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Brief communication (Original)

Prevalence of class 1 and 2 integrons among the multidrug resistant uropathogenic strains of *Escherichia coli*

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Background: Multidrug resistance is a serious problem in the treatment of urinary tract infections. Horizontal gene transfer, directed by strong selective pressure of antibiotics, has resulted in the widespread distribution of multiple antibiotic resistance genes. The dissemination of resistance genes is enhanced when they are trapped in integrons.
Objectives: To determine the prevalence of integrons among multidrug resistant *Escherichia coli* strains collected from regional hospitals and private clinical laboratories in Alborz province.
Methods: The susceptibility of 111 clinical *Escherichia coli* isolates was tested using a Kirby–Bauer disk diffusion method for common antibiotics. Isolates were screened for the production of extended spectrum β-lactamases (ESBLs) using a double disk synergy test. The existence of integrons was confirmed by amplification of the integrase gene and their class determined via analysis of PCR products by PCR-RFLP.
Results: Isolates showed the highest resistance to amoxicillin. Nitrofurantoin, amikacin, and ceftizoxime were the most effective antibiotics in vitro. Eighty-eight isolates of 111 (79%) were resistant to more than three unrelated drugs. We found 30% of the multidrug resistant isolates harbor integrons. Class 1 and 2 integrons were detected in 25 and 1 isolates, respectively. ESBL screening of strains showed 45 isolates (40%) were positive; 22% of the ESBL-positive isolates carried class 1 integrons and the frequency of MDR in ESBL-positive isolates was 93%.
Conclusion: The existence of integrons in only 29.5% of multidrug resistant isolates showed that besides integrons, antibiotic resistance genes were probably carried on other transferable elements lacking integrons, such as transposons or plasmids.

Keywords: ESBL, *Escherichia coli*, integron, multidrug resistance, PCR-RFLP

In recent years, because of excessive and unregulated use of antibiotics, the threat of acquisition of antibiotic resistance by pathogens is growing [1]. Misuse and overuse of antibiotics have played a substantial role in the development of multidrug resistant (MDR) bacteria. Multidrug resistance is a serious problem in therapy of patients with urinary tract infections. Horizontal transfer of antibiotic resistance genes has led to the rapid emergence of antibiotic resistance among clinical isolates of bacteria [2] and resulted in the widespread distribution of multiple antibiotic resistance genes on plasmids and transposons among many gram-negative isolates. The dissemination of resistance genes is greatly enhanced when they are trapped in a mobile gene cassette, the so-called integron [3]. Integrons are conserved DNA sequences that provide an efficient means for capturing and spreading of antimicrobial resistance genes [4] and are carried on episomal genetics structures. The essential components of an integron include the integrase gene (*intI*), the attachment site (*attI*), and the promoter (P_{ant}), which promotes the expression of any suitably integrated gene(s). Integrase is a member of the tyrosine site-specific recombinase family that catalyzes the excision and integration of DNA units by performing two consecutive strand breakages and rejoining steps [5]. Four classes of integrons so far identified are

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distinguished by their respective integrase (*int*) genes. Most of the resistance integrons found in clinical isolates of *Enterobacteriaceae* are class 1 integrons, which are highly associated with resistance to antimicrobial agents [6]. Integrons are of clinical importance, because the use of only one antibiotic may activate the expression of a whole gene cassette.

Because there is not much published data available on the detection of integrons in MDR isolates of uropathogenic *Escherichia coli* from Iranian medical centers, the aim of this study was to survey the prevalence of MDR and the frequency of class 1 and 2 integrons by restriction fragment length polymorphism analysis of PCR products (PCR-RFLP) of clinical isolates of *E. coli* in the immigration friendly city of Karaj, Iran and to investigate associations between MDR and existence of integrons.

Materials and methods

Bacterial strains

Between April and July 2013, 111 anonymized nonduplicate *E. coli* isolates from urine samples were collected from 4 hospitals and 2 private clinical laboratories of Alborz province, Karaj city. Isolates were identified as *E. coli* based on standard biochemical tests [7].

