

Sulfonamide Resistance Genes (*sul*) M in Extended Spectrum Beta Lactamase (ESBL) and Non-ESBL Producing *Escherichia coli* Isolated From Iranian Hospitals

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Background: Extensive use of cotrimoxazole has been associated with increasing level of *Escherichia coli* resistance.

Objectives: In the current study, we focused on assessing the prevalence of *E. coli* resistance to cotrimoxazole and frequency of its associated genes.

Materials and Methods: One-hundred and forty-four *E. coli* isolates were identified during March 2007 to April 2012 at Ilam hospitals and Milad (Tehran) hospital. Antibiotic susceptibility for screening of resistance isolates was done by the Kirby-Bauer method. The *sul1*, *sul2*, *sul3*, *dfiA1*, *dfiA5*, *int1*, *blaTEM*, *blaSHV* and *CTX-M* genes were detected by polymerase chain reaction (PCR) amplification. Plasmid curing was done for identifying correlations between resistance genes and plasmids.

Results: Amongst the 144 *E. coli* isolates, seventy-two (50%) Extended Spectrum Beta Lactamase (ESBL)-producing and seventy-two (50%) non-ESBL-producing *E. coli* isolates were identified; eighty-seven isolates (60.41%) were resistant to cotrimoxazole. Frequencies of *sul1*, *sul2* and *sul3*, were 81% (116 isolates), 67% (96 isolates) and 2.29% (three isolates), respectively. Furthermore, 50.57% (72 isolates) had *sul1* and *sul2*, 2.29% (3 isolates) contained *sul2* and *sul3*, and 2.29% (three isolates) contained *sul1*, *sul2* and *sul3* genes, simultaneously. Thirty-four (39.1%) of the isolates had the *dfiA1* gene. Five (5.7%) of the isolates had the *dfiA5* gene. Sixty-eight (78.2%) strains contained the *int1* gene. Furthermore, *dfiA1* and *dfiA5* were present in three (3.4%) of the isolates. The results showed that of the ESBL-producing isolates, 85.2% (n = 122), 53.2% (n = 76) and 26.1% (n = 37) were *blaTEM*, *blaSHV* and *CTX-M* harboring isolates, respectively.

Conclusions: Our study indicated a high frequency of cotrimoxazole resistance gene in *E. coli* isolates from Ilam and Tehran (Milad) hospitals, and *sul* genes had a major role in cotrimoxazole resistance of these isolates.

Keywords: Trimethoprim-Sulfamethoxazole Combination; Iran; Resistance; *Escherichia coli*

1. Background

Escherichia coli is one of the important members of Enterobacteriaceae, which causes some of the common bacterial infections such as urinary tract, bacteremia and traveler's disease (1). Because of colonization in the intestine, *E. coli* is considered as enteric bacteria (2, 3). Chromosomal mutation is one of the mechanisms of antibiotic resistance that occurs in the absence of antibiotic, yet the secondary resistance mechanism, is genetic interchanges that is mainly conducted by plasmids. This kind of resistance in bacteria has also been reported against sulfonamides (4, 5).

Sulfonamides are structural analogues of para amino benzoic acid (PABA), which competitively inhibit dihydropteroate synthetase activity. Simultaneous prescription of dihydrofolate reductase (trimethoprim) with sul-

fonamides creates a synergistic antimicrobial activity in bacterial infections. Combination of trimethoprim and sulfamethoxazole with the trade name of cotrimoxazole is the first antibiotic that has been used for the treatment of urinary tract infections (5, 6).

Dihydropteroate synthetase with low affinity for sulfonamides is encoded on a plasmid, which has high-specific transfer potential to other organisms. Furthermore, *sul1*, *sul2*, and *sul3* are known plasmid encoded sulfonamide resistance genes, which produce dihydropteroate synthetase (DHPs) and induce resistance against sulfonamides (6, 7). High prevalence of sulfonamide resistance between Gram-negative bacteria, including *E. coli* isolated from human and animal resources, have been reported worldwide (8-10).

