regions; thereby, indicating variability in their origin and selection were recorded in this study. Association of gene cassette with *dfrA17* and *dfrA12* were the most prevalent in *Enterobactericeae* and frequently observed to be disseminated through horizontal gene transfer [6]. Gene cassette such as *dfrA1-aadA1, dfrA12-orfF-aadA2, dfrA17-aadA5*were first observed in 1990 [14,15]. The present study however, showed that the presence of association with integron mediated *dfrA30*, which was first identified in *Klebsiella pneumonia* in 2011[15] could not be established. The high correlation between *dfrA* gene and integron was also reported in different studies [6,14,16,17]. The wide dissemination of *dfrA17* variants were found along with *aadA5* gene over the course of time [18] and new variants dfrA30 was found associated with integron system. The presence study could highlight parallel existence of five different cassette array systems in a single hospital setting with multiple variants of same resistance gene types (*dfr*). This kind of study is unique of its own where diverse gene cassettes are maintained in a hospital setting where antibiotic pressure is high.

Conclusion

In conclusion, this study demonstrated the importance of class 1 integrons for acquisition of resistance gene with special reference to trimethoprim, among *Enterobacteriaceae*. The wide dissemination of *dfrA17* variants were found along with *aadA5*

Table 4 Characterization and screening of Integron in Clinical Isolates.

Clinical specimen	*Int*1	*Int* 2	*Int* 1 and *int*2
Escherichia coli= 174	98	11	23
Klebsiella.pneumoniae =71	44	2	4
Proteus mirabilis=23	7	2	5
Total=	149	15	32

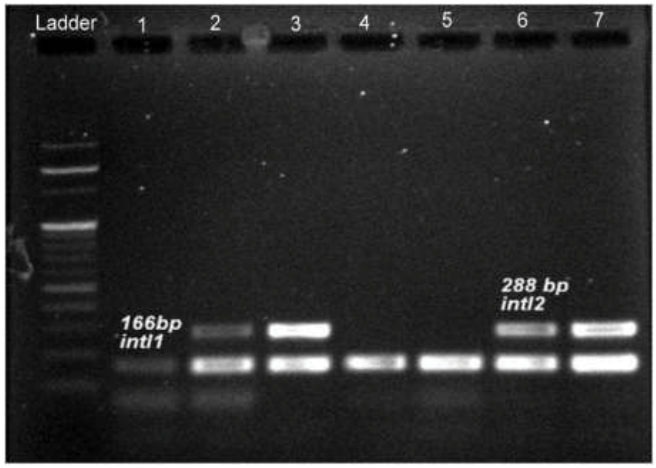

Figure 1 Characterization of class I and class II integron among bacterial isolates; lane 1, lane 3, lane 4 showing only class 1integron and lane 2, lane 3, lane 6 and 7 showing amplification of both class I and class II integrons.

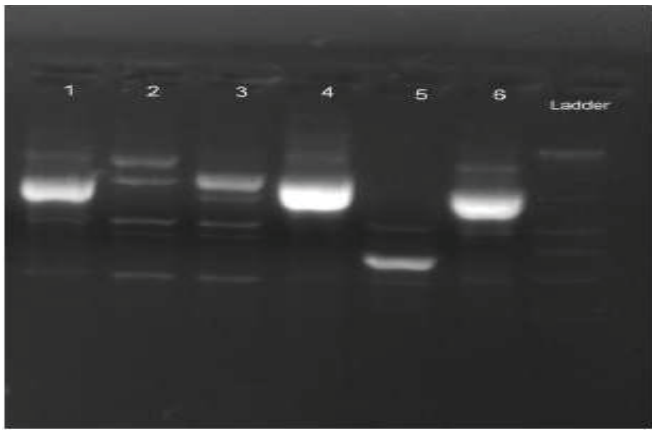

Figure 2 Amplification of class 1 integron; lane 1, 2, 4 and 6 showing amplification with 1.5 kb and other ranges from 800 bp.

gene over the course of time and new variants *dfrA30* was found associated with integrons system. By using the PCR we have determined the content and order of the antibiotic resistance genes inserted between the conserved regions in the integron system of clinical settings. In the form of gene cassette, selective pressure from one antibiotic can simultaneously select genes when physically linked with other genes. Therefore, PCR mapping with 5´-CS and 3´-CS reveals the position and orientation of inserted gene cassette in integron which can be useful to study the evolution and dissemination of multidrug resistance genes in bacterial population.

