Short Communication

Assay for Integrons and Pattern of Antibiotic Resistance in Clinical Escherichia coli Strains by PCR-RFLP in Southern Iran

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(Received August 24, 2007. Accepted November 26, 2007)

SUMMARY: The purpose of this study was to determine the prevalence of multidrug-resistant Escherichia coli in clinical specimens. In addition, the existence of integrons in resistant isolates was assessed by amplification of integrase genes. Susceptibility of 200 isolates from five Shiraz hospitals and health centers to 13 antibiotics was determined by the Kirby-Bauer disk diffusion method. The majority of the bacteria were isolated from urine (70.5%) and stool (25.5%) specimens. Antibiotic resistance patterns were observed as follows: amoxicillin 63%, tetracycline 57.5%, co-trimoxazole 48%, cephalotin 40%, nalidixic acid 36%, ciprofloxacin 21%, nitrofurantoin 25%, norfloxacin 20.5%, gentamicin 18%, chloramphenicol 18%, ceftazidime 14%, amikacin 8.5% and imipenem 2%. Of 200 isolates tested, 165 (82.5%) were multidrug resistant. The frequency of multidrug resistance to more than 5 antibiotics was 24.2%. The existence of integrons was confirmed in 44.8% of isolates. Significant association between resistance to gentamicin, amikacin, cephalotin, nalidixic acid, ciprofloxacin, norfloxacin and co-trimoxazole with the existence of integrons was obtained by the PCR-RFLP method. These results showed that integrons may be partly responsible for multidrug resistance. Imipenem, amikacin and ceftazidime were the most effective antibiotics in vitro; however, the clinical efficacy of these antibiotics remains to be assessed.

In recent years, the threat caused by the acquisition of antibiotic resistance by pathogenic bacteria has been growing. Antibiotic resistance and, in particular, multidrug resistance (MDR) are major problems for the empirical treatment of patients. Increased antimicrobial usage is the main driving force leading to the evolution of drug-resistant bacteria (1). At the same time, resistance to drugs such as co-trimoxazole, a combination of sulfanomide and trimethoprim, which are anti-folate drugs used to combat urinary tract infection (UTI), has remained high in spite of currently reduced use (2). Studies also show that, once evolved, resistance genes can spread through the world’s bacterial populations irrespective of the pattern of antimicrobial use in an area (3). Therefore, mechanisms other than selection pressure might exist for maintaining a resistant bacterial pool. MDR may be developing through the acquisition of resistance genes or an array of resistance genes by horizontal transfer. Dissemination of antibiotic resistance genes by horizontal transfer has led to the rapid emergence of antibiotic resistance among clinical isolates of bacteria (4). Four classes of integron so far identified (classes 1, 2, 3 and 4) are distinguished by their respective integrase (int) genes (5-7). Class 1 integrons, located on plasmids and transposons, make up the majority of the integrons found in clinical isolates and are associated with the MDR seen in the hospital environment (8-10).

Polymerase chain reaction (PCR) is now broadly applied for the molecular analysis of antibiotic resistance genes in bacteriology (4). PCR-restriction fragment length polymorphism (RFLP) is a two-step version of PCR in which PCR products are digested with appropriate restriction endonuclease enzymes. This could reveal minor differences within sequences of amplified PCR products by the generation of bands with different sizes in agarose gel (11). Escherichia coli is frequently isolated at our hospitals and medical centers from patients with urinary or gastrointestinal infections. However, so far there is no publicized information available on the prevalence of integron classes. Therefore, in the present study, we assessed the prevalence of MDR in E. coli and the existence of integrons in resistant isolates by PCR-RFLP.

Between April and November 2005, 200 E. coli isolates from different patients were obtained from three hospitals and two health centers affiliated with Shiraz University of Medical Sciences. Isolates were identified as E. coli based on standard biochemical tests (12). The isolates were screened for antimicrobial susceptibility using Kirby-Bauer disk diffusion methodology (13). The zone of inhibition of each isolate was tested on Mueller-Hinton agar medium (Oxoid Unipath, Ltd., Hampshire, UK) with a commercial disk (Mast, Co., Merseyside, UK). The group of antimicrobials tested were aminoglycosides (gentamicin, 10 μg; amikacin, 30 μg), betalactams (amoxicillin, 10 μg; cefazidime, 30 μg; cephalotin, 30 μg; imipenem, 10 μg), quinolones (nalidixic acid, 30 μg; ciprofloxacin, 5 μg; norfloxacin, 10 μg; anti-folate (co-trimoxazole, 25 μg), tetracycline, 30 μg; chloramphenicol, 30 μg and nitrofurantoin, 300 μg. E. coli (ATCC 25922) was used as a control for antibiotic susceptibility determination. Antibacterial susceptibility was confirmed by the standard disk diffusion method (13). MDR was defined as resistance in an isolate to more than two unrelated drugs.