Antimicrobial susceptibility assay

The susceptibilities of all isolates to 19 different antibiotics were determined using a Kirby–Bauer disk diffusion method, as suggested by the Clinical and Laboratory Standards Institute [8]. The zone of inhibition of each isolate was tested on Muller–Hinton agar medium with commercial antimicrobial disks (Padtan Teb Co, Tehran, Iran). The antibiotic disks used in this study were gentamicin (10 μg), amikacin (30 μg), amoxicillin (10 μg), ceftazidime (30 μg), cephalothin (30 μg), cephalexin (30 μg), ceftizoxime (30 μg), ceftriaxone (30 μg), imipenem (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), co-trimoxazole (25 μg), tetracycline (30 μg), chloramphenicol (30 μg), ofloxacin (5 μg), levofloxacin (5 μg), amoxicillin/clavulanic acid (20/10 μg), and nitrofurantoin (300 μg). *E. coli* (American Type Culture Collection (ATCC) No. 25922) was used as the reference strain for the antibiotic susceptibility tests.

Extended spectrum β-lactamase detection

Using β-lactam and β-lactamase-inhibitor disks is a widely accepted method of detecting extended-spectrum β-lactamase (ESBL)-producing gram-negative bacilli. Isolates were screened for ESBL production using a double disk synergy test. The disks of extended-spectrum cephalosporins (cefotaxime, ceftazidime, ceftriaxone, and ceftizoxime) were placed around an amoxicillin (20 μg)–clavulanate (10 μg) disk at a distance of 25–30 mm center to center. Plates were incubated at 37°C for 18 hrs. ESBL production was deduced when the zone of cephalosporins was expanded by clavulanate [9].

K. pneumoniae (ATCC No. 700603) and *E. coli* (ATCC No. 35218) were used as positive and negative ESBL controls respectively.

Detection of class 1, 2 and 3 integrons by PCR-RFLP

Genomic DNA of the isolates was extracted using a boiling method [10]. Integrons were detected by PCR with degenerate primers hep35 (5′-TGCGGG TYAARGATBTKGATTT-3′) and hep36 (5′-CARC ACATGCGTRTARAT-3′), where B = C or G or T, K = G or T, R = A or G and Y = C or T, which hybridize to conserved regions of integron-encoded integrase genes *intI1*, *intI2*, and *intI3* [11]. To amplify the integrase gene, 1 μL of template DNA was added to 25 μl of PCR mixture. Each reaction contained 2.5 μL of 10× PCR buffer, 0.5 μL of 10 mM dNTPs, 0.7 μL of each primers (20 pM), 2 μL of template DNA, and 0.2 μL of 5U/μl Taq DNA polymerase. Amplification conditions were as follows: early denaturation at 95°C for 3 min followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 90 s, with a final elongation step of 10 min at 72°C. The length of the expected amplified fragment was about 491 bp. Clinical isolates of *E. coli* harboring integron class 1 and 2 genes (kindly provided from Dr. Alebouyeh, Research Center for Gastroenterology and Liver Diseases, Iran) were used as positive controls. A tube including the PCR reaction mix with no DNA template was used as negative control for all PCRs.

Determination of integron classes

To determine the integron classes, PCR products were digested with *Rsa*I and *Hinf*I restriction enzymes (Cinagene, Iran). The products of each distinct RFLP were analyzed by electrophoresis in 1.3% agarose gel [5].

Statistical analysis

Chi-square and Fisher exact tests were used to

calculate the association between antibiotic resistance and presence of integron. $P < 0.05$ was considered significant.

Results

This study was performed on 111 anonymized uropathogenic *E. coli* strain isolates from urine samples of hospitalized patients and from private clinical laboratories of Alborz province, Karaj city. From 111 clinical *E. coli* strains, 77 (70%) were isolated from female and 34 (30%) from male patients. Isolates were identified as *E. coli* based on standard biochemical tests. All isolates were subjected to an antibiotic susceptibility test using the Kirby–Bauer disk diffusion method. Resistance to amoxicillin was observed in 79% of isolates, to tetracycline in 64%, co-trimoxazole in 61%, cephalexin in 59%, nalidixic acid in 60%, cephalothin in 49%, ceftriaxone in 41%, cefotaxime in 41%, ciprofloxacin in 33%, norfloxacin in 32%, ofloxacin in 31%, levofloxacin in 31%, ceftazidime in 30%, imipenem in 26%, ceftizoxime in 24%, gentamycin in 23%, chloramphenicol in 17%, amikacin in 14%, and nitrofurantoin in 7% (**Table 1**). Eighty-eight isolates of the 111 were resistant to more than 3 unrelated antibiotics. Frequencies of MDR to 4, 5, and 6 or more antibiotics were 75%, 72%, and 59% respectively. By amplification of the integrase gene, it was found that 26 multidrug resistant isolates (30%) harbor antibiotic resistance integrons. By PCR-RFLP, class 1 and 2 integrons were found in 25 isolates and 1 isolate respectively (**Figure 1**). No class 3 integron was observed among our isolates and 62 MDR isolates did not harbor any integrons.

