

Full Length Research Paper

Integron and genotype patterns of quinolones-resistant uropathogenic *Escherichia coli*

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Uropathogenic *Escherichia coli* are the most common cause of urinary tract infections, and quinolones-resistant strains cause growing concern in developing countries. This study provides relevant data in relation to the molecular epidemiology of these isolates with respect to the distribution of integron among them and in doing so, to control the infections and adopt efficient strategies. This study was performed on 96 strains of *E. coli* isolated from patients with community acquired urinary tract infections in Jahrom, Iran. Having determined the antibiotic susceptibility patterns, isolates were resistant to quinolones (Ciprofloxacin, Norfloxacin and Nalidixic acid) screened for integron classes by polymerase chain reaction (PCR). Then the genetic patterns of the strains were compared by pulsed field gel electrophoresis. Of the 96 samples, 30 were resistant to at least one of the above mentioned antibiotics. Integrons were detected in 20.8, 25 and 25% of the Nalidixic acid, Ciprofloxacin and Norfloxacin resistant isolates, respectively. By using pulsed field gel electrophoresis among the 30 samples, we obtained 30 genetic patterns. The patterns revealed no clonal relationships between the strains. The gene cassettes observed in class 1 and 2 integrons did not correlate completely with the quinolone resistance observed in these isolates.

Key words: Quinolones-resistant Uropathogenic *Escherichia coli*, pulsed field gel electrophoresis, integrons.

INTRODUCTION

Urinary tract infections (UTIs) are among the most frequently community acquired bacterial infections. It is associated with significant morbidity and mortality especially in children (Paterson, 2004; Polwichai et al., 2009). The pathogenic potential of *Escherichia coli* strains is thought to be dependent on the presence of virulence factors (VFs) (Johnson, 1991; Morin and Hopkins, 2002; Vila et al., 2002).

As extra intestinal pathogenic *E. coli*, including uropathogenic *E. coli* (UPEC) bacteria are the most etiologic agent; they constitute a major target of antimicrobial therapy (Rijavec et al., 2006). In fact, antimicrobial is the main method of treatment. Different antibiotics can be used to treat UTI infections, but quinolones are an important class of antibiotics, because

they have wide spectrum activities with excellent bioavailability, good penetration and low incidence of side effect (Polwichai et al., 2009; Sharma et al., 2009; McDonald et al., 2001). The more these antibiotics are prescribed to combat a variety of UTI infections, the higher the rate of resistance among bacterial UPEC (Akram et al., 2007). This resistance has become a major trouble in the treatment and management of the infections in different countries (Guidoni et al., 2008; Li et al., 2007). Resistance genes are disseminated by plasmids or by transposons and also can be integrated into DNA elements designated integrons (Olsson-Liljequist et al., 1997). Integrons are mobile genetic elements thought to play an important role in the dissemination and accumulation of resistance genes in bacteria. The presences of integrons are associated with antimicrobial resistance and are being increasingly reported worldwide (Fluit and Schmitz, 1999; Lee et al., 2001; Salem et al., 2010). Due to Lack of sufficient data about Quinolones-resistant uropathogenic *E. coli*

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(QRUPEC) and the corresponding integron gene cassettes distributions in Iran, has prompted the present study which seeks to investigate these issues in patients with urinary tract infections in Jahrom, southern Iran.

To this end, we also used Pulsed field gel electrophoresis (PFGE), the most effective epidemiologic molecular method, to verify the molecular relatedness of the strains (Arbeit, 1999). Many researchers have utilized this method to study the clonal relationship of the strains (Kawamori et al., 2008; Watabe et al., 2008; Seto et al., 2007). Therefore the present study was intended to determine whether the infections caused by QRUPEC isolates were caused by the clonal spread of a small number of related isolates, or by many genetically diverse strains of *E. coli*.

MATERIALS AND METHODS

Bacterial isolation

During a period of one year from 2008 to 2009, 96 strains of UPEC were isolated from the urine samples of children who referred to Motahhari teaching hospital, affiliated with Jahrom University of medical sciences, southern Iran.

UTI diagnosis was established by the hospital physicians based on the clinical symptoms and laboratory findings. *E. coli* isolates were identified by standard methods (Farmer, 1999). Positive urine cultures were defined by a bacterial growth more than 10^5 colony forming unit per milliliter. As the cases considered in this study were only the patients with community acquired UTI, the exclusion criteria were recent antibiotic use during the last 15 days and nosocomial infections defined as the infections noted 48 hr after admission or within 4 weeks after a previous discharge.

