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ORIGINAL ARTICLE

High Prevalence of Class 1 to 3 Integrons Among Multidrug-Resistant Diarrheagenic *Escherichia coli* in Southwest of Iran

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Abstract
Objectives: Horizontal transfer of integrons is one of the important factors that can contribute to the occurrence of multidrug-resistant (MDR) bacteria. This study aimed to determine the prevalence of integrons among MDR *Escherichia coli* strains isolated from stool specimens and investigate the associations between the existence of integrons and MDR properties in the southwest of Iran.
Methods: There were 164 *E. coli* strains isolated from January 2012 to June 2012. Fecal specimens identified as *E. coli* by the conventional methods. Subsequently the antibiotic resistance was assessed using Clinical and Laboratory Standard Institute criteria. The presence of class 1–3 integrons and embedded gene cassettes was verified using specific primers by multiplex polymerase chain reaction assay.
Results: Among a total of 164 studied samples, 69 (42.07%) isolates were multidrug resistant. Class 1 and class 2 integrons were present in 78.26% and 76.81% MDR isolates, respectively. For the first time in Iran, class 3 integron was observed in 26.09% MDR isolates. Significant correlations were identified between: class 1 integron and resistance to amikacin, gentamicin, chloramphenicol, ampicillin, tetracycline, nalidixic acid, and co-trimoxazole; class 2 integron and resistance to aminoglycosides, co-trimoxazole, cefalexin, ampicillin, and chloramphenicol; and class 3 integron and resistance to gentamicin, kanamycin, and streptomycin.
Conclusion: Our results indicate that integrons are common among MDR isolates and they can be used as a marker for the identification of MDR isolates. Therefore, due to the possibility of a widespread outbreak of MDR isolates, molecular surveillance and sequencing of the integrons in other parts of the country is recommended.

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1. Introduction

The appearance of resistance to various antimicrobial agents in pathogenic bacteria has become an important public health threat. Isolates are categorized as: pandrug resistance, extensive drug-resistance, multidrug resistance (MDR), and nonmultiresistant, if resistance is found to all, to all except 1 or 2, to ≥3, and to <3 antibiotic classes, respectively [1,2].

The phenomenon of MDR is a major health care problem among pathogenic bacteria such as *Escherichia coli,* where it is associated with increased mortality and morbidity, worldwide [3,4]. Although it is clear that antibiotic utilization is pivotal in the selection of bacterial resistance, the facile spread of resistance genes has been a fundamental force in the rapid evolution of resistance to a wide range of unrelated antibiotics among diverse bacteria.

The spread of antimicrobial resistance in bacteria is a complex process involving a diversity of different mechanisms. Susceptible bacteria may obtain resistance through mutations or the transfer of resistance genes located on mobile DNA elements such as integrons [5,6]. MDR in intestinal bacteria such as *Escherichia coli* is known to be associated with integrons [7].

Integrons were defined by Hall and Collis as DNA elements that function as gene-capture and expression systems [8,9,12]. This element contains three necessary components located within the 5′ conserved segment include: an integrase gene (*IntI*), which encodes a site-specific recombinase enzyme; an *attI* site [8], which is recognized by the integrase and acts as an acceptor for gene cassettes; and a promoter region (PC) [7,10,11]. Gene cassettes become a part of the integron when integrated [12−14].

Although integrons are not mobile, they can be transferred between bacteria by transposons or plasmids in which they are present. Accordingly, integrons are a major mechanism for the spread of multidrug resistance [15]. Three types of integrons, each with different *int* genes have been identified (*IntI1*, *IntI2*, and *IntI3*) that are known to be associated with antibiotic resistance [7].