2. Objectives

Because of increasing antibiotic consumption and in turn antibiotic resistance, determination of antibiotic susceptibility patterns of *E. coli* against cotrimoxazole and study of related genes are necessary for elucidating molecular and epidemiological mechanisms of resistance.

3. Materials and Methods

3.1. Bacterial Isolates

One-hundred and forty-four (144) clinical strains of *E. coli* were collected from hospitals of Ilam and the Milad Hospital of Tehran. All of the antibiotic discs used in this study were from Mast, England. Antibiotic susceptibility for ampicillin, tetracycline, ciprofloxacin and cotrimoxazole was done by the disk diffusion method based on recommendations of the national clinical laboratory standards (NCLS) (11, 12).

3.2. Phenotypic Detection of Extended Spectrum Beta Lactamase

Extended spectrum beta lactamase (ESBL) producers were confirmed by a disc diffusion method. The pairs of discs tested included cefotaxime-clavulanic acid (30/10 µg) and cefotaxime (30 µg) discs, and ceftazidime-clavulanic acid (30/10 µg) and ceftazidime (30 µg) discs. An increase in the zone diameter of ≥ 5 mm for the clavulanic acid-supplemented discs compared to the zone diameter for the plain discs was considered as an indication of an ESBL-producer. Culture media, agarose and ethidium bromide used in the study were purchased from Merck, US. *Escherichia coli* ATCC 700603 was used as the ESBL-positive control (13).

3.3. DNA Extraction and Polymerase Chain Reaction Studies

Cotrimoxazole resistance genes *sul1*, *sul2*, *sul3*, *dfrA1*, *dfrA5*, *int1*, *blaTEM*, *blaSHV* and *CTX-M* were detected by PCR. The presence of class 1 integrons (*int1*) in each strain was assessed by using class 1 specific primers. Extended Spectrum Beta Lactamase genes *blaTEM*, *blaSHV* and *CTX-M* were detected by PCR. A fresh bacterial colony was suspended in 100 mL of sterile distilled water and boiled at 100°C for 10 minutes. After centrifugation, 3 µL of supernatant was used for PCR assays with the primers described in Table 1. Amplification of DNA was performed in a thermal cycler (Eppendorf, Germany). All of the enzymes of the study were purchased from Thermo, US. Polymerase Chain Reaction elongation times and temperature conditions are described in Table 1. The PCR products were electrophoresed on 1.5% agarose gel and visualized under UV light (Biorad, US) (14).

3.4. Determination of Cotrimoxazole Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) values for cotrimoxazole were determined by broth macrodilution of cotrimoxazole-resistant isolates (n = 30). Only isolates that showed resistance to cotrimoxazole (irrespective of resistance to other antibiotics) were selected for MIC determination. *Escherichia coli* ATCC 25922 was used as the control strain in both Kirby-Bauer disk diffusion and broth macrodilution tests (12).

3.5. Curing of Plasmids by Ethidium Bromide

Techniques were similar as that described by Ansary (16). The R plasmids were studied for curability in cotrimoxazole resistant *E. coli* isolates. Nutrient broth (5 mL) tubes containing 100 µg/mL concentration of ethidium bromide were incubated with log phase cultures of *E. coli* host bearing R plasmids to give a 20-fold dilution. A control tube lacking ethidium bromide was always included. All the tubes were incubated over night at 37°C. The contents of the control tube and of the ethidium bromide-containing tubes were cultured on Muller Hinton Agar (MHA), to obtain isolated colonies, while appropriate dilutions of the culture were inoculated on MHA to obtain single colony isolates. After overnight incubation at 37°C, the resulting colonies were tested for loss of antibiotic resistance on MHA plates containing subMIC concentrations of cotrimoxazole (17).