Antibiotic susceptibility

Susceptibility of all the isolates to different antibiotics was determined by the disk diffusion method, as recommended by National Committee for Clinical Laboratory Standards (CLSI, 2006) with commercial antimicrobial disks (Mast. Co.UK). Norfloxacin, nalidixic acid and ciprofloxacin were also used to determine the QRUPEC. *E. coli* ATCC 25922 was used for quality control purposes.

DNA extraction and polymerase chain reaction (PCR) amplification

DNA to be amplified was extracted from intact organisms by boiling. Bacteria were harvested from 1.5 ml of an overnight Luria-Bertani broth culture, suspended in sterile distilled water, and incubated at 95°C for 10 min. Following centrifugation of the lysate, the supernatant was stored at -20°C as a template DNA stock.

Integrons were detected using PCR with degenerate primers designed to hybridize to conserved regions of integron encoded integrase genes *int1*, *int2* and *int3*. The sequences of the primers were as follows: *hep35*, 5' TGC GGG TYA ARG ATB TKG ATT T 3' and *hep36*, 5' CAR CAC ATG CGT RTA RAT 3', where B=C or G or T, K=G or T, R=A or G and Y=C or T (C). The primers sequences were previously reported (Arisoy et al., 2006) and obtained from TIB MOLBIOL Syntheselabor GmbH (Berlin, Germany)

PCR amplification were carried out in 50 µl reaction mixtures containing 5 µl DNA template, 50 pmol of each primer, 1.5 mM MgCl₂, 0.2 nM of dNTPs and 2.5 U Taq polymerase. PCR was

performed as follow: initial denaturation at 94°C for 5 min followed by 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s and final extension at 72°C for 10 min. Expected sizes of the amplicons were ascertained by electrophoresis in 1.5% agarose gel with an appropriate molecular size marker (100-bp DNA ladder, MBI, Fermentas, Lithuania).

Detection of integron classes by RFLP- PCR

The classes of the integrons were determined by analyzing integrase PCR products by restriction fragment length polymorphism (RFLP) following digestion using either RsaI or HinfI restriction enzyme according to the manufacture instruction (MBI, Fermentas, Lithuania).

Statistical analysis

Statistical analysis was performed using SPSS software for Windows, version 11.5 (SPSS). Student T test, *Chi square* and logistic regression were done for the evaluation of variables correlation. P value less than 0.05 was considered as significant result.

Pulsed field gel electrophoresis

This procedure was designed based on previously reported protocol by Ejnreas et al. (Ejrnaes et al., 2006) with some modifications. Briefly, the isolates were grown overnight on blood agar plates at 37°C. In order to protect the DNA against breakage, the bacteria were incorporated into agarose plugs, as described below. About three loops of bacteria were washed in 1 milliliter saline to obtain an optical density of 0.7 at wavelength of 610 nm and resuspended in 1 milliliter TE buffer (10 mM Tris HCl [pH 8.00], 100mM EDTA) and incubated at 50°C in a water bath for maximum 15 min. Chromosomal DNA was prepared in solid agarose plugs by mixing 1 ml of bacterial cell suspension with an equal volume of 2% low melting agarose (Fermentase, Lithuania). Following the overnight incubation at 54°C in lysis buffer (50 mM Tris HCl [pH 8.00], 50 mM EDTA, 1% laurylsarcosine, 1 mg/ml of proteinase K), the DNA plugs were washed four times in TE buffer for 30 min at 50°C and three times in distilled water. One third of each plug was cut and transferred to a tube containing *Xba*I restriction enzyme (Fermentase, Lithuania) according to the manufacture's instruction and remained overnight at 37°C.

DNA preparations were put in the wells of an agarose gel (molecular grade, Amersham Bioscience, Sweden), and covered with 0.5X TBE buffer and then were run in a homogenous electric field (Amersham Bioscience, Sweden). The electrophoretic conditions used were as follows; initial switch time: 5 s, second switch time: 20 s, final switch time: 40 s, temperature: 12°C, run time: 33 h, angle: 120°, gradient 6 v/cm. In each set, 1000 bp lambda ladder (Biolabs, New England) was used as molecular marker. After electrophoresis, the gel was stained in ethidium bromide and then photographed.

Investigation of similarity among the isolates

Photocapt software was used to determine the molecular weights of the sample profiles. The sizes of DNA fragments were determined according to the DNA marker. In each profile the bands were recorded as number 1 for present or zero for absent. Consequently, the data set was used to calculate pair-wise similarity coefficient following the Jaccard method. To generate a dendrogram using

Table 1. Relationship between Quinolone-sensitivity and integron among *E. coli* strains isolated from children with UTI.

