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Sulphonamide Resistance in Clinical Isolates of *Escherichia coli* and their Association with Class I Integron: A Study from India

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Abstract

The aim of the study was to assess the prevalence of sulfonamide resistance and their association with integron among *Escherichia coli* from hospital patients of Silchar Medical College. Out of 177 consecutive, non- duplicate clinical isolates of Enterobacteriaceae resistance pattern against 5 antimicrobial agents assessed by disc diffusion and minimum inhibition concentration. Presence of class I integron-associated integrase (intI) gene, as well as the presence of multiple sul genes was detected using gene specific PCR. 60 isolates were resistant to one or more of the tested antimicrobial drugs, with highest resistance (94.4%) observed against co-trimoxazole.Integrase PCR showed 90 isolates harboring class I. Among the test isolates 57 isolates were found carry both *sul1* and *sul2* whereas *sul3* gene was present only in 3 isolates. This study could conclude that genetic background of sulphonamide resistance is diverse within single hospital setting in our area.

Keywords: Enterobacteriaceae; Escherichia coli; Integron; Sulphonamide resistant gene (sul)

Introduction

Co-trimoxazole, a combination of two synthetic antibiotics sulfamethoxazole and trimethoprimcame into practice in 1970 and since then being a low cost drug, has been used effectively to treat urinary tract infection evidently used in animals also [1-3]. However, resistance against it developed very quickly within the members of Enterobacteriaceae, which resulted in the massive reduction in susceptibility rate [1]. Sulphonamide resistance is commonly contributed through three resistant genes namely *sul1, sul2* and *sul3* encoding sulphonamide resistant dihydropterote synthase enzyme [1,2,4]. Most of the genes for sulphonamide resistance are spread by the integron [5]. Amongst them *sul1* is the most prevalent and also located in the 3' conserved region of class I integron, but not as a gene cassette [6]. *Sul2* is generally not considered as a part of a distinct genetic element and associated with streptomycin resistance gene [2,6-8]. Whereas, *sul3* has been occasionally linked with non-classic Class I integron without 3´ conserved sequence (3´CS) [9].

It is reported earlier that sulphonamide resistance genes can be horizontally transferred through integron, transposons and plasmids from commensal bacteria to a virulent one in human intestine [3,10]. It is also hypothesised that prolonged use of co-trimoxazole therapy is responsible for selection of integron positive Enterobacteriaceae and in turn responsible for sulphonamide resistance [11].

In India there is paucity of data regarding status of transmission and genetic basis of sulphonamide resistance while studies have reported high prevalence of sulphonamide resistance based on phenotypic screening [9].

In the present study, molecular basis of sulphonamide resistance was assessed among clinical isolates of *Escherichia coli* in tertiary referral hospital of India.

Materials and Methods

Bacterial strains

A total of 177 consecutive, non-duplicate isolates of *Escherichia coli* were collected from patients admitted or attended in the clinic of Silchar Medical College and Hospital, Silchar, India for a period of 1year (February 2012 - January 2013). Isolates were identified using standard biochemical norms [12].

Phenotypic screening of MDR strains

All the isolates were screened for susceptibility 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 interpreted as per CLSI criteria [13]. *E. coli* ATCC 25922 was taken as negative control.

Phenotypic screening of sulphonamide resistance isolates

All the Co-trimoxazole resistant isolates were further subjected to susceptibility testing against trimethoprim (5 mcg) and sulphafurazole (300 mcg) independently [Hi-Media, Mumbai, India] separately. Minimum inhibition concentration (MIC) for sulphafurazole and trimethoprim were also determined with Hi-comb MIC test strip [Hi-Media, Mumbai,India] the breakpoint used was the one defined by the CLSI [13] for the family *Enterobacteriaceae.*

PCR amplification of Sul gene

Amplification was carried out by heating for 3 minutes at 95˚C, followed by 34 cycles at 95˚C for 20 seconds; 58˚C for 1 minute 72˚C for 45 seconds followed by 72˚C for 5 minutes. PCR reaction was performed using primers for *sul1, sul2* and *sul35* **(Table 1).**