4. Results

A total of 144 clinical isolates were collected from patients admitted to different wards of the Milad Hospital (Tehran) and various hospitals of Ilam (Imam khomani and Shahid Mostafa), Iran. Out of 144 strains of *E. coli* isolated from urinary tract infections 87 (60.41%), 117 (81.25%), 84 (58.33%), 5 (3.47%) and 59 (40.97%) were resistant to cotrimoxazole, ampicillin, tetracycline, nitrofurantoin and ceftriaxone, respectively (Table 2). Out of 87 cotrimoxazole resistant isolates, 71 (81%) had the *sul1* gene (Figure 1). Fifty-nine (67%) and two (2.29%) of the strains had the *sul2* and *sul3* genes, respectively (Figure 1). Forty-four (50.57%) strains had the *sul2* and *sul1* genes, simultaneously (Figure 1). Two (2.29%) of the isolates possessed all of the studied genes, namely *sul1*, *sul2* and *sul3* (Figure 1). Thirty-four (39.1%) and five (5.7%) of the isolates had the *dfrA1* and *dfrA5* gene, respectively. Sixty-eight (78.2%) strains had the *int1* gene. Furthermore, *dfrA1* and *dfrA5* were present in three (3.4%) of the isolates.

4.1. Polymerase Chain Reaction for the Detection of *blaTEM*, *blaSHV* and *CTX-M* Genes

Among the 144 *E. coli* isolates, seventy-two (50%) ESBL producing and seventy-two (50%) non-ESBL producing *E. coli* isolates were identified. All of the ESBL-producing *E. coli* isolates were tested for detection of *blaTEM*, *blaSHV*

and CTX-M. Our results showed that 85.2% (n = 76), 53.2% (n = 48) and 26.1% (n = 24) of the tested *E. coli* were positive for *blaTEM*, *blaSHV* and *CTX-M*, respectively. Furthermore, 21.4% (n=19), 16% (n=14) and 9% (n=8) of the isolates had the following gene combinations *blaTEM* and *blaSHV*, *blaTEM* and *CTX-M*, and *blaSHV* and *CTX-M*, respectively. Five percent (n = 6) of the isolates had three genes, simultaneously.

4.2. Plasmid Mediated Resistance

The MIC of 30 cotrimoxazole resistant isolates by broth dilution was 700 µg/mL. Curing of plasmids by ethidium bromide in 30 isolates caused disability of bacterial growth on nutrient agar containing sub-MIC concentrations of cotrimoxazole.

Table 1. Primers Used for Polymerase Chain Reaction Detection of *sul1*, *sul2*, *sul3*, *dfrA1*, *dfrA5*, *int1*, *blaTEM*, *blaSHV* and *CTX-M* Genes

Gene	Primer	Size, bp	Annealing Temperature, °C	Reference
<i>sul1</i>	F: 5'-CGGCGTGGGCTACCTGAACG-3'	432	55	Present study
	R: 5'-GCCGATCGCGTGAAGTCCG-3'			
<i>sul2</i>	F: 5'-GCGCTCAAGGCAGATGGCATT-3'	293	53	Present study
	R: 5'-GCGTTTGATACCGGCACCCG-3'			
<i>sul3</i>	F: 5-CAGATAAGGCAATTGAGCATGCTCTGC-3	569	55	Present study
	R: 5-GATTTCCGTGACACTGCAATCATT-3?			
<i>dfrA5</i>	F: 5-ACGGAGTGATTGGTTGCGG-3	279	53	(15)
	R: 5-CTCTGTAATCTCCCCGCC-3			
<i>dfrA1</i>	F: 5-TGGAGTTATCGGGAATGGC-3	334		Present study
	R: 5-AACATCACCTTCCGGCTCG-3			
<i>int1</i>	F: 5-GCCGTTCGGTTCGTAAGCT-3	585	56	(15)
	R: 5-CGGATGTTGCGATTACTTCG-3			
<i>blaTEM</i>	F: 5-ATG AGT ATT CAA CAT TTC CGT-3	861	53	Present study
	R: 5-TTA CCA ATG CTT AAT CAG TGA-3			
<i>blaSHV</i>	F: 5-GGG TTA TTC TTA TTT GTC GC-3	927	53	Present study
	R: 5-TTA GCG TTG CCA GTG CTC-3			
<i>CTX-M</i>	F: 5-ACGCTGTTGTTAGGAAGTG-3	759	55	Present study
	R: 5-TTGAGGCTGGGTGAAGT-3			