Class 1 integrons have been reported in many Gram-negative bacteria, including *Acinetobacter*, *Vibrio*, *Aeromonas*, *Proteus*, *Burkholderia*, *Alcaligenes*, *Campylobacter*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Mycobacterium, Pseudomonas*, *Serratia*, *Salmonella, Shigella*, and *Escherichia* [14]. Class 2 integrons are embedded in the Tn7 family of transposons and have been found in *Salmonella*, *Acinetobacter, Escherichia, Shigella*, *Aeromonas*, and *Morganella* [13]. Class 3 integrons appear to be much less common and so are less involved in the spread of multidrug resistance. Class 3 integrons have been described in *Acinetobacter* spp., *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Escherichia coli, Serratia marcescens*, *Alcaligenes xylosoxidans*, *Pseudomonas putida, Salmonella* spp., *Klebsiella pneumonia*, and *Delftia* spp. [13,14,16].

Several studies have investigated prevalence of integrons in MDR *Escherichia coli* isolates around the world. These studies found a substantial association between the presence of integrons and antibiotic resistance. However, there is not enough information available on spread of class 1−3 integrons and their association with MDR in diarrheagenic *Escherichia coli* in our area of research. This study aimed to assess the prevalence of three classes of integrons in a MDR diarrheagenic *Escherichia coli* strains isolated from children <5 years in the southwest of Iran and investigates associations between MDR and the existence of integrons.

2. Materials and methods

2.1. Type of study and microorganism identification

This descriptive cross-sectional study was performed on a total of 164 fecal samples were recovered from children aged between 1 month and 5 years, referred to Yasouj Hospital (Emam Sajad Hospital, Dena and Yazdanpanah private laboratories, Yasouj-Iran), in a period of 5 months (January 2012 to June 2012). Fecal samples were seeded on eosin methylene blue agar plates and incubated at 37°C for 24 hours. One colony from each sample with a typical *Escherichia coli* morphology was recovered and verified by standard biochemical tests (Triple Sugar Iron, Sulfide indole motility, Methyl Red/Voges-Proskauer, Lysine Iron Agar, citrate, urea). After identification, the isolates were subcultured in tryptic soy broth (Oxoid, UK) and were then stored at −70°C for further investigations.

2.2. Antibiotic susceptibility testing

Antimicrobial susceptibility of all isolates was determined using the standard Kirby−Bauer disk diffusion method according to Clinical and Laboratory Standard Institute guidelines [17].Antimicrobial agents tested were, ampicillin, nalidixic acid, cefalexin, sulfamethoxazole-trimethoprim, gentamicin, tetracycline, streptomycin, kanamycin, chloramphenicol, and amikacin (Padtanteb, Tehran-Iran). The *E. coli* ATCC 25922 strain was used as a reference isolate. Intermediate sensitivity was considered as resistance.

2.3. Polymerase chain reaction

Extraction of genomic DNA from isolates was performed by the boiling method [3]. Briefly, a single colony of each organism was inoculated from a blood agar plate into 5 mL of Luria−Bertani broth (Sigma−Aldrich, Munich, Germany) and incubated for 20 hours at 37°C. Cells from the overnight culture were harvested by centrifugation at 12,000 × *g* for 5 minutes. Then the supernatant was decanted and the pellet was

resuspended in 300−400 μL of sterile distilled water. Later the cells were lysed via heating at 95°C for 10 minutes and any cell debris was removed by centrifugation for 5 minutes at 12,000 × *g*. The supernatant was stored at −20°C and used as the source of the template for amplification.

The presence of class 1−3 integrons in all MDR *E. coli* isolates was tested by multiplex polymerase chain reaction (PCR) using primers specific for integrases genes of the integron, *intI1*, *intI2*, and *intI3* (Table 1). The size of variable regions of class 1 and 2 integrons was determined by PCR assay. Primer sequences, sizes of PCR products, and PCR conditions are shown in Table 1. A tube containing PCR reaction without any DNA template was used as a negative control. All primers were obtained by Cinnagene Co. (Tehran, Iran).

