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Antimicrobial Susceptibility and Molecular Detection of Integrons, Sulfonamides and Trimethoprim Resistance of Extra Drug-Resistant *Escherichia coli* Isolates

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Abstract

Background: *Escherichia coli*, a gram-negative bacterium, is the causative agent for approximately 80% of urinary tract infections (UTIs). UTI treatment has resulted in the overuse of antibiotics in hospitals and communities, and subsequently the increase of antimicrobial resistance. The emergence of extensively drug resistance (XDR) strains has become a costly and dangerous challenge in the treatment of most bacterial infections and UTIs.

Objective: This study aimed to determine the frequency of XDR isolates and investigate the distribution of common sulfonamide- *(sul1, sul2, & sul3)* and trimethoprim *(dfrA1, dfrA12, & dfrA14)*-related resistance genes among *E. coli* isolates from UTI patients. Furthermore, the isolates were sought for the presence of class 1 and class 2 integrons *(Int1 & Int2)* among XDR *E. coli* isolates.

Materials and Methods: 120 uropathogenic-*E. coli* isolates recovered from UTI cases in Mashhad were assessed in 2017-2019. Overall, 39 out of 120 isolates were identified as XDR *isolates* as they were resistant to all classes of tested antibiotics, except for two or fewer comprising quinolones (first and second generation), cephalosporins (first and third generation), penicillins, tetracyclines, and sulfonamide-trimethoprim.

Results: The antimicrobial susceptibility testing (AST) results determined a substantial resistance rate against cloxacillin (98.3%), oxacillin (98.3%), and cephalexin (94.17%). According to polymerase-chain reaction results, *sul1* and *dfrA14* genes with the frequency of 35 (89.74%) and 28 (71.79%) were identified as the most prevalent resistant genes among XDR isolates. In addition, *int1* and *int2* genes were detected among 23 (58.9%) and 8 (20.5%) XDR isolates, respectively. In conclusion, the substantial distribution of *sul1* and *dfrA14* genes was highlighted among XDR *E. coli* isolates recovered from UTI.

Conclusion: Based on the present research findings, class I integrons play a major role in the dissemination of resistance gene cassettes, including *sul* and *dfr* in XDR isolates, and should be investigated in the future.

Keywords: Antimicrobial resistance, *Escherichia coli*, XDR, Urinary tract infections, *sul* genes, *dfr* genes, integrons

Background

Urinary tract infections (UTIs) have been recognized as one of the most common infections that result in the increased use of antibiotics worldwide.[1] As a gram-negative bacterium, *Escherichia coli* is isolated from approximately 80% of UTIs that introduce this bacterium as a significant etiologic pathogen.[2] Several systematic review studies have reported that 50% of women and 12% of men suffer from UTIs at least once, while recurrent UTIs have been found in approximately 30% of women.[3,4] Although UTIs are often considered self-limiting infections, it is recommended that an appropriate antibiotics be administered to avoid complicated UTIs; therefore, the lack of precise diagnosis and inappropriate antibiotic therapy are significant risk factors causing complicated UTIs and increasing antimicrobial resistance.[5,6]

However, UTI treatment results in overusing antibiotics in hospitals and communities; subsequently, increasing the multi-drug resistant (MDR), extensively drug resistance (XDR), and pan-drug resistant (PDR) strains have become a serious worldwide concern.[7] A global review of evidence about antimicrobial resistance has revealed 700 000 annual mortalities due to antibiotic-resistant infections which is probably rising to 10 million by 2050.[8,9] This has led to the restriction of, the treatment of UTIs using available antibiotics; to the latest guidelines by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Disease, the first-line treatment of uncomplicated UTIs

consists of nitrofurantoin monohydrate/macrocrystals and trimethoprim-sulfamethoxazole.[10] Patients should be treated with first-line antibiotics that among the local *Escherichia coli* population, have a good effect on preventing further development of MDR, XDR, and PDR. However, to make the best possible empirical choice, it is crucial to periodically assess antibiotic susceptibility in commonly treated populations with UTIs.[2,11]

On the other hand, the mainly acquired resistance mechanisms against sulfonamide and trimethoprim have been defined through mutations and alterations in genes *encoding* the dihydropteroate synthase or dihydrofolate reductase as target enzymes or by harboring the responsible genes for encoding dihydropteroate synthetases (*sul*) or insensitive dihydrofolate reductases (*dfr*) that are insensitive to sulfonamide and trimethoprim, respectively.[12] Further, integrons (*Int*) are highly reported as important factors that carry resistance gene cassettes that increase the dissemination of antimicrobial resistance genes.[13] Thus, multiple *sul* and/or *dfr* genes on integrons contribute to spreading resistance.[12]