Table 2. Frequency of Cotrimoxazole Resistance Genes in *Escherichia coli* Isolates^a

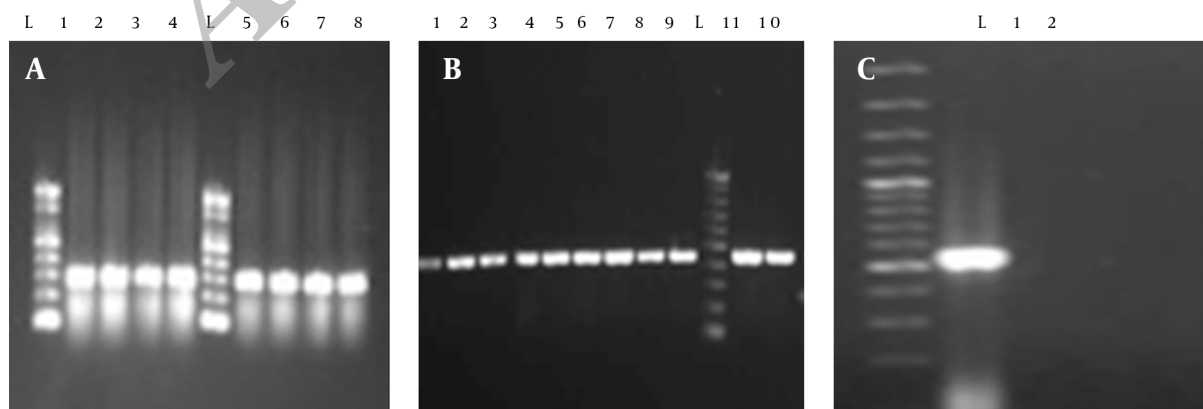
Cotrimoxazole Resistance Genes	Isolates
<i>sul1</i>	71 (81.6)
<i>sul2</i>	58 (66.7)
<i>sul3</i>	2 (2.3)
<i>sul1 + sul2</i>	44 (50.6)
<i>sul1 + sul3</i>	2 (2.3)
<i>sul2 + sul3</i>	2 (2.3)
<i>sul1 + sul2 + sul3</i>	2 (2.3)
<i>dfrA1</i>	34 (39.1)
<i>dfrA5</i>	5 (5.7)
<i>int1</i>	68 (78.2)
<i>dfrA1 + dfrA5</i>	3 (3.4)
<i>dfrA1 + int1</i>	27 (30.3)
<i>int1 + dfrA5</i>	5 (5.7)
<i>dfrA1 + dfrA5 + int1</i>	3 (3.4)
<i>sul1 + sul2 + int1</i>	36 (41.1)
<i>sul1 + int1</i>	25 (28.7)
<i>sul1 + int1 + dfrA1</i>	7 (8.0)
<i>sul1 + dfrA1</i>	26 (29.9)
<i>sul2 + int1</i>	42 (48.3)
<i>sul2 + dfrA1</i>	26 (29.9)
<i>sul2 + int1 + dfrA1</i>	22 (25.3)
<i>int1 + sul1 + sul2 + dfrA1 + dfrA5</i>	1 (1.1)
<i>sul1 + sul2 + sul3 + dfrA1 + dfrA5 + int1</i>	0 (0)
<i>sul3 + int1</i>	2 (2.3)
<i>sul3 + dfrA1</i>	1 (1.1)
<i>sul3 + dfrA5</i>	0 (0)
<i>sul3 + int1 + dfrA1</i>	1 (1.1)
<i>sul3 + dfrA1 + dfrA5 + int1</i>	0 (0)

^a Data are presented as No. (%).