Antibiotic	Total resistant n (%)	Positive integron n (%)	P value	Positive integron n (%)		P value
				Class I	Class II	
Nalidixic acid	24(25)	5(20.8)	0.17	2(33.3)	3(30)	0.6
Ciprofloxacin	8(8.3)	2(25)	0.23	2(33.3)	0(0.0)	0.35
Norfloxacin	8(8.3)	2(25)	0.62	2(33.3)	0(0.0)	0.45

average linkage procedure, the analysis of the similarity coefficients matrices were performed using unweighted pair-group method analysis (UPGMA). To calculate correlations among the variables, the standardized data matrices were used. These correlations were subjected to Eigen Vector analysis to evince the first three uttermost elucidative principal components. To study the patterns of variations which were observed among the isolates, the three principal components were plotted. The software NTSYSpc version 2.02i (Exeter software, New York) was used to conduct all the numerical analyses.

RESULTS

Bacterial strains and antibiotic susceptibility

Totally, 96 strains of *E. coli* were isolated from the urine samples of children with community acquired UTI, aged 1 month to 14 years (average 21.8 ± 26.9 months). Antibiotic susceptibility patterns of the isolates were determined by standard disc diffusion methods. Among the drugs under study, ampicillin, co-trimoxazole and tetracycline showed the least antimicrobial effects. No resistance to imipenem was seen among the strains. Seventy seven percent of the isolates were resistant to three or more antibiotics which were designated as multidrug resistant (MDR). The patterns for all the isolates have been presented in a previous report (Farshad et al., 2010). Of the 96 samples, 30 were resistant to at least one of the quinolones and all of them were MDR. The resistance rates to quinolones are shown in Table 1.

Prevalence of integron among the QRUPEC

The integron proportions in the isolates resistant to Ciprofloxacin, Norfloxacin and Nalidixic acid were 25, 25 and 20.8%, respectively. No *intI3* was detected in the isolates. Among resistant strains no significant relationships between carrying *intI1* and *intI2* with resistance to antibiotics was observed. The incidence of class 1 and class 2 integrons among (QRUPEC) isolates from urine specimens collected in this study is shown in Table 1.

PFGE

Thirty PFGE profiles were obtained from the genome of *E. coli* strains based on drawn dendrogram. At similarity

value of 89%, 6 clusters were obtained (Figure 1). These strains were introduced as E1 to E30 and majority of the strains showed 12 to 13 bands and the patterns with 9 or 17 bands had the lowest percentage. XbaI restriction of 30 strains of QRUPEC generated 10 to 480 kbp fragments. All of the strains had the 10, 45, 295, 290 and 382 kbp fragment, except for Norfloxacin-resistant strains, in which the 290 and 382 kbp fragment was not detected.

Figure 2 shows a PFGE pattern of QRUPEC. Each lane represents one strain of *E. coli* DNA. Lane 2 and 3 have indistinguishable banding patterns and are thus considered the same strains.

DISCUSSION

Urinary tract infections, including cystitis and pyelonephritis are the most common human extra intestinal infections. Heterogeneous group of UPEC is the most frequent cause of UTI. In fact, the most UPEC isolates commonly represent multiple VFs simultaneously. This genetic diversity has hindered the recognition of UTI genes (Johnson and Russo, 2005; Zhang et al., 2000).

Furthermore, resistance to quinolones is an increasing issue causing concern in many parts of the world (McDonald et al., 2001; Yang et al., 2010). In this study, 30 out of 96 strains (31%) exhibited resistance to quinolones. This ratio is comparable to other studies in different countries. In 2001, McDonald et al showed that 11.3% of *E. coli* isolates were resistant to fluoroquinolones in Taiwan (McDonald et al., 2001). In another study by Karlowsky, the rate of resistance to quinolones was reported to be between 1.9 to 2.5 percent (Karlowsky et al., 2003). A high rate of resistance was observed among the community of Greece, Saudi Arabia and Senegal (Chaniotaki et al., 2004; Al-Tawfiq, 2006; Sire et al., 2007).

Resistance to Nalidixic acid in our study was 25% that is lower than that of reported in other parts of the world (Rijavec et al., 2006; Mathai et al., 2004) which could be explained by low prescription of these antibiotics in our region.