Chromosomal and plasmid DNA were extracted as described by Enne et al. (2). Integrons were detected with degenerated primers to conserve regions of integron-encoded integrase genes intI1, intI2 and intI3. Primers used were hep35...
5'-TGCGGGTYAARGATBTKGATTT-3' and hep36 5'-CAR CACATTGCTGYRARAT-3', where B = C or G or T, K = G or T, R = A or G and Y = C or T (11). The primers were obtained from TIB MOLBIOL Syntheselabor GmbH (Berlin, Germany). PCR amplification was carried out in 50 µl reaction mixtures containing 5 µl DNA, 50 pm of each oligonucleotide primer, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.01% (v/v) Triton X-100, 1.5 mM MgCl₂, 0.2 mM dNTPs (dATP, dCTP, dGPT, dTTP) and 2.5 U Taq polymerase (MBI Fermentas, Vilnius, Lithuania). PCR assay was performed for 30 cycles as follows: initial denaturation at 94°C for 5 min and cycles consisting of denaturing at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and then a final extension at 72°C for 10 min. The expected size (491 bp) was ascertained by electrophoresis in a 1.5% agarose gel with appropriate molecular size markers (100-bp DNA ladder; MBI Fermentas). To detect integron classes, the amplified fragments were digested with two restriction enzymes, *RsaI* and *HinfI* (MBI Fermentas). The size and number of generated fragments are shown in Table 1 and Figure 1. Association between antibiotic resistance and the existence of the integrase gene by PCR was calculated by chi-square and Fisher exact tests, respectively. The significance level was defined as *P* < 0.05.

One hundred nine (54.5%) and 91 (45.5%) *E. coli* bacteria were isolated from adult females and males, respectively. The bacteria were isolated from urine (141; 70.5%), stool (51; 25.5%), blood (3; 1.5%), wounds (3; 1.5%), eye (1; 0.5%) and sputum (1; 0.5%) samples. Resistance to amoxicillin was observed in 126 isolates (63%), tetracycline in 115 (57.5%), co-trimoxazole in 96 (48%), cephalotin in 80 (40%), ciprofloxacin in 42 (21%), norfloxacin in 41 (20.5%), gentamicin in 36 (18%), nitrofurantoin in 50 (25%), nalidixic acid in 72 (36%), and imipenem in 4 (2%). Of 200 isolates tested, 165 (82.5%) were multidrug resistant. Frequencies of MDR to three, four, five and six or more antibiotics were 37 (22.4%), 34 (20.7%), 12 (7.3%) and 40 (24.2%), respectively. The existence of integrons was confirmed for 74 (44.8%) of the isolates by PCR-RFLP (Table 2). Class 1 and 2 integrons were detected in 55 (33.3%) and 11 (6.7%) isolates, respectively. PCR-RFLP products of the integrase gene are shown in Fig 1. Coexistence of class 1 and 2 integrons was detected in 8 isolates (4.8%). No class 3 integrons were observed among our samples. Ninety-one isolates (55.2%) did not harbor integrons (Table 2). Associations of drug resistance to gentamicin, amikacin, cephalotin, ciprofloxacin, nalidixic acid, norfloxacin and co-trimoxazole with the presence of integrons were statistically significant (Table 3).

Integrons play an important role in antibiotic resistance of *E. coli* strains because they are able to capture, integrate and express gene cassettes encoding antibiotic resistance (6). The prevalence of integrons ranging from 22 to 59% has been reported in clinical *E. coli* (14,15). The existence of integrons was confirmed in 74 (44.8%) of our multidrug-resistant isolates, indicating the prevalence values is in the high range. As previously noted, integrons of class 1 were more prevalent than those of class 2 (14,15). In addition, consistent with other reported studies, we detected no class 3 integrons (7,11). It is well known that integrons carry and transfer MDR genes in bacteria (4,11). In our isolates integrons were significantly associated with resistance to certain antibiotics, including gentamicin, amikacin, cephalotin, ciprofloxacin, nalidixic acid, norfloxacin and co-trimoxazole (Table 3). Nevertheless, to genetically confirm this association, sequencing and amplification of class 1 and 2 integrons cassette regions should to be performed (11).