5. Discussion

This is the first report of *sul* genes from Iranian *E. coli* isolates. The overall resistance percentage of isolates against trimethoprim/sulfamethoxazole, ampicillin, tetracycline and ciprofloxacin was very high, and the highest resistance was estimated against ampicillin (81.25%), followed by cotrimoxazole (60.41%). Based on our results the *sul1* gene has the highest prevalence in *E. coli* strains resistant to cotrimoxazole. Frequency of *sul1* (81%) was higher than *sul2* (67%) and *sul3* (2.29%), which is in accordance with other studies conducted worldwide (9, 10). Our observed trends in sulfamethoxazole-resistant allele distributions (*sul1* > *sul3* > *sul2*) were different from previous studies (9, 15, 18, 19). There have been a few studies of the genetic distributions underlying trimethoprim resistance in the world. Our results agree with four European studies, which have found *dfrA1* to be very common in Europe (20, 21). Conversely, studies performed in Korea and Australia found *dfrA17* and *dfrA12* to be the most common alleles (21-23). In this study prevalence of *dfrA1* was more than *dfrA5*.

Existence of *sul* genes in different kinds of clinical and environmental isolates indicates that these genes have a universal function of carrying and spreading sulfonamide resistance in bacteria (6, 18, 24-26). For example in a study of 100 environmental *E. coli* strains, it was revealed that *sul1* (50%) and *sul2* (60%) genes were present in these isolates at a high percentage (18). The *sul1* gene is part of class I integrons in many sulfonamide resistant bacteria (25, 27-30). Class 1 integrons play an important role in antibiotic resistance dissemination in many Multi-drug Resistant (MDR) Gram-negative bacteria, including many zoonotic serovars of *Salmonella enteric* and other enterobacteriaceae (27). We observed regional variation in integron distributions. Prevalence of *int1* was positive in 78% (68) of isolates; coexistence of *dfrA1* and *dfrA5* with *int1* was found in 30% and 5.7% of the isolates, respectively. This indicates a correlation between *Int1* and *dfr1*.

Figure 1. Electrophoresis of the Polymerase Chain Reaction Products of the *Sul* Genes on 1% Agarose Gel

A, L (ladder 100 bp), *sul1* = 432 bp (lane 1-7), lane 8, positive control; B, L (ladder 100 bp), *sul2* = 293 bp (lane 1-9); C, L (ladder 100 bp), lanes 10 and 11, positive control, *sul3* = 569 bp (lane 1), lane 2, negative control.

Other genetic mobile elements have also been found to act as sources of *sul* genes. For example, in SMX resistant *Vibrio cholerae* serogroup O139, it was reported that the *sul2* gene was part of a cluster located on a newly discovered genetic element of the integrative conjugative element group named SXT. The resistance genes of SXT exist in a composite transposon-like structure and were probably acquired recently (30). It is important to mention that frequency and distribution of *sul* genes and trimethoprim/sulfamethoxazole resistance in various bacteria indicates that these genes are transferred mainly by rapid methods including horizontal routes between *E. coli* and other bacteria. Many studies have referred to this point (27, 29).

Resistance to cotrimoxazole was observed in ESBL-producing *E. coli* more than non-ESBL producing *E. coli* isolates. Frequency of *bla*TEM was higher than *bla*SHV and *CTX-M*. Coexistence of ESBL genes with *sul* genes in some of the strains probably indicated that these genes are present on one plasmid. *Escherichia coli* isolates with both *sul1* and *sul2* were found in ESBL-producing, and non-ESBL producing *E. coli* isolates. The majority of resistance genes in Gram-negative bacteria exist on cytoplasmic plasmids that spread antibiotic resistance in epidemics. Our results indicated that all of the resistant isolates had *sul* genes and lost cotrimoxazole resistance after plasmid curing. Sulfonamides are broad-spectrum antimicrobials against Gram-negative and Gram-positive organisms, and have bacteriostatic activities (31).