Cloning of *Sul* gene

In order to determine the sul gene functionality new sets of sul primers were designed **(Table 1).** Amplified products were cloned using pGEM-T vector [Promega, Madison, USA] and transformed into E.coli, JM107. Transformants were confirmed for the presence of sul genes by PCR. The PCR conditions were 94°C for 2 minutes, followed by 35 cycles of 94°C for 15 seconds, 52°C for 20 seconds, 72°C for 1.3 minutes and final extension at 72°C for 7 minutes. The transformants were further subjected to MIC determination against trimethoprim and sulphafurazole.

Characterization of Integron

Presence of integrons among the isolates was further detected by amplification aided with primers *int1* and *int2* **(Table 1)** [14]. 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.

Typing of isolates harbouring *sul* gene

Isolates were typed by pulse field gel electrophoresis where genomic DNA was prepared in agarose blocks and digested with the restriction enzyme *XbaI* [Promega, Madison, USA] and then the DNA fragments were separated with a CHEF DRIII apparatus [BIO-RAD, USA] for 22 hours at 4 V/cm.

Results

Among the isolates tested, 60 were found to be resistant to all the antibiotics. High resistance was found against co-trimoxazole (94.4%), followed by ampicillin (80.2%) and ciprofloxacin (70.6%), whereas gentamicin and cefepime were found to be less resistant **(Table 2)**. Co-trimoxazole resistance was observed in 167 isolates. Among these sulphafurazole and trimethoprim resistance were observed in isolates 90 and 51.5 % respectively. Integrase gene PCR results showed that 90 isolates were harbouring class I and 8 were carrying class II integron, while presence of both class I and class II integrons were observed in 12 isolates **(Figure 1).** While performing multiple PCR for sulphonamide resistance, three isolates were found to harbour single *sul3* gene **(Figure 2)**. However, in 57 isolates both *sul1* and *sul2* genes were observed. Cloning of all the individual genes **(Figures 3 and 4)** from each isolates was attempted where the MIC value for *sul2* and *sul3* against sulphonamide were in resistant range for both parent strains and their clones **(Figure 5).** However, for *sul1* gene variable MIC value was noticed for clones, where half of the clones showed the MIC range below break point **(Table 3).** On performing PFGE 18 pulsotypes of *E. coli* was observed.

Discussion and Conclusion

It is known that the sulphonamide resistance determinant (*sul1*) is located within integron and also established that integrons were selected during use of trimethoprim/ sulfomethoxazole in the intestinal flora [6,11]. However, in our study *sul1* gene was found in integron- negative isolates as well. Thus, extra integron existence of *sul1* gene also contributed phenotypic sulphonamide resistance, which too was evident by MIC study. This indicates that sulphonamide resistance is not originated from 3´CS region of Integron. In our study presence of other sulfonamide resistance genes Viz; *sul2* and *sul3* were also responsible confering resistance.

This study also underlines presence of three sulphonamide resisatnce genes in a single- center study with a single isolate harboring more than one type of sul gene. These genes were probably selected during course of co-trimoxazole therapy which is very common in community-acquired infection in this region, and also maintained in the subsequent generation. Current study, probably the first study from India describing genotypic background of sulphonamide resistant. Further investigation is needed for assessment of their acquisition and expansion when co-trimoxazole pressure is withdrawn and their persistance through Class I integron within enteric pathogen.

