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DETECTION OF INTEGRONS AMONG MULTI-DRUG RESISTANT (MDR) *ESCHERICHIA COLI* STRAINS ISOLATED FROM CLINICAL SPECIMENS IN NORTHERN WEST OF IRAN

Mohammad Ahangarzadeh Rezaee[1]*, Vajihe Sheikhalizadeh[2], Alka Hasani[3]

[1]Research Center of Infectious Diseases and Tropical Medicine, Department of Medical Microbiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran; [2]Department of Medical Microbiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran; [3]Research Center of Infectious Diseases and Tropical Medicine, Department of Medical Microbiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.

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ABSTRACT

Transference of resistance determinants by integrons is one of the important factors that can contribute to the increase in multi-resistant bacteria. We determined the prevalence and class of integrons among multi-drug resistant (MDR) *Escherichia coli* strains isolated from clinical specimens in Tabriz teaching hospitals. Firstly, susceptibility of 140 isolates to 13 antibiotics was determined using the disc diffusion method. Then, prevalence and class of integrons was detected in MDR strains by PCR-RFLP. One hundred five (75%) of total 140 isolates were uropathogenic *Escherichia coli* (UPEC). Other pathotypes included were: diarrheagenic *Escherichia coli* (13; 9.3%), sepsis-associated *E. coli* (5; 3.6%) and newborn meningitis-associated *E. coli* (2; 1.4%). Antibiotic resistance patterns were as follows: amoxicillin 99.3%, gentamicin 33.6%, tetracycline 72.8%, ceftazidime 46.4%, co-trimoxazole 75%, imipenem 1.4%, ciprofloxacin 47.6%, norfloxacin 50.7%, cephalothin 77.8%, amikacin 12.1%, nitrofurantoin 12.9%, chloramphenicol 20.7% and nalidixic acid 60.7%. One hundred eighteen (84.2%) of tested isolates were multi-drug resistant. Prevalence of integrons was confirmed in 27.1% of MDR isolates. *intI1* and *intI2* were detected respectively in 22.05% and 5.08% of MDR strains. No *intI3* was detected. Resistance to gentamicin, amikacin and chloramphenicol was significantly associated with the presence of integrons. These results showed high resistance of *E. coli* to routine antibiotics, however, in consideration of low prevalence of integrons among these strains, we can conclude that antibiotic resistance genes in these strains presumably carried on elements other than integrons.

Key words: Integron, *Escherichia coli*, Multi-drug resistance

***Corresponding Author. Mailing address:** Department of Medical Microbiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.; Tel/Fax.: +98-411-3364661.; E-mail: rezaee@tbzmed.ac.ir

INTRODUCTION

Escherichia coli is the head of the large bacterial family, *Enterobacteriaceae*. Based on the diversity in pathogenicity and related clinical symptoms, *E. coli* strains are categorized into intestinal and extraintestinal pathotypes. Intestinal pathogenic *E. coli* pathotypes causing diarrheal diseases, DEC, include: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adhering *E. coli* (DAEC). Moreover, extraintestinal *E. coli* pathotypes cause infections outside of the gastrointestinal tract and include: uropathogenic *E. coli* (UPEC); newborn meningitis-associated *E. coli* (NMEC) and sepsis-associated *E. coli* (SEPEC) (4).

Todays, multi-drug resistance (MDR) among clinical isolates of bacteria such as *E. coli* pathotypes, is major healthcare problem and is associated with increased morbidity and mortality, worldwide (9). Although classically acquired and spread through chromosomal mutations, resistance genes can be disseminated by extrachromosomal elements acquired from other bacteria. These include different types of mobile DNA segments, such as plasmids, transposons, and integrons (1). Integrons are genetic structures capable of integrating or mobilizing gene cassettes encoding antibiotic resistance determinants (1, 5).

The key 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 catalyze the excision and integration of DNA units (1, 18). There are four different classes of integrons in bacteria carrying genes for antimicrobial resistance, each integron with a distinct integrase gene. Nearly all known gene cassettes from class 1, 2 and 3 integrons encode resistance to antibiotics or disinfectants (2, 11). Class 1 integrons are the most prevalent and well characterized (8, 11, 12). Class 4 is a distinctive class of integrons located in the *Vibrio cholerae* genome and is not known to be associated with antibiotic resistance (19).

Several studies have examined integron distributions in multi-drug resistant *Escherichia coli* strains around the world (2, 6, 12, 14, 21). However, no publicized information is available on detection of integrons in MDR isolates of pathogenic *E. coli* from northern west of Iran. The aim of this study was to define the current prevalence and phenotypes of multi-drug resistant *E. coli* isolated from clinical specimens in northern west of Iran and to investigate associations between multi-drug resistance and existence of integrons.

MATERIALS AND METHODS

Bacteria and clinical specimens

The study investigated 140 isolates of *Escherichia coli* obtained from various clinical specimens collected during April to September 2009 from teaching hospitals in Tabriz, northern west of Iran. Clinical specimens included: urine, stool, blood, vaginal discharge, catheter, eye swab and CSF (Cerebrospinal fluid). The bacteria were isolated and identified by standard bacteriological procedures (10). After identification, each strain was subcultured in 20% glycerol in Tryptone Soya Broth (Oxoid, UK) as frozen stock at -70˚C.