It has also been shown that resistance rate to ciprofloxacin and norfloxacin was 8.3%. It was comparatively low among the UPEC isolates in this study. However, some studies have shown a sharp increase in

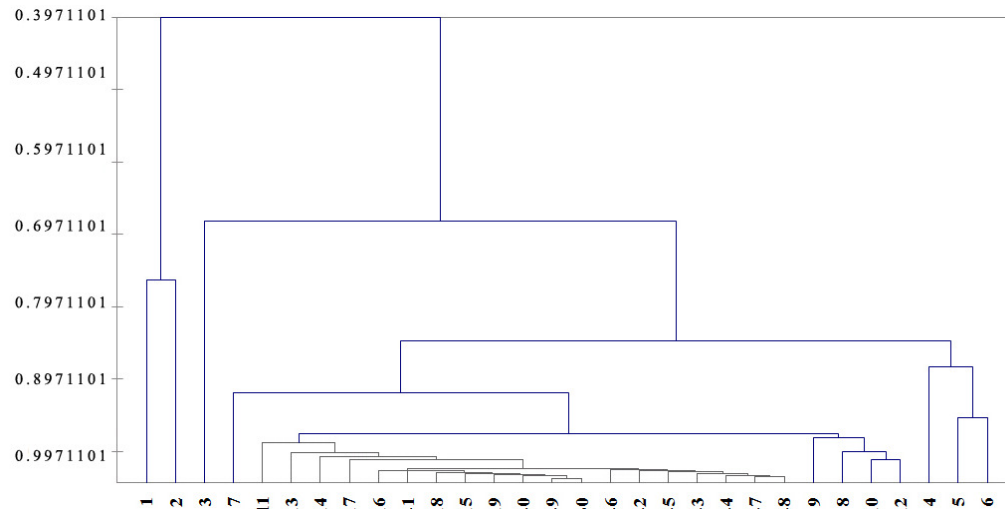


Figure 1. A dendrogram showing relationships among *Quinolones resistant UPEC* isolates from children with pyelonephritis (P) and cystitis(C).

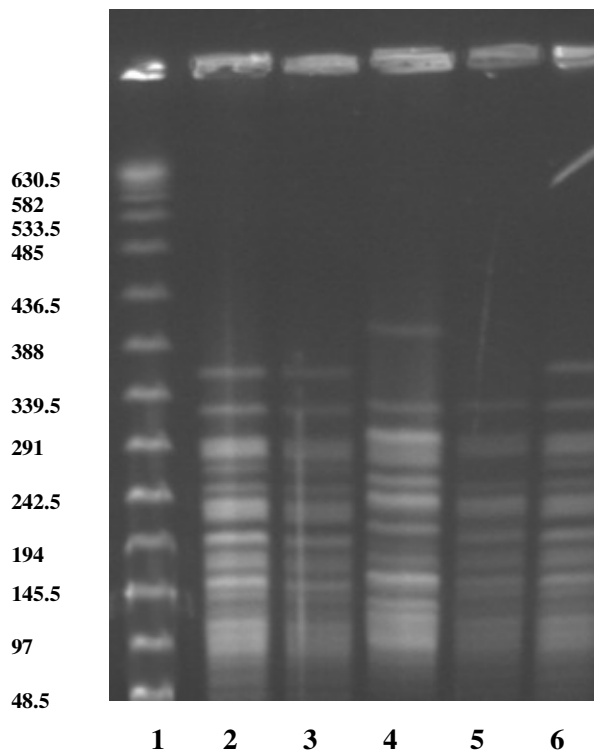


Figure 2. One representative PFGE profile showing *XbaI*-digested genomic DNA of *E. coli* strains isolated from the patients with urinary tract infections (lane 2-6). PFM is lambda ladder which is used as molecular size marker (lane 1).

ciprofloxacin resistance among UPEC (Mathai et al., 2004; Warren et al., 1999; Arredono-Garcia and Amabile-Cuevas, 2008). Of course in some countries like the

United States and Canada, the resistance remains low (Manges et al., 2006; Manges et al., 2008).

As we evaluated the sensitivity of QRUPEC by other antibiotics, all of them were MDR. As Jahrom is a small city and people living there are not well-educated about the drug consumption and provide antibiotics over the counter and take the medications on their own, MDR is more remarkable. Moreover, the over consumption of quinolones in food animal industries can contribute to the growing quinolone resistance. So, quinolones should be used for the treatment of animals only when all other therapies have failed and not to help it grow or improve the way it digests its feed. This is an urgent public health issue.

Resistance to quinolones is a growing concern among the health authorities in Iran. This increased resistance is multi-factorial and may be due to lack of proper policy to antibiotics usage and transfer of resistance genes by transportation tools such as plasmids, bacteriophage, and integrons.