ESBL screening of strains by double disk diffusion showed that of the 111 *E. coli* isolates, 45 (40%) were ESBL positive and 22% of the ESBL-positive isolates carried class 1 integrons. The frequency of MDR in ESBL positive isolates was 93%.

Table 1. Association between antibiotic resistance and presence of integrons in *E. coli* isolates

Antibiotic	Resistant int-positive* (Number of isolates)	Resistant int-negative** (Number of isolates)	Total number of isolates	Association with integron
Nitrofurantoin	1	9	10	<0.12
Levofloxacin	10	24	34	<0.59
Amikacin	3	12	15	<0.22
Chloramphenicol	4	15	19	<0.87
Gentamycin	6	20	26	<0.72
Nalidixic acid	15	52	67	<0.44
Ciprofloxacin	10	27	37	<0.23
Norfloxacin	9	26	35	<0.23
Ofloxacin	9	25	34	<0.32
Tetracycline	15	56	71	<0.35
Amoxicillin	20	68	88	<0.97
Co-trimoxazole	19	49	68	<0.048
Imipenem	8	21	29	<0.93
Cephalexin	16	49	65	<0.37
Ceftriaxone	11	34	45	<0.76
Cefotaxime	11	35	46	<0.77
Cephalothin	12	42	54	<0.74
Ceftazidime	6	27	33	<0.34
Ceftizoxime	5	22	27	<0.74

*int-positive = Integron positive, **int-negative = Integron negative

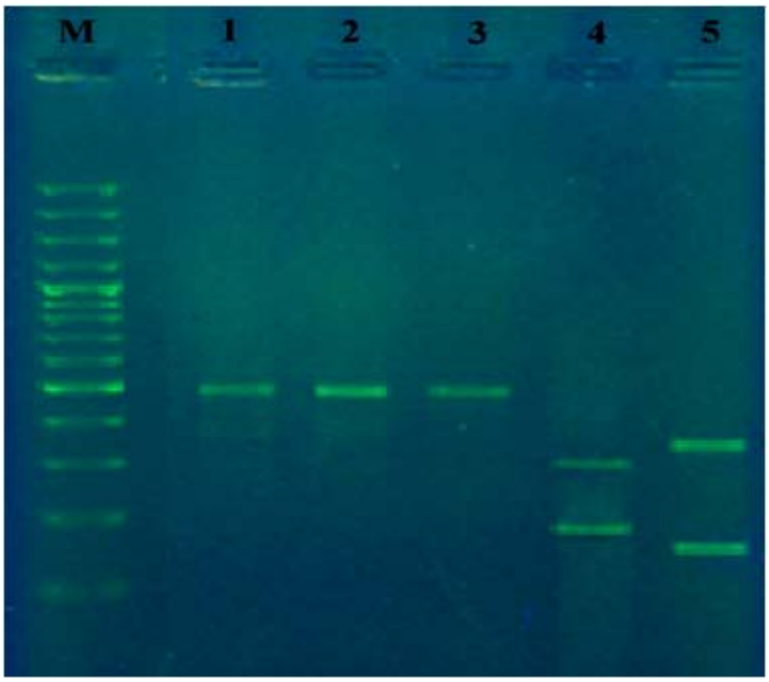

Figure 1. PCR analysis and restriction fragment length polymorphism analysis of PCR products of *E. coli* antibiotic resistance integrons. M: 100 bp ladder, Lane 1: positive control (491 bp), Lane 2: Class 1 integron (PCR product treated by *Hinf*I, a 491 bp fragment), Lane 3: Class 1 integron (PCR product treated by *Rsa*I, a 491 bp fragment), Lane 4: Class 2 integron (PCR product treated by *Hinf*I, 300 and 191 bp fragments), Lane 5: Class 2 integron (PCR product treated by *Rsa*I, 334 and 157 bp fragments).