The multiplex PCR assay was performed as follows. Each 50 μL of reaction mixture contained 0.5 μL dNTPs, 0.75 μL of each primer, 1.5 mM $MgCl_2$, 2.5 μL 10× reaction buffer (10 mM Tris-HCl, 50 mM KCl), 0.25 μL of *Taq*DNA polymerase, and 5 μL of template DNA. The expected amplicons were analyzed by electrophoresis on 1.5% w/v agarose gel in TBE buffer.

2.4. Statistical analysis

Statistical analyses were performed by SPSS software version 15 (SPSS Inc., Chicago, IL, USA). The Chi-square test was used to calculate the association between antibiotic resistance and integration existence. The significance level was defined as $p < 0.05$.

3. Results

E. coli isolates were collected from 164 clinical specimens of patients referred to Yasouj Hospital. The isolates were obtained from children aged <5 years. Sixty-nine isolated strains (42.07%) were designated as MDR, with 23 (33.33%) isolates from female and 46 (66.67%) from male patients. The percentage of the resistant isolates to the tested antimicrobials is presented in Figure 1. Of the 69 MDR *E. coli* isolates, 54 (78.26%) isolates were identified as being positive for class 1 integron. PCR amplification of the integron cassette region occurred in 48 (69.56%) class 1 integron-containing isolates (Table 2). Class 2 integron was detected in 53 (76.81%) isolates. The integron cassette region could not be amplified by PCR in 24 (45.28%) of the class 2 integron-containing isolates (Table 3). The frequency of simultaneous occurrence of integrons is depicted in Figure 2. For the first time in Iran, class 3 integron was observed in 18 (26.09%) isolates. A significant correlation was revealed between class 1 integron and the gene cassettes with resistance to amikacin ($p = 0.027$), gentamicin ($p = 0.040$, $p = 0.001$), chloramphenicol ($p = 0.026$), ampicillin ($p = 0.018$, $p = 0.000$), tetracycline ($p < 0.001$), nalidixic acid ($p = 0.026$), and co-trimoxazole ($p = 0.032$, $p = 0.015$); and also between class 2 integron and gene cassette with resistance to kanamycin ($p = 0.006$), streptomycin ($p = 0.006$), amikacin ($p = 0.005$), gentamicin ($p = 0.000$), cefalexin ($p = 0.029$), co-

Table 1. Primers and PCR conditions used in this study.

Gene	Primer sequence	Size of product (bp)	PCR conditions	Reference
IntI1-F	GGT CAA GGA TCT GGA TTT CG	436 bp	5 min at 94°C; 32 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C; 10 min at 72°C	Machado, 2005
IntI-R	ACA TGC GTG TAA ATC ATC GTC			
IntI2-F	CAC GGA TAT GCG ACA AAA AGG	788 bp	5 min at 94°C; 32 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C; 10 min at 72°C	Machado, 2005
IntI2-R	TGTA GCA AAC GAG TGA CGA AAT G			
IntI3-F	AGT GGG TGG CGA ATG AGT G	600 bp	5 min at 94°C; 32 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C; 10 min at 72°C	Machado, 2005
IntI3-R	TGT TCT TGT ATC GGC AGG TG			
5'CS	GGC ATC CAA GCA GCA AG	Variable	5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 58°C, 2 min at 72°C; 10 min at 72°C	Machado, 2005
3'CS	AAG CAG ACT TGA CCT GA			
attI2-F	GAC GGC ATG CAC GAT TTG TA	Variable	5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 58°C, 2 min at 72°C; 10 min at 72°C	Machado, 2005
orfX-R	GAT GCC ATC GCA AGT ACG AG			