Nowadays, physicians in Iran rarely prescribe first-line treatment alternatives, which may worsen resistance development and limit our choice to save new drugs for the last resort treatment in complicated cases. As a result, it appears necessary to evaluate whether first-line alternatives such as sulfonamides and trimethoprim are still effective against uropathogenic *E. coli*. Therefore, the current study sought to determine the frequency of XDR isolates and the rate of common sulfonamide-(*sul1, sul2, & sul3*) and trimethoprim (*dfra1, dfra12, & dfra14*)-related resistance genes in *E. coli* isolates from UTI patients in Mashhad, Iran in 2017-2019. In addition, class 1 and class 2 integrons (*Int1 & Int2*) were looked for in XDR *E. coli* isolates.

Materials and Methods

Bacterial Sample Collection

The current study was conducted on 120 uropathogenic-*E. coli* (UPEC) isolates recovered from uncomplicated UTI cases. All isolates were collected from patients with clinical UTIs in 2017-2019. The etiologic pathogen was then identified as *E. coli* using the biochemical standard tests and detecting a specific gene, namely, *cdgR* (a cyclic di-GMP regulator) gene for *E. coli* according to previous research [14]. Eventually, the identified *E. coli* isolates were stored at a -70°C refrigerator in trypticase soy broth, and 15% glycerol was added to them.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of isolates was evaluated against nine antibiotics using the Kirby-Bauer disk diffusion method as recommended by Clinical & Laboratory Standards Institute (CLSI) 2021.[15] The nine tested antibiotics were cefalexin (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), nitrofurantoin (300 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), doxycycline (30 µg), and trimethoprim-sulphamethoxazole (1.25/23.75 µg). *E. coli* ATCC 25922 was implied as a positive control strain. The interpretation of antimicrobial susceptibility testing (AST) was accomplished according to the CLSI 2021. The XDR isolates were determined regarding non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates remained susceptible to only one or two categories) for the investigation of the frequency of resistance genes.[16]

Molecular Detection of Antimicrobial Resistance

First, the DNA extraction of sulfonamide resistance isolates was performed using the boiling method based on an earlier study.[17] The multiplex-polymerase chain reaction (m-PCR) was set up for the molecular determination of sulfonamide resistance genes (*sul1 & sul2*); moreover, another sulfonamide resistance gene (*sul3)*, the integrons (*Int1, 2*), and trimethoprim resistance genes (*dfrA1, dfrA12, & dfrA14*) were detected through uniplex-PCR (u-PCR). The PCR was set up at the final 25 µL, consisting of 12.5 µL of PCR 2×Master Mix (Amplicon, Denmark) containing Taq DNA Polymerase, reaction buffer (including Tris-HCL, potassium chloride, and magnesium chloride), and dNTPs mixture, a protein stabilizer, and the convenience for use was optimized by adding sediment for electrophoresis and 2×solution of loading dye, 0.5 µL of each primer (2 µM), 2 µL of template DNA, and up to 25 µL the final volume used nuclease-free water. Further PCR information, including the oligonucleotide primer sequences and annealing temperature, is listed in Table 1.

Statistical Analysis

Analyses were accomplished using SPSSTM software, version 22.0 (IBM Corp., USA). The results are presented as descriptive statistics in terms of the relative frequency. Values were expressed as the mean ± standard deviation or group percentages (categorical variables). Fisher's exact statistical test was performed to analyze the data, and $P \leq 0.05$ was considered statistically significant.

Results

Molecular Confirmation of Escherichia coli Strains

All 120 isolates were confirmed by amplifying the *cdgR* (cyclic di-GMP regulator) gene using the PCR method.

Antimicrobial Susceptibility Testing

Accordingly, to the antimicrobial resistance patterns, 39 out of 120 isolates were identified as XDR *isolates* (Table 2). The AST results revealed a substantial resistance rate against cloxacillin, oxacillin, and cephalexin (98.3%, 98.3%, and 94.17%), respectively, followed by nalidixic