Antibiotic susceptibility tests

All isolates were examined for resistance to routine antimicrobial agents by standard disk diffusion method (15). The antibiotics tested were gentamicin, amikacin, amoxicillin, ceftazidime, cephalothin, imipenem, nalidixic acid, ciprofloxacin, norfloxacin, co-trimoxazole, tetracycline, chloramphenicol and nitrofurantoin (Mast Co, UK). *E. coli* ATCC 25922 was used as a control strain. MDR was defined as resistance to 3 or more unrelated antibiotics (2).

Template DNA preparation

Template DNA for PCR was prepared by boiling method

(21). Briefly, the organisms were inoculated into 1.5 ml of Luria Bertani broth (Sigma Aldrich, Germany) and incubated for 20 h at 37°C. The bacterial cells were then harvested by centrifugation at 10,000 ×*g* for 5 min. The pellet was resuspended in 300-400 µl of sterile distilled water, then boiled for 10 min and any cell debris was removed by centrifugation for 5 min at 11,500 ×*g*. The supernatant was stored at -20˚C and used as template DNA stock.

PCR amplification for integrase genes

To determine whether the *E. coli* isolates carried integron(s), the conserved regions of integron-encoded integrase genes *intI1*, *intI2*, and *intI3* were amplified with the degenerate primer pair hep35: 5′-TGCGGGTYAARGATBTKGATTT-3′ and hep36**:** 5′-CARCACATGCGTRTARAT-3′, where B = C or G or T, K = G or T, R = A or G and Y = C or T (19). Primers were provided by Alpha DNA (Canada). The PCR was performed in 25 µl reaction mixture containing 2 µl of DNA template, 50 pm of each oligonucleotide primer, 0.2 mM of deoxynucleoside triphosphates sets (TAKARA, Japan), 1.5 mM of $MgCl_2$ (TAKARA, Japan), 2.5 µl of 10X PCR buffer (100 mM Tris-HCl, pH 8.3 and 500 mM KCl) and 2.5 U of *Taq* polymerase (TAKARA, Japan). PCR was performed as follows: initial denaturation at 94˚C for 10 min followed by 30 cycles of denaturation at 94˚C for 30 s, annealing at 55˚C for 30 s, and extension at 72˚C for 45 s, with a final extension step at 72˚C for 10 min. Plasmid pUB2401::Tn*21* carrying the Tn*21* integron and *E. coli* 96K062 were used as positive controls for the class 1 and Class 2 integrons, respectively. Moreover, a tube includes PCR reaction with no DNA template was used as negative control for all PCRs.

The expected amplicons (491 bp) were analyzed by electrophoresis on agarose 1.5% w/v gels in TAE buffer at 100-120 V for 30-55 min.

Determination of integron classes

To determine integron classes, the PCR products were further analyzed by restriction fragment length polymorphism (RFLP) following digestion using either *Rsa*I or *Hinf*I (MBI, Fermentas, Lithuania). For restriction digestion, 10 µl of the PCR products was digested in a 32 µl reaction mixture containing 2 µl of *Rsa*I (or *Hinf*I) and 2 µl of the appropriate restriction buffer, as well as 18 µl of deionized distilled water at 37˚C for 16 h. The size and number of generated fragments are shown in Table 1.

Table 1. Classification of integrase PCR product by RFLP

PCR product	Enzyme	No. of fragment	Fragment size (bp)
*IntI*1	*Hinf* I	1	491
	*Rsa*I	1	491
*IntI*2	*Hinf* I	2	334, 157
	*Rsa*I	2	300, 191
*IntI*3	*Hinf* I	3	97, 104, 290
	*Rsa*I	2	119, 372

Statistical analysis

Analyses were performed by SPSS software version 13. Chi-square test was used to calculate association between antibiotic resistance and integron existence. The significance level was defined as $P < 0.05$.

RESULTS

Bacteria

Eighty-six (61.4%) and 54 (38.6%) *E. coli* strains were obtained from female and males, respectively. The bacteria

were isolated from urine (105; 75%), stool (13; 9.3%), blood (5; 3.6%), vaginal discharge (4; 2.9%), catheter (3; 2.1%), eye swab and CSF (2; 1.4% each one).

Antimicrobial Susceptibility

One hundred eighteen (84.2%) of tested strains were observed as MDR. Nearly all isolates (139; 99.3%) were resistant to amoxicillin. The resistance percentages of all strains to tested antibiotics were as follows: gentamicin 33.6%, tetracycline 72.8%, ceftazidime % 46.4, co-trimoxazole %75, imipenem 1.4%, ciprofloxacin 47.6%, norfloxacin 50.7%, cephalothin 77.8%, amikacin 12.1%, nitrofurantoin 12.9%, chloramphenicol 20.7% and nalidixic acid 60.7%.