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Conflicts of interest

None to declare

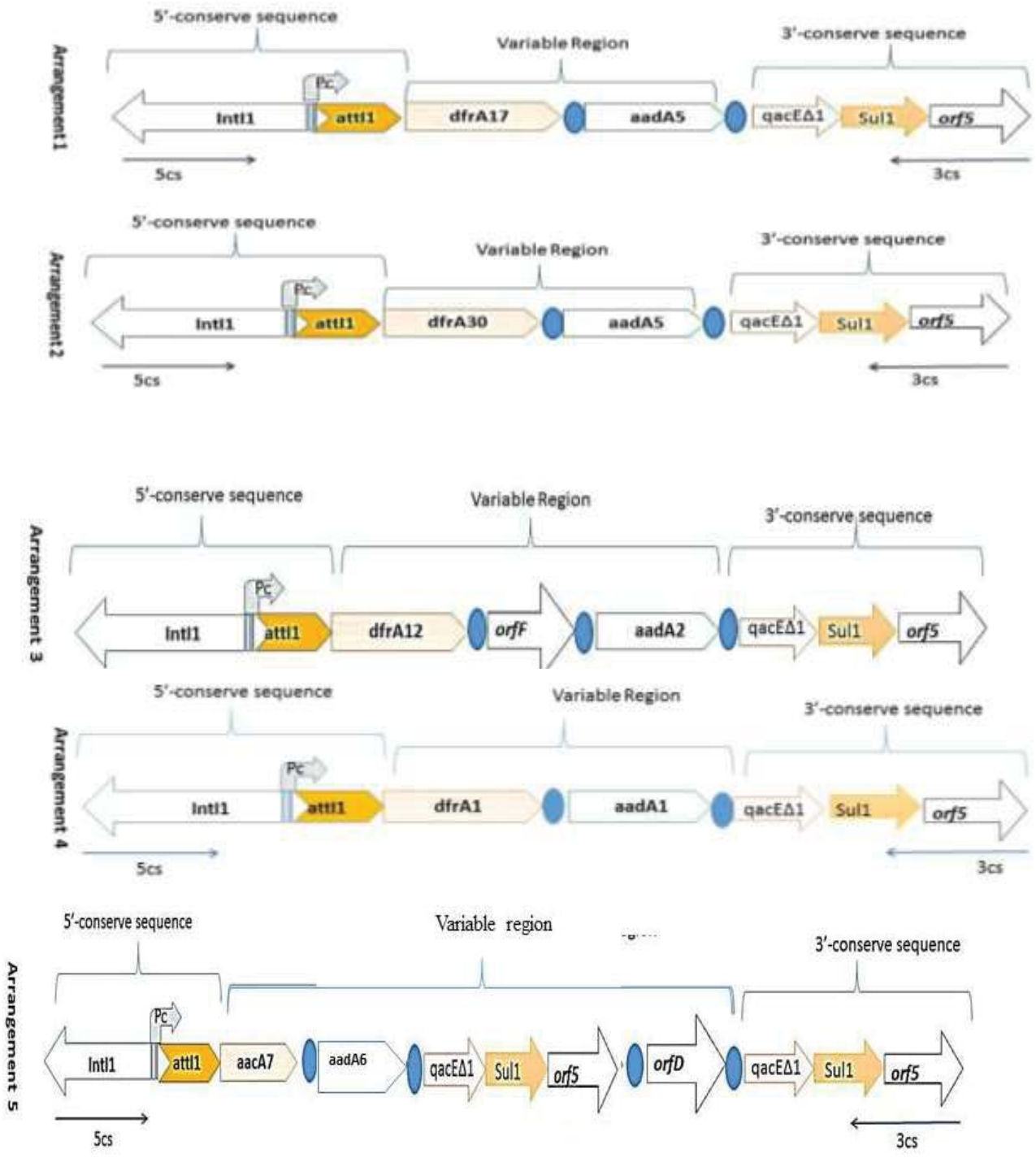

Figure 3 Schematic representation of variable region in class 1 integron, gene cassette are shown as a boxes, arrow represents the way of transcription and blue circle represents the *attC* site. **Arrangement 1:** showing the chronological arrangement of class I integron and cassette carrying *dfrA17* and *aadA*5; **Arrangment 2:** showing the class I integron gene cassette along with *dfrA30* and *aadA5*; **Arrangement 3:** showing the trimethoprim determinant *dfrA*12 followed by *orfF*, hypothetical protein and *aadA*2; **Arrangement 4:** showing class 1 integron associated with *dfrA*1 and *aadA*1; **Arrangement 5:** showing the variable region with *aacA*7 and *aadA*6.

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