Table 1. The Sequence of Oligonucleotides Used as Primers

Primer's Name	Primer Sequence (5´-3´)	Product Size (bp)	Annealing Temperature (°C)	References
sul1	F- CGGCGTGGGCTACCTGAACG R- GCCGATCGCGTGAAGTTCCG	433	67	[18]
sul2	F- GCGCTCAAGGCAGATGGCATT R- GCGTTTGA-TACCGGCACCCG	285	67	[18]
sul3	F- GAGCAAGATTTTTGGAATCG R- CTAACCTAGGGCTTTGGATAT	790	53	[18]
dfrA1	F- TGGTAGCTATATCGAAGAATGGAGT R- TATGTTAGAGGCGAAGTCTTGGGTA	425	52	[19]
dfrA12	F- TTTATCTCGTTGCTGCGATG R- TAAACGGAGTGGGTGTACGG	457	58	[20]
dfrA14	F- GTTGCGGTCCAGACATAC R- CCGCCACCAGACACTA	253	54	[20]
int1	F- GGCATCCAAGCAGCAAG R- AAGCAGACTTGACCTGA	Variable	58	[21]
int2	F- CGGGATCCCGGACGGCATGCACGATTTGTA R- GATGCCATCGCAAGTACGAG	Variable	56.5	[21]
cdgR	F- CCAGGCAAAGAGTTTATGTTGA R- GCTATTTCCTGCCGATAAGAGA	212	57	[14]

Table 2. The Antimicrobial Resistance Patterns of XDR *Escherichia coli* Isolates From UTI Patients (n = 39)

Antimicrobial Category	Antimicrobial Agent	No. of Resistance (%)		No. of Intermediate (%)		No. of Sensitive (%)	
		None XDR Isolates: n = 81	XDR Isolates: n = 39	None XDR Isolates: n = 81	XDR Isolates: n = 39	None XDR Isolates: n = 81	XDR Isolates: n = 39
Quinolones; 1st generation	Nalidixic acid	47 (58%)	39 (100%)	2 (2.46%)	0	32 (39.5%)	0
Quinolones; 2nd generation	Ciprofloxacin	29 (35.8%)	38 (97.4%)	1 (1.23%)	1 (2.56%)	51 (62.96%)	0
	Norfloxacin	29 (35.8%)	38 (97.4%)	2 (2.46%)	1 (2.56%)	50 (61.73%)	0
Cephalosporins; 1st generation	Cephalexin	74 (91.35%)	39 (100%)	0	0	7 (8.64%)	0
Cephalosporins; 3rd generation	Ceftriaxone	33 (40.74%)	38 (97.4%)	4 (4.93%)	1 (2.56%)	44 (54.32%)	0
	Cefotaxime	40 (49.38%)	39 (100%)	15 (18.51%)	0	26 (32.1%)	0
Penicillins	Cloxacillin	79 (97.5%)	39 (100%)	2 (2.46%)	0	0	0
	Oxacillin	79 (97.5%)	39 (100%)	1 (1.23%)	0	1 (1.23%)	0
Tetracycline	Doxycycline	36 (44.44%)	35 (89.74%)	17 (20.98%)	4 (10.26%)	28 (34.56%)	0
Sulphonamide-trimethoprim	Sulfamethoxazole	25 (30.86%)	39 (100%)	4 (4.93%)	0	52 (64.19%)	0

Note. XDR: Extra drug resistance; *E. coli: Escherichia coli*; UTI: Urinary tract infection.

acid (71.7%). Furthermore, the most effective ones were trimethoprim-sulphamethoxazole (43.3%), ciprofloxacin (42.5%), and norfloxacin (41.7%), respectively. The additional information on the resistance patterns of isolates is depicted in Figure 1.

Prevalence of sul, dfr, Int Class1, and Int Class 2 Genes

All XDR isolates (39 out of 120) were investigated for demonstrating the distribution of *sul1, sul2, sul3, dfra1, dfra12, dfra14, Int1, and Int2 genes*. According to the results, *sul1* and *dfrA14* genes, with a frequency of 35 (89.74%) and 28 (71.79%), were identified as the most resistant genes among XDR isolates. Additionally, the co-harboring of resistance genes was observed for *sul1* and *sul2* genes within 15 (38.46%) and *dfrA1* and *dfrA14* genes within 8 (20.51%) of XDR isolates. In addition, *int1* and *int2* genes were detected among 23 (58.9%) and 8 (20.5%) XDR isolates, respectively; further, five isolates (12.8%) contained both (Figure 2). Furthermore, 95.65% of *int1* positive isolates (22/23) were positive for harboring *sul1* or *sul2* genes; moreover, 100% of *Int2* positive isolates (8/8) contained the *dfra14* gene. The statistical analysis evaluated the comparison of *sul* and *dfr* genes within the *Int1* and *Int2 positive isolates*. As a result, only the prevalence of *sul2* positive isolates among the *Int1* harboring was significant ($P = 0.043$; Table 3).