Integrons carriage among the isolates

A positive test result for integrons was found in 32 (27.1%) of 118 MDR isolates screened, including 26 (22.03%) of class 1 and 6 (5.08%) of class 2 integrons. No integron class 3 was detected in any of the isolates. Moreover, no strain was found to contain both class 1 and 2 integrons. The association of resistance to antibiotics and integrons is shown in Table 2.

Table 2. Association between antibiotic resistance and integron existence in MDR isolates.

Antibiotics	No. (%) of resistant isolates with *int.* genes	No. (%) of resistant isolates with *intI*1 genes	No. (%) of resistant isolates with *intI*2 genes	No. (%) of total resistant isolates	Association of resistance with integron
Gentamicin	10 (8.4)	7 (5.9)	3 (2.5)	47 (39.8)	*P=0.01**
Tetracycline	29 (27)	24 (20.3)	5 (4.2)	101 (85.6)	*P=0.47*
Ceftazidime	19 (16.1)	15 (12.7)	4 (3.4)	65 (55.1)	*P=0.84*
Cotrimoxazole	26 (22)	21 (17.8)	6 (5.1)	97 (82.2)	*P=0.86*
Imipenem	1 (0.8)	0 (0)	1 (0.8)	1 (0.8)	*P=0.10*
Ciprofloxacin	15 (12.7)	12 (17.8)	3 (2.5)	67 (56.8)	*P=0.18*
Norfloxacin	16 (13.5)	12 (17.8)	4 (3.4)	70 (59.3)	*P=0.20*
Cephalothin	28 (24)	23 (19.5)	7 (5.9)	102 (86.4)	*P=0.21*
Amikacin	7 (5.9)	6 (5)	1 (0.8)	17 (14.4)	*P=0.02**
Amoxicillin	32 (27.1)	26 (22)	6 (5.1)	117 (99.2)	*P=0.54*
Nitrofurantoin	5 (4.2)	4 (3.4)	1 (0.8)	17 (14.4)	*P=0.83*
Chloramphenicol	5 (4.2)	2 (1.6)	3 (2.5)	30 (25.4)	*P=0.02**
Nalidixic acid	22 (18.6)	17 (14.4)	5 (4.2)	83 (70.3)	*P=0.49*

*significant values

DISCUSSION

The emergence of *Escherichia coli* isolates with multiple-antibiotic-resistant phenotypes, has been previously reported and is considered a serious health concern (17).

In this study, MDR *E. coli* isolates with resistance to three or more different antibiotics were common. One hundred eighteen isolates (82.4%) had MDR phenotype, which is similar to the rate of multi-drug resistance reported in *E. coli* isolates by Japoni *et al*.(6).

E. coli isolates in our study were extremely resistant (99.3%) to amoxicillin. High resistance of *E. coli* isolates to amoxicillin (83.7%) has earlier been reported by Umolu *et al.* (19) from Nigeria.

In the present study, resistance rate to quinolones (nalidixic acid, ciprofloxacin and norfloxacin) were 60.7%, 47.6% and 50.7%, respectively. Japoni *et al*. (6) conducted similar study in Shiraz, South Iran and reported 71%, 21% and 20.5% resistance rate to these antibiotics.

Ciprofloxacin resistance in USA, Spain and Italy was 6%

(2), 19.3% (14) and 5.3% (14), respectively while in Korea it was 8.3% (20).

Seventy-five percent of our isolates were resistant to co-trimoxazole. Japoni *et al*. (6) reported 48% resistance rate to this antibiotic, while Oteo *et al*. (14) showed 32.6% resistance in their isolates.

Multi-drug resistance encoded by resistance genes clustered in integrons, which are potentially mobile genetic elements, considered to be involved in the transfer of MDR (3).

Of all *E. coli* isolates collected in our study, 27.1% of MDR isolates were positive for integron(s). The prevalence of class 1, 2 were 22.05% and 5.05%, respectively. The prevalence of integrons in our study was lower than that reported by Japoni et al. (6) who observed 44.8% of *E. coli* isolates carried integrons. Class 3 integrons was not found in any of our isolates that agree with other investigators reports (5, 6,20).

In the present study, only resistance to gentamicin, amikacin and chloramphenicol was found to be integron mediated. No significant association between quinolone compounds resistance and integron present was not surprising, because resistance to quinolone compounds is derived through chromosomal point mutations rather than being carried on any mobile genetic elements (11). Significant association between resistance to aminoglycosides tested (gentamicin, amikacin) and integron existence was also explainable because many aminoglycoside resistance genes have been reported within integron structures, including *aadA, aadB, aadA7, aacA4* and *aacA1* (11).

This study showed high prevalence of resistance rates with low distribution of integrons among clinical isolates of *E. coli* in northern west of Iran compared with similar study in south Iran in 2008. It is unclear why *E. coli* from this area manifests such high rates of resistance to antibiotics. However, it may be because of differences in antimicrobial usage, infection control practices, and unrecognized factors. Low prevalence of integrons among multi-drug resistant *E. coli* isolates suggests that the antibiotic resistance genes in these strains presumably carried on other elements such as transposons or plasmids.

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