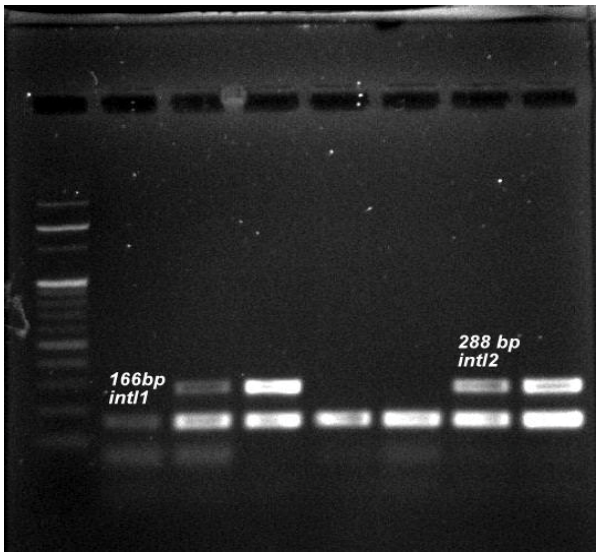

Figure 1 Characterization of class 1 and class 2 intgron among transformants; lane 2, lane 3, lane 6 and lane 7 showing both class 1 and class 2 integron; lane 1,lane 4 and lane 5 showing only class 1 integron.

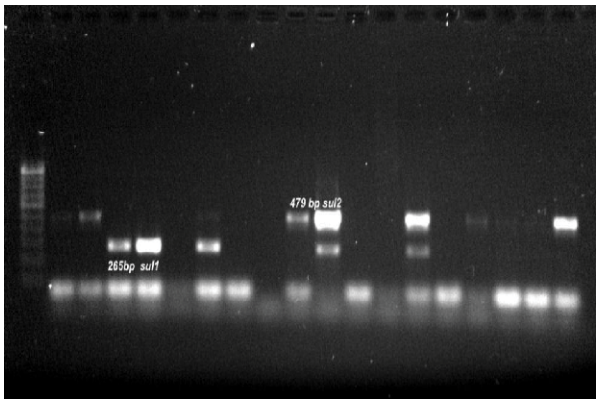

Figure 2 Detection of *Sul* gene; lane 1, lane 2, lane 9, lane 15, lane 8 amplifies only *sul2*; lane 10, lane 13, amplify both *sul1* and *sul2* gene; lane 3, lane4, lane 6, showing only *sul1* gene.

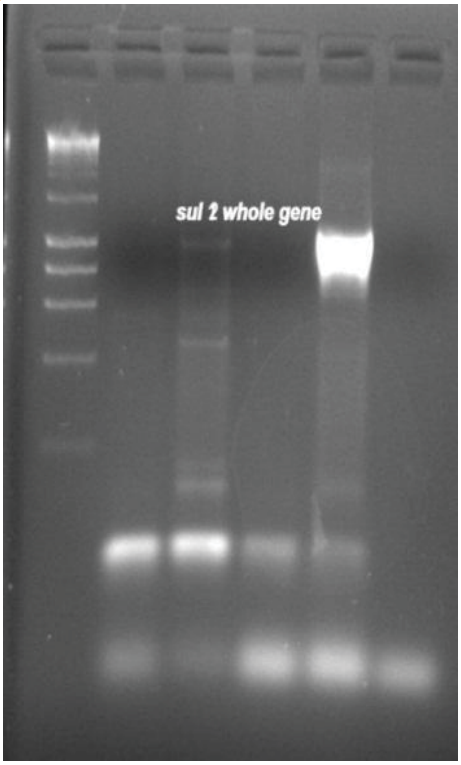

Figure 3 Detection of *Sul2* whole gene; lane 2 and lane 4 showing *sul2* whole gene.

Figure 4 Detection of *Sul3* whole gene: lane 1 showing *Sul2* whole gene.