Isolates in this study were highly sensitive (196; 98%) to imipenem. Extreme sensitivity of *E. coli* isolates to imipenem has earlier been reported by Tariq et al. (16). Furthermore, this antibiotic has been recently administrated in our clinical practice. Nowadays in our clinics, quinolones (nalidixic acid, ciprofloxacin and norfloxacin) are often prescribed to treat gastrointestinal and UTIs. Therefore, it could be expected that the prevalence of strains resistant to these antibiotics is increasing gradually. A consistent stepwise increase in *E. coli* resistance to ciprofloxacin was observed from 1995 (0.7%) to 2001 (2.5%) by Bolon et al. (17). Ciprofloxacin resistance in Portugal was 25.8% and in Italy was 24.3%, while in Germany and The Netherlands it was 15.2 and 6.8%, respectively (18). Ciprofloxacin resistance in our isolates was 21%, which was similar to that in southern European countries.

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### Table 1. RFLP classification of integrase PCR products

<table>
<thead>
<tr>
<th>PCR product</th>
<th>Enzyme</th>
<th>No. of fragment</th>
<th>Fragment size(s) (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IntI1</td>
<td><em>RsaI</em></td>
<td>1</td>
<td>491</td>
</tr>
<tr>
<td></td>
<td><em>HinfI</em></td>
<td>1</td>
<td>491</td>
</tr>
<tr>
<td>IntI2</td>
<td><em>RsaI</em></td>
<td>2</td>
<td>334, 157</td>
</tr>
<tr>
<td></td>
<td><em>HinfI</em></td>
<td>2</td>
<td>300, 191</td>
</tr>
<tr>
<td>IntI3</td>
<td><em>RsaI</em></td>
<td>3</td>
<td>97, 104, 290</td>
</tr>
<tr>
<td></td>
<td><em>HinfI</em></td>
<td>2</td>
<td>119, 372</td>
</tr>
</tbody>
</table>

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### Table 2. Frequency of integron in 165 multidrug-resistant isolates of *E. coli* by PCR-RFLP method

<table>
<thead>
<tr>
<th>Integron class</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integron class 1</td>
<td>55 (33.3)</td>
</tr>
<tr>
<td>Integron class 2</td>
<td>11 (6.7)</td>
</tr>
<tr>
<td>Integron class 1, 2</td>
<td>8 (4.8)</td>
</tr>
<tr>
<td>Integron class 3</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Without integron</td>
<td>91 (55.2)</td>
</tr>
<tr>
<td>Total</td>
<td>165 (100)</td>
</tr>
</tbody>
</table>

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![Fig. 1. PCR-RFLP of integrase gene products. Lane 1, PCR products of conserve regions of integrase; lane 2, *HinfI* treated of products represent of class 1 integron; lane 3, *RsaI* treated of amplified products represent class 1 integron; lane 4, *HinfI* digested of products represent class 2 integrons; lane 5, *RsaI* digested of amplified products represent class 2 integrons. M, molecular marker (100 base pair ladder).](image-url)
percentage of ofloxacin resistance observed in this study was 20.5%, which is on the high side. Similar high resistance of *E. coli* to ofloxacin has also been documented by Alex et al. (19), who observed that 24% of 189 *E. coli* isolates were resistant to ofloxacin. MDR among UTI isolates in the United States was reported to be 7.1% in 2000 (20). Such MDR has serious implications for the empirical treatment of infections caused by *E. coli* and for the possible co-selection of antimicrobial resistance mediated by MDR plasmids (21).

Multidrug-resistant *E. coli*, i.e., isolates resistant to three or more unrelated antibiotics, were quite common in our study. One hundred sixty-five isolates (82.5%) showed MDR. This implies that integrons in our isolates are evolving, and the capture of larger cassettes, with 6 to 12 resistance determinants, confirms this allegation. Evidently there is a positive correlation between antibiotic resistance and class 1 and 2 integrons. However, a significant association between resistant isolates to nearly half of the antibiotics including amoxicillin and tetracycline and the presence of integrons was not found statistically (Table 3). Nevertheless, mobilization of antibiotic resistance determinants by elements such as plasmids or transposons would be alternative approaches (22,23). Imipenem, amikacin and ceftazidime were the most effective antibiotics in vitro. Nevertheless, the clinical efficacy of monotherapy or combined administration of these antibiotics remains to be assessed.

**ACKNOWLEDGMENTS**

This study was granted by Professor Alborzi Clinical Microbiology Research Center (PACMRC) and Department of Bacteriology, Shiraz University of Medical Sciences, Iran with grant number 84-3.

**REFERENCES**