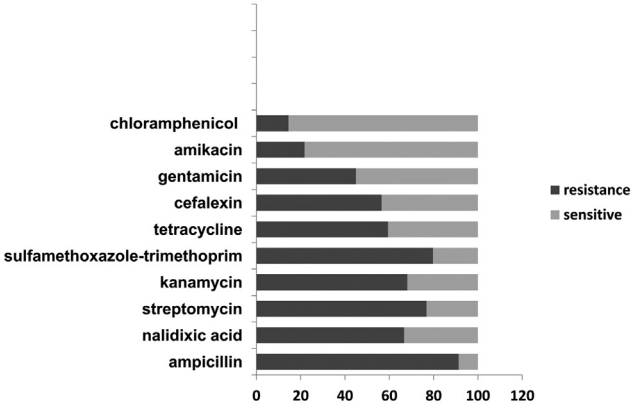

Figure 1. The percentages of antimicrobial resistance detected among multidrug-resistant *E. coli* isolates.

trimoxazole ($p = 0.021$), ampicillin ($p = 0.029$), and chloramphenicol ($p = 0.009$). In addition, a significant association was found between class 3 integron and resistance to gentamicin ($p = 0.031$), kanamycin ($p = 0.039$), and streptomycin ($p = 0.007$).

4. Discussion

Acceleration of the frequency and spectrum of antimicrobial resistant infections in recent years is a major public health concern. Investigations have suggested that regardless of antibiotic consumption pattern, resistance genes could be transferred between bacterial populations. The acquisition of resistance genes by horizontal transfer is currently thought to play a major role in the development of MDR strains [18].This study aimed to investigate the role of class 1−3 integrons in antibiotic resistance MDR diarrheagenic *E. coli* isolates.

In this study, MDR *E. coli* isolates with resistance to three or more different antibiotics were common. Sixty-nine isolates (42.07%) had the MDR phenotype, which is similar to the rate of MDR reported in *E. coli* isolates by Rezaee et al [3]. MDR *E. coli* isolates in our study were highly resistant to ampicillin (91.30%). High-level resistance to ampicillin (100%) among *E. coli* strains isolated from children has also been documented in Marvdasht, Iran [19]. Also, in the present study, resistance to sulfamethoxazole-trimethoprim, streptomycin, kanamycin, nalidixic acid, tetracycline, cefalexin, gentamicin, amikacin and chloramphenicol in MDR *E. coli* isolates were 79.71%, 76.81%, 68.12%, 66.67%, 59.42%, 56.52%, 44.93%, 21.74%, and 14.49, respectively.

However, Jones et al [20] reported a resistance to ampicillin 100%, cefalexin and streptomycin 89%, kanamycin and chloramphenicol 84%, gentamicin and tetracycline 79%, nalidixic acid 68%, and amikacin 68%. In another article by Murshed et al [21], resistance rates to cefalexin, co-trimoxazole, gentamicin, chloramphenicol, tetracycline, nalidixic acid, and ampicillin were noted as 79%, 75%, 54%, 50%, 77%, 83%, and 96%, respectively.

The various percentages of resistance in different parts of the world are due to differences in the prevalence of antibiotic consumption in each country [22]. In this study frequency of integrons of class 1, 2 and 3 were estimated as 78.26%, 76.81%, and 26.09%, respectively. The integron prevalence was relatively higher than in other investigations; for example, Jones et al [20] reported that 47% of MDR isolates carried class 1 and 2 integrons, whereas no integron class 3 was detected. Furthermore, research by Farshad et al [15] found frequencies of 25.6% for class 1 integron and 41.10% for class 2 integron, whereas Rezaee et al [3] reported 26.03% and 5.08% of class 1 and 2 integrons, respectively. In other research by Ranjbaran et al [23], a class 1 and 2 integron prevalence of

Table 2. Sizes of variable regions of integron class I cassettes in *intI1* positive isolates.

Pattern of integron I cassettes bands (pb)	No. of isolates (%)
750	2 (3.70)
800	16 (29.63)
1000	2 (3.70)
1200	2 (3.70)
1500	25 (46.30)
2000	1 (1.85)
Without PCR product	6 (11.12)
Total no. of *intI1* positive isolates	54 (100)

Table 3. Sizes of variable regions of integron class 2 cassettes in *intI2* positive isolates.