Discussion

Over 150 million cases of UTI diseases per year have been reported worldwide, subsequently causing a 6-billion-US dollar cost for treatment.[22,23] UTIs have been managed using oral antibiotics consisting of cephalosporins,

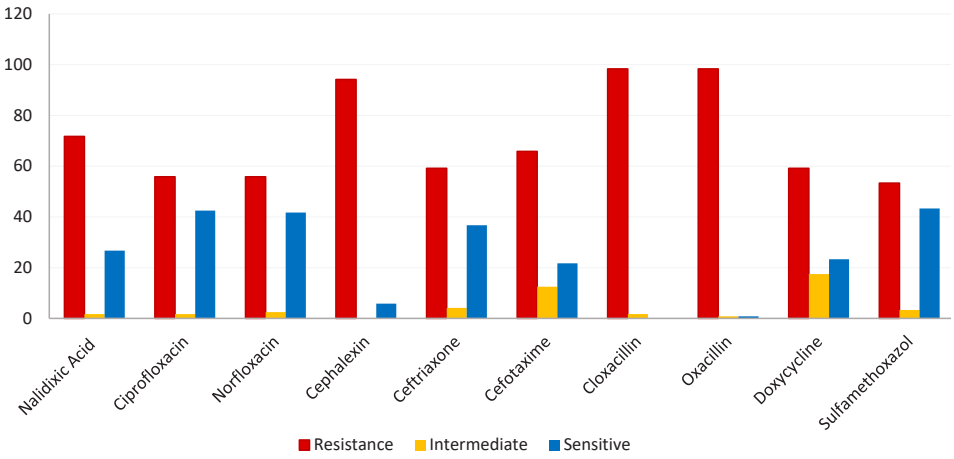

Figure 1. The Antimicrobial Susceptibility Patterns of *Escherichia coli* Isolates From UTI Patients (n = 120). *Note.* UTI: Urinary tract infection

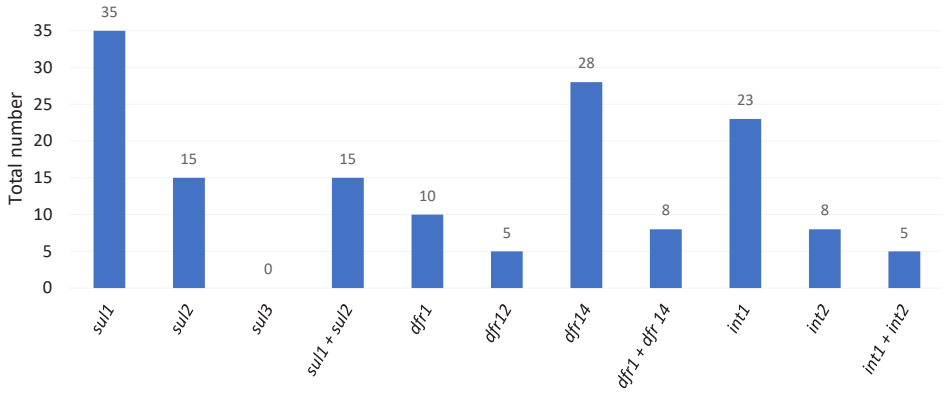

Figure 2. The Frequency of *sul* and *dfr* Genes Among XDR *Escherichia coli* Isolates From UTI Patients (n = 39). *Note.* XDR: Extra drug resistance; UTI: Urinary tract infection

Table 3. Prevalence of *sul, dfr, Int* class 1 and *Int* Class 2 and the Association of Class 1 and 2 Integrons With *sul 1, 2* and *dfrA 1, 12, 14* Resistance Genes Among XDR *Escherichia coli*

Genes		Integron 1	*P* Value	Integron 2	*P* Value
	Total (%)	23		8	
sul1	35 (89.74)	21	>0.99	3	>0.99
sul2	15 (38.46)	13	0.043 *	4	0.69
drf1	10 (25.64)	4	>0.149	4	0.17
dfr12	5 (12.82)	1	>0.068	0	0.5
dfr14	28 (71.79)	16	0.7	8	0.07

Abbreviation: XDR: Extra drug resistance.
*P values (by Fisher's exact test) are shown when <0.05.

trimethoprim-sulfamethoxazole, and fluoroquinolones.[24] However, overusing these antibiotics has recently developed resistant strains against mentioned antibiotics that restricted antibiotic therapy.[25] Indeed, the appropriate treatment of UTIs is one of the most serious problems regarding raising MDR, XDR, and PDR strains.