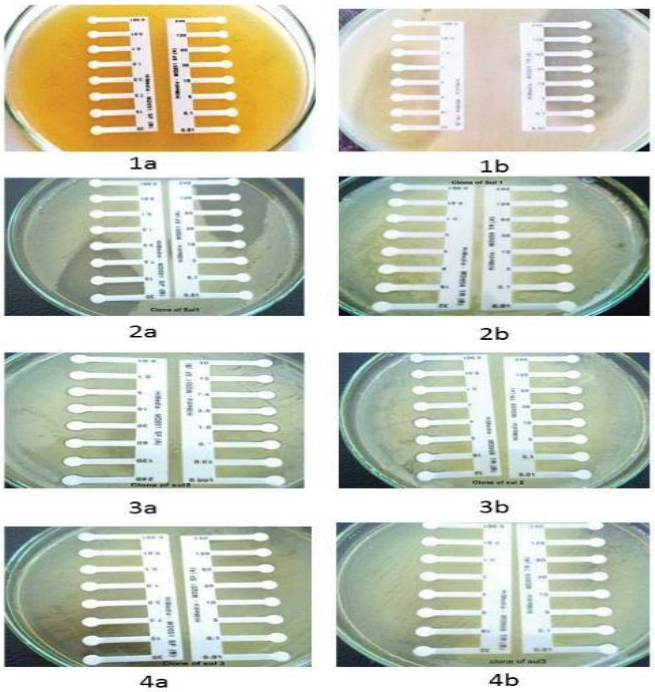

Figure 5 MIC panel of sul whole gene: 1a and 1b showing the wild type *E. coli* strains against TMP and SUL respectively; 2a and 2b showing the cloned *Sul1 E. coli* strains against TMP and SUL respectively; 3a and 3b showing the cloned *Sul2 E. coli* strains against TMP and SUL respectively; 4a and 4b showing the cloned *Sul3 E. coli* strains against TMP and SUL respectively.

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Conflicts of interest

None to declare

Table 1: List of primers used in this study.

Primer	Neucleotide Sequence (5' to 3')	Product size (bp)	Target site	Reference
IntI 1 F	CAG TGG ACA TAA GCC TGT TC	160	*Int I1*gene	Koeleman et al. *J Clin Microbiol* 2001 [14]
IntI 1 R	CAG TGG ACA TAA GCC TGT TC			
IntI 2 F	TTG CGA GTA TCC ATA ACC TG	288	*Int I2* gene	Koeleman et al. *J Clin Microbiol* 2001 [14]
IntI 2 R	TTA CCT GCA CTG GAT TAA GC			
Sul 1 F	CCG ATA TTG CTG AGG CGG	265	*Sul 1* gene	Bean et al. *AAC* 2009 [5]
Sul 1 R	CCA ACG CCG ACT TCA GCT			
Sul 2 F	TCG TCA ACA TAA CCT CGG ACA G	479	*Sul2* gene	Bean et al. *AAC* 2009 [5]
Sul 2 R	GTT GCG TTT GAT ACC GGC AC			
Sul 3 F	GAG CAA GAT TTT TGG AAT CG	790	*Sul3* gene	Bean et al. *AAC* 2009 [5]
Sul 3 R	CAT CTG CAG CTA ACC TAG GGC TTT GGA			
Sul 1 XF	AGT TGG CGA AGT AAT CGC AAC	1300	*Sul1* whole gene	This study
Sul 1 XR	ACG CAC AGT CAA CTT ATT GGA TG			
Sul 2 YF	ATT GCC TAC TGA GCG CTG CC	1051	*Sul2* whole gene	This study
Sul 2YR	CTT CAG TTT TCT GAT GAA GCG			
Sul 3ZF	CAG CGC ATT TTT AAT GCA AAG G	1374	*Sul3* whole gene	This study
Sul 3ZR	CAA GTA CGC CAA CAC AAC TTC AG			

Table 2: Antibiotic susceptibility profiling.

Antibiotic tested	Resistant isolates		Co-trimoxazole resistance isolates n =167			
			Trimethoprim		Sulphaafurazole	
	n	%	n	%	n	%
Co-trimoxazole	167	94.4%	86	51.5%	151	90%
Gentamicin	88	49.7%				
Ciprofloxacin	125	70.6%				
Cefepime	83	53%				
Ampicillin	142	80.2%				
Five or more Antibiotics	60	33.9%				

n= number of resistant isolates, % = percentage of resistance

Table 3: MIC status of cloned *sul* against wild type.

Strains	Sulphafurazole	
	MIC 50	MIC90
Wild type	>256	>256
Clone of *Sul1*	10	>256
Clone of *Sul2*	>256	>256
Clone of *Sul3*	>256	>256

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