Pattern of integron 2 cassettes bands (pb)	No. of isolates (%)
800	11 (20.76)
1300	2 (3.77)
1500	2 (3.77)
2000	14 (26.42)
Without PCR product	24 (45.28)
Total no. of *intI2* positive isolates	53 (100)

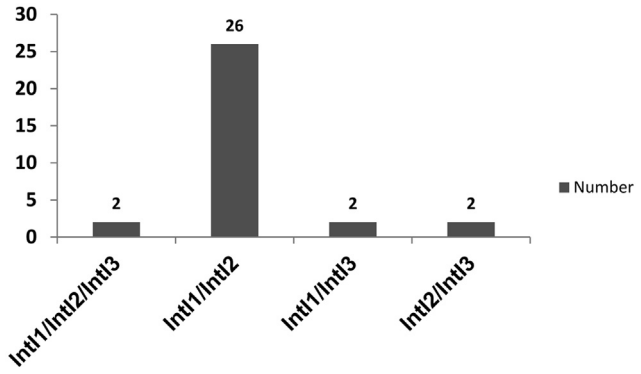

Figure 2. Frequency of simultaneous occurrence of integrons in multidrug-resistant isolates.

86% and 8% was reported, respectively. In contrast to investigations in Australia [24], Korea [25], France [26], Spain [27], China [28], Iran [3,15,23,29], Taiwan [9], Malaysia [30], and Pakistan [31], that did not detect any class 3, in the current study we found class 3 integron at a frequency of 26.09% in *E. coli* isolates, for the first time in Iran. In addition, here we studied the existence and the sizes of variable regions of class 1 and 2 integrons, using their specific primers by PCR technique, as we detected 48 (69.56%) and 29 (42.03%) isolates containing the gene cassette among 54 (78.26%) and 53 (76.81%) class 1 and 2 integron-bearing isolates, respectively. The gene cassettes ranged between 750 base pairs and 2000 base pairs, and amplicons of 1500 base pairs and 2000 base pairs were the most common gene cassette regions harbored in class 1 and 2 integrons, respectively. Moreover Machado et al 2007 [32] and Bakhshi et al 2012 [33] published similar results confirming our investigation.

As previously noted, the presence of integrons is closely related to resistance to quinolones, aminoglycoside compounds, trimethoprim, chloramphenicol, and β-lactam antibiotics [28,34−37]. We also detected a substantial correlation between class 1 integron, embedded gene cassette and resistance to amikacin, gentamicin, chloramphenicol, ampicillin, tetracycline, nalidixic acid, and co-trimoxazole, and also between class 2 integron, correspondent gene cassette and resistance to aminoglycosides, co-trimoxazole, cefalexin, ampicillin, and chloramphenicol. We also found a significant relationship between integron class 3 and the resistance to gentamicin, kanamycin, and streptomycin. We also confirmed that among a total of 69 MDR strains in this research, none of them contained unaccompanied class 3 integron, but it was found with high frequency co-occurring with class 1 and 2 integrons (Figure 2).

Considering the results obtained from our study, it was determined that some strains, despite being a MDR and having the integrase gene do not encompass the variable region. As Dawes et al [38] and Japoni-Nejad et al [39] reported, the differences in PCR results of integron identification, in the samples, could be due to variation in the 3′ region of the integron and the primer-binding site or the extensive size of the gene cassette. Also, as can be seen from Tables 2 and 3 and Figure 2, there are some strains containing multiple integrons and gene cassettes; therefore, it is concluded that there are multiple integrons present at the different chromosomal regions of the isolates.

In conclusion, recent investigations suggest that integrons are common among MDR isolates and they can be used as a marker for the identification of MDR isolates. This could lead to a serious threat of an outbreak of antimicrobial resistance development, which complicates the treatment of infections in the future, therefore precautionary measurements must be adopted to prevent the spread of these integrons. Eventually, considering the number of isolates bearing class 1 and class 2 integrons, lacking any gene cassette, it is suggested that the integron structures should be further investigated at the preserved regions in future researches.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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