In the current study, 39 out of 120 (32.5%) *E. coli* isolates were XDR, which is higher than in prior studies in Iran and other regions (14-24.3%).[25-27] However, Yuan et al reported a high frequency of XDR (64%) in China.[28] Interestingly, in a comprehensive Australian laboratory-based retrospective assessment, Fasugba et al demonstrated the 0.2% XDR rate among UTI *E. coli* isolates during a 5-year assessment.[29] This comparison has shown that observation and appropriate antibiotic supervision can reduce and control increased antimicrobial resistance.

Considering the AST results, a significantly resistant rate of UTI isolates was revealed against β-lactam families (oxacillin, cephalexin, and cloxacillin, >94%). On the other hand, the highest activity was observed for co-trimoxazole, ciprofloxacin, and norfloxacin with a sensitivity of 43.3%, 42.5%, and 41.7%, respectively. These findings are correlated with those of a recent meta-analysis study by Jabalameli et al, representing the high distribution of extended-spectrum β-lactamase producing *E. coli* in Iran.[30] However, the rate of resistance against β-lactams families was reported as the lowest one in another meta-analysis survey by Bunduki et al, analyzing the AST reports of UPEC worldwide.[13] Several reasons such as differences in tested antibiotics and sample

sizes can explain this controversy. Regarding the present findings, co-trimoxazole, ciprofloxacin, and norfloxacin might be practical choices for empirical therapy.

The PCR results confirmed a co-harboring of *sul1* and *sul2* genes among 15 (38.4%) XDR isolates. In contradiction with earlier studies that reported 44.150.6%, respectively,[31,32] our finding is in line with that of another study in southwest Iran by Boroumand et al, reporting the frequency of co-existing *sul1* and *sul2* genes by 37.3%.[33] Surprisingly, the *dfrA14* gene was the predominant gene (71.7%) among different tested variants of *dfrA* genes. As reported in prior surveys, the *dfrA14* gene was common within isolates from animals[20,34]; consequently, the *dfrA14* gene is disseminated among *E. coli* strains in the environment, which raises resistance strains.

As a classical structure, integrons are involved in the bacteria evolution through reading cassette frameworks.[35] In the current survey, 26 out of 39 XDR isolates (66.6%) contained either *Int1* or/and *Int2*; this supported the statement that integrons exert a highlighted role in developing antimicrobial-resistant, particularly among Gram-negative bacteria.[36] No significant difference was found with the earlier experiment that reported 76.7% positive isolates for the presence of integrons[37]; Further, the high prevalence of *intl1* was observed among isolates in several studies.[38-40] However, the present study reported a lower incidence of *intl1* compared with the mentioned studies. Nevertheless, as previously reported by Khamesipour and Tajbakhsh, class 1 integrons with different gene cassette arrays and *sul1* genes were highly common in Enterobacteriaceae; likewise, the remarkable co-existence of *sul1* or *sul2* genes among *Int1* positive isolates has been determined in a currently running survey.[41] This observation has been confirmed with earlier studies.[32,42] Although all *Int2* positive isolates were *dfra14* gene positive as well, there was no significant relationship between the presence of these genes. However, a literature review study by Sabbagh et al introduced the *dfra14* gene as a new cassette gene for *Int2*.[35] The most important reason for these differences in the obtained results was the difference in the number of tested strains. In most studies, more than 100 strains have been used, while in the present study, the number of strains was 39.

Further experiments are essential for analyzing and characterizing integrons' structures and determining the relationship of cassette genes with integrons.

Conclusion

The results have outlined the most activity for co-trimoxazole and quinolones among tested antibiotics, indicating that they can still be helpful as first-line treatment. However, a significant rate of XDR phenotype among *E. coli* isolates from UTI patients was detected as well. The highlighted prevalence was demonstrated for *sul1* and *dfrA14* genes among XDR isolates. Regarding the high distribution of integrons within tested isolates, it can be concluded that integrons influence the dissemination of the *dfrA14* gene among *E. coli* isolates from different sources.

Author Contributions

The study was conceptualized by: Zahra Sabeti, Gholamreza Hashemitabar, Mahdi Askari Badouei, Vahid Soheili. The experiments and data curation was conducted by Zahra Sabeti. The data analyzed by Zahra Sabeti, Fatemeh Aflakian, Vahid Soheili, Gholamreza Hashemitabar, Mahdi Askari Badouei. The project was supervised by Gholamreza Hashemitabar and Mahdi Askari Badouei.

The first draft was written by Zahra Sabeti and Fatemeh Aflakian. All authors edited the manuscript and approved the final version.

Conflict of Interest Disclosures

The authors declare that they have no competing interests.

Ethical Approval

The isolates were from the bacterial collection. Few isolates were obtained upon the written consent.

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