High prevalence of sulfonamide resistance has been observed in Gram-negatives isolated from human and animals, worldwide (32). According to previous studies, *sul1* and *sul2* genes equally exist in sulfonamide resistant isolates. However, in Denmark, it was revealed that the prevalence of *sul2* is higher than *sul1* gene in *E. coli* strains isolated from humans (24). Grape et al. (25), in their study on *sul3* gene in *E. coli* strains isolated from human resources, reported that out of 64 sulfonamide resistant isolates, 39 and 48 isolates carried *sul1* and *sul2* genes, respectively. In contrast, 25 isolate simultaneously had *sul1* and *sul2* genes, while two strains were lacking both of these genes (25). The *sul2* gene has been observed with high prevalence in *E. coli* strains isolated from pig, poultry, cows, human feces and urinary tract infections (6). According to the studies of Wu et al. (7), *sul3* gene is least prevalent in *E. coli* isolates from human and animal resources. Out of 501 isolates, 109 cases were resistant to sulfonamides. Relative prevalence of *sul2*, *sul1* and *sul3* genes in this study were 65%, 45% and 12%, respectively, which is different with our results. However, co-existence of class I integrons also existing in plasmids carrying *sul1*, *sul2* and *sul3* genes was found in 80%, 100% and 5% of isolates, respectively; this finding is similar with our results, yet the prevalence of this coexistence in our study was lower than that of Wu et al. (7).

Medina et al. (33) in their study of the resistance pattern of more than 400 pathogenic *E. coli* from ruminants re-

ported very high percentage of resistance, and in their studied strains resistance rate was 76.3% (*dhfr1*), 60% (*sul1*) and 63.3% (*sul2*) for trimethoprim and sulfamethoxazole. Al-Agamy (34) in a comprehensive study of susceptibility patterns of 100 uropathogenic *E. coli* isolates showed that resistance rates for ampicillin, tetracycline, trimethoprim, sulfamethoxazole/trimethoprim and ciprofloxacin, were 90%, 85%, 70%, 62% and 24%, respectively. The prevalence of the antibiotic resistance genes for *sul1*, *sul2* and *sul3* were 68.18%, 86.36% and 5.5%, respectively. The *sul2* was the most prevalent resistance gene. The prevalence of class 1 integron was reported to be 95.45% in this study (34). Infante et al. (26) in their study of sulfonamide resistance genes of 20 cotrimoxazole resistant *E. coli* isolates from children's feces found that 19 out of 20 strains carried at least one *sul* gene. Furthermore, *sul1* and *sul2* genes were detected in 13 isolates, while one isolate carried *sul3* and *sul2* genes. The *sul2* gene was the most prevalent and was observed in 11 strains, while *sul1* was detected in four strains (26). In another study of 350 uropathogenic *E. coli* isolates from Europe and Canada, *sul2* (77.9%) was reported as the most common sulfamethoxazole resistant gene in sulfamethoxazole-resistant isolates (28). Recently, numerous studies have been conducted in Iran on cotrimoxazole resistance. Results of such reports indicated that high rate of resistance against cotrimoxazole are present in different genera including *E. coli* (35).

High frequency of *sul* genes, plasmid related resistance and high prevalence of SXT resistance in *E. coli* isolates indicates that continuous surveillance programs should be implemented in hospital and clinical settings to better control and treat related diseases, and monitor the trends of SXT resistance in Gram-negative bacteria.

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Authors' Contributions

Authors' Contributions: Study concept and design: Iraj Pakzad; acquisition of data: Hadis Arabi, and Naser Samadi; analysis and interpretation of data: Ayat Nasrollahi, Morovvat Taherikalani, and Farid Azizi Jalilian; drafting of the manuscript: Hasan Hosainzadegan; critical revision of the manuscript for important intellectual content: Hasan Hosainzadegan; statistical analysis: Ayat Nasrollahi, Morovvat Taherikalani, and Farid Azizi Jalilian; administrative, technical, and material support: Alireza Monadi Sefidan; study supervision: Iraj Pakzad.

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