Discussion

Isolates in this study were highly resistant to amoxicillin (79%) followed by tetracycline (64%) and co-trimoxazole (61%). Resistance to other antimicrobial agents were as follows: nitrofurantoin (7%) and amikacin (14%). These finding are comparable to those of Mansouri et al. who have reported high resistance of enteric bacteria to trimethoprim/sulfamethoxazole, amoxicillin, and tetracycline and higher sensitivity to imipenem and ceftizoxime in Kerman city [12]. In 2008, Japoni et al. reported high resistance of clinical *E. coli* isolates in southern Iran to amoxicillin, tetracycline, and co-trimoxazole. Amikacin and imipenem have been demonstrated as the most effective drugs [1]. Extreme sensitivity to imipenem (100%) and amikacin (99.7%) has been reported by Oteo et al. [13]. These findings suggest that antibiotics like ampicillin, tetracycline and co-trimoxazole should be prescribed cautiously. Currently, in most of clinics in our city, ciprofloxacin is the first antibiotic that is prescribed to treat urinary tract infections (UTIs). A survey in the United States found that ciprofloxacin was the only agent studied for which the resistance of clinical *E. coli* isolates has been steadily increasing from 1995 (0.7%) to 2001 (2.5%). Ciprofloxacin resistance in Germany was 15.2%, and in The Netherlands was 6.8%, while in Portugal it was 25.8%, and Italy was 24.3% [13]. In our study, the resistance to ciprofloxacin was 33%, which is considerably higher than in all of the previous reports. This may be in part because of excessive and unregulated use of this antibiotic. In all reports noted above, imipenem has appeared to be an efficient drug, but in our study resistance to imipenem (26%) was relatively high. It seems that antibiotics like ciprofloxacin and imipenem should be prescribed in the light of antibiograms and maybe the strategy for treatment of patients in our region with *E. coli* infections needs to be revised.

The major mechanism of resistance to β-lactams, particularly in gram-negative bacteria, is the production of ESBLs [14]. These enzymes are encoded on chromosomes or plasmids, and are associated with mobile genetic elements such as transposons or integrons, carrying genes that encode resistance to other antimicrobial agents. We found that our ESBL-positive isolates show higher rates of resistance to β-lactam antibiotics and 93% of them

are MDR, which indicates that the incidence of MDR and ESBL producing enteropathogenic *E. coli* is high in Karaj and is cause for concern. Moreover, 22% of the ESBL-positive and 24% of non-ESBL isolates in this study carried class 1 integrons. These findings are comparable to those of Machado et al. [15] who have studied integron content of ESBL producing *E. coli* strains over 12 years in a single hospital in Madrid, and reported that class 1 integron occurrence was similar in ESBL-positive (47%) and ESBL-negative (40%) groups.

The results of this study indicate that MDR is a serious therapeutic concern. Multidrug resistance in the United States was 7.1% in 2000 [16] and in Spain it was 17.1% and increased by 50% between 2001 and 2003 [13]. The rate of MDR in the study of Mansouri et al. for enteric bacteria in the pediatric wards of regional hospitals was 23.4% (16). Japoni et al. have reported that 82.5% of their *E. coli* isolates showed a MDR phenotype [1]. Capture of larger cassettes, with 7 to17 resistance determinates, by our integron-positive multidrug resistant isolates and widespread distribution of MDR phenotype (79%) in our study suggest that integrons in our isolates are evolving.

Integrons play an important role in the antibiotic resistance of clinical *E. coli* strains because they are able to capture, integrate, and express gene cassettes encoding antibiotic resistance. It is well known that integrons carry and transfer MDR genes in bacteria. The prevalence of integrons ranging from 22% to 59% has been reported in clinical *E. coli* [1]. In our present study, we found that 26 multidrug resistant isolates (30%) harbor antibiotic resistance integrons. The prevalence of integrons in our study was lower than that reported by Japoni et al. [1] and was similar to that in study performed by Ahangarzadeh et al. [5] who observed 27.1% of MDR isolates were positive for integron(s). The relatively low rate of class 1 integrons among multidrug resistant *E. coli* isolates suggests that the antibiotic resistance genes in these strains were more likely to be carried on transferable elements lacking integrons. We only found a significant correlation between resistance to co-trimoxazole and integrons, but not between resistance to all of the other antibiotics tested (**Table 1**). However, mobilization of antibiotic resistance determinants by plasmids or transposons would be alternative approaches [1]. Findings of this study suggest that nitrofurantoin and amikacin are the most effective drugs in vitro, but clinical adequacy of monotherapy or combined administration of these antibiotics should be appraised. As regional variation in resistance patterns is usual, local surveillance of antimicrobial resistance is recommended.

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