In this study, we examined the role of the integrons in the prevailing quinolone resistance situation in south of Iran. Only a few studies have made systematic surveys of integron distribution. One of the first studies was by Sallen who showed integrons in 59% of the isolates belonging to six different species of Enterobacteriaceae (Sallen et al., 1995). Some of these isolates carried multiple integrons. However, the prevalence of integrons ranges from 22 to 59% has been reported in clinical *E. coli* isolates (Fluit and Schmitz, 1999; Martinez-Freijo et al., 1998; Solberg et al., 2006). All these data suggest that integrons are common worldwide, especially in Enterobacteriaceae, and that they contribute to resistance.

Considering the low prevalence of integrons among

QRUPEC, it seems that the antibiotic resistance cassettes in these strains presumably are mostly carried on the other transposable elements rather than integrons.

As VFs are located on large plasmids, pathogenicity islands and chromosomes (Hacker et al., 2003), so genotyping of the whole genome by PFGE is an effective method to determine the genetic relatedness of the QRUPEC (Arbeit, 1999).

As shown in Figure 1, 30 PFGE patterns were identified among the 30 quinolones resistant isolates. These diverse patterns have also been seen in other studies. Yang et al. obtained 69 patterns among the 74 quinolones-resistance *E. coli* isolates (Yang et al., 2010). In another study carried out in Brazil, the patterns of ciprofloxacin resistant *E. coli* by PFGE were evaluated and 46 profiles among 95 isolates were obtained (Pereira et al., 2007). In the present study, we obtained 9-15 bands, and the molecular weights of the bands vary between 10 to 480 kbp. Other studies also have shown different bands with high diversity of molecular weights. Ejrnaes et al. revealed 15-20 distinct bands with fragments of 50-1200 kbp in molecular typing of UPEC strains (Ejrnaes et al., 2006). These different results in different geographic areas imply the great diversity of *E. coli* strains.

It should be noted that all of the QRUPEC isolates have been multidrug resistant and some of them belong to a similar clone defined by a distinct pulsed-field gel electrophoresis (PFGE). The comparison of the quinolones resistant UPEC and sensitive strains, revealed the difference only in a couple of bands for some cases. Bearing in mind that mutation is an important factor in emerging quinolones resistance (Hooper, 2001); we can suggest that PFGE profiles alteration seen in the patterns could be due to mutations (insertion and deletion of DNA). These mutations occur in a region known as the quinolone resistance-determining region (Ito et al., 2008). Mutations also could be declared the relationship between quinolone resistance and low prevalence of virulence genes.

For some cases with different antibiogram patterns, maximum similarity bands with similar molecular weights were observed by PFGE methods. The data suggest that integrons may contribute the horizontal transfer of antimicrobial resistance genes among bacterial species and it forces in the emergence of new bacterial variants.

On the other hand, integrons were not seen in the strains with more than 99% similarity in their PFGE patterns. As these elements promote genetic diversity so the greater similarity is detected among the strains, the less involvement of integrons is observed.

As noted before, different PFGE patterns are seen in different regions. One of the most important reasons could be spontaneous point mutations and the acquisition of specific resistance genes by means of plasmids and transposons from other strains or even other species (Arbeit, 1999; Tenover et al., 1995). Point mutation, gene

rearrangement, or loss of genetic information also plays crucial roles in producing of different patterns. Considering these points, we can suggest these random genetic events cause resistance through the organisms which could enter the fragments in the restriction site or emit them and these agents also cause the high variety of the genome of *E. coli* strains.

Another reason for these variations in laboratories is technical difficulties such as incomplete lysis, inadequate washing or incomplete digestion, unsuitable flowing of buffer in electrophoresis chamber, fluctuation of buffer temperature during run and existence of any contamination. It also depends on other factors which cause the production of different patterns, for instance, each individual person, laboratory set up and equipment, reagent being used, quality of water, interruptions or distractions and other unknown reasons.

To the best of our knowledge, this is the first report of applying the PFGE genotyping method to the study of a molecular epidemiology of QRUPEC infection in Iran. In general, this report showed no close relationship between QRUPEC strains among a community population in southeast Iran. Of course, additional PFGE studies and novel combined methods are necessary both to early detection of the genes and their mutations which may occur in the genome of quinolones-resistant strains. Besides, resistance to the antibiotics is multi factorial and according to the low prevalence of integrons among the QRUPEC strains under the study and the MDR patterns, suggesting the minor importance of integron class in the acquisition of multidrug resistance by the UPEC.

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