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Study of class 1 integrons and plasmid profile among multiple drug resistant uropathogenic *Escherichia coli*

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Abstract: The emergence of multidrug resistance in uropathogenic *Escherichia coli* (UPEC) is associated with the presence of drug resistant plasmids and integrons which facilitate horizontal gene transfer which impose serious challenges in patients with urinary tract infections (UTIs). The proposed research study is designed to determine emerging antibiotic resistance trends and the presence of plasmids and class 1 integron in UPEC. A total 74 strains of urinary pathogens were procured among them 50 UPEC isolates were selected and their antibiotic resistance pattern was performed by CLSI guidelines. Plasmid DNA of UPEC strains was extracted by kit method and profiling was done using gel electrophoresis. Class 1 integron genes *intI1, sul1* and *qacEΔ1* were detected by multiplex PCR in UPEC. Among gram negative urinary isolates, 50 (68%) isolates were *E. coli*, while the rest were Klebsiella, Pseudomonas, Enterobacter etc. All the tested UPEC were totally resistant to quinolones while sensitive to fosfomycin, imipenem and colistin antibiotics. Majority of multidrug resistant UPEC showed common resistant phenotype of fluoroquinolones, cephalosporins, sulfamethoxazole/trimethoprim and aminoglycosides. Out of the 50 UPEC isolates 46 (92%) were multi-drug resistant having one to three plasmids of more than 1kb and 41 (82%) possessed class 1 integron genes. Over all association between antibiotic resistance and presence of class 1 integron genes showed statistically significant results ($p<0.05$). Our results also depict a strong correlation between multidrug resistance and presence of class 1 integron in UPEC isolates ($p<0.05$). The presence of multiple plasmid bands in MDR *E. coli* strains and high prevalence of class 1 integrons indicate the role of plasmids and integrons in the horizontal transmission of antibiotic resistance genes in UPEC.

Keywords: Class 1 integron, multiple drug resistance, plasmid profiling, Uropathogenic *E. coli* (UPEC)

INTRODUCTION

Escherichia coli is responsible for 90% community acquired and 50% nosocomial UTIs (Wireko *et al.,* 2017). Approximately 150 million individuals are identified with UTIs resulting in 6 billion US dollars health care expenditures (Ramírez-Castillo *et al.* 2018). It is the third most detected bacterial infection and a crucial health problem following respiratory and gastrointestinal tract infections (Nandihal 2015). Antibiotics have been successfully used to cure such infections unfortunately the development of multiple drug resistance (MDR) has become a major public health issue associated with considerable morbidity and motility especially in developing countries. Antimicrobial resistance is responsible for 700,000 deaths each year worldwide and about 10 million people will be at risk of death by 2050 (Scarafile 2016).

Integrons are mobile genetic elements responsible for the transfer of antibiotic resistance genes. Class 1 integrons are associated with multidrug resistance to different antibiotics like cephalosporins, aminoglycosides, sulfamethoxazole/trimethoprim and quaternary ammonium compounds (Poey and Laviña 2018). The 5′ conserved region of class 1 integron encodes a site-specific recombinase gene, integrase, *intI1* and a strong promoter that ensure expression of the integrated resistance gene cassettes. The 3′ conserved segment carries *qacEΔ1* gene that confers resistance to quaternary ammonium compounds (disinfectants) and *sul1* responsible for sulfonamide resistance (Canal *et al.,* 2016).

In gram negative uropathogens drug resistance character is frequently encoded on plasmids or transposons i.e. integrons which can simply be transferred among other isolates via transformation, conjugation or transduction (Lina *et al.* 2007). Moreover, Gram-negative bacteria often transfer genes that confer high levels of resistance to various other antibiotics e.g. fluoroquinolones and aminoglycosides (Adamus-Białek *et al.* 2017).

In Pakistan the problem of antibiotic resistance is alarming because of the overuse and misuse of antibiotics along with insufficient data and the absence of systematic national surveillance program. Studies on the prevalence and resistance to antibiotics have been reported from Pakistan but limited data regarding presence of class 1 integron genes and plasmid profiling of uropathogens emphasizes the need for more studies at molecular level. Therefore, the aim of the current study was to monitor the emerging drug resistance against commonly used

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antibiotics and the presence of class 1 integron genes and plasmids in UPEC. This research study would help us to understand the emergence of MDR strains and the nature of drug resistance in uropathogenic *E. coli* because presence of resistant determinants on plasmids or integron has been a major issue due to its role in the spread of antimicrobial drug resistance especially with regards to extended spectrum β-lactamase producing gram negative pathogens.

MATERIALS AND METHODS

Isolation and identification of uropathogens

The study included 50 non duplicate UPEC isolates received at the Department of Microbiology, University of Karachi. The isolates were originally obtained from urine specimens with bacterial count $\geq 10^5$ CFU/ml submitted to clinical laboratory of tertiary care hospital in Karachi, Pakistan for routine culture and antimicrobial susceptibility testing. The isolates were further purified on MacConkey‘s agar (Oxoid) and subjected to confirmation by conventional biochemical tests (Holt *et al*. 1994). All UPEC isolates were preserved in 20% glycerol and kept in freezing condition for further studies.

Determination of antimicrobial susceptibility

UPEC isolates were examined for their susceptibility to 16 different antibiotics belonging to following classes: Quinolone/Fluoroquinolones; nalidixic acid (30µg), ofloxacin (5µg), ciprofloxacin (5µg), levofloxacin (5µg). Beta-lactams; cefoxitin (30µg), cefotaxime (30µg), ceftriaxone (30µg), imipenem (10µg), aztreonam (30µg), co-amoxiclav (20µg/10µg). Aminoglycosides: gentamicin (10µg), amikacin (30µg). Sulfonamides: sulfamethoxazole/trimethoprim (25µg), Nitrofuran derivatives; nitrofurantoin (300µg), Phosphonic acid derivative: fosfomycin (50µg), and Polymixin; colistin (10µg). All these antibiotics were used to observe their spectrum of activity by disk diffusion assay (Kirby-Bauer 1996) as per the criteria specified by the Clinical Laboratory Standards Institute. *E. coli* (ATCC 25922) was used as control strain.

Plasmid DNA isolation and profiling by gel electrophoresis

UPEC were grown in nutrient broth containing sub inhibitory concentrations of ciprofloxacin and cefotaxime in order to increase the copy no of plasmids and plasmid DNA was extracted using kit (Promega, USA). Gel electrophoresis was performed according to Shehabi *et al.* (2004) with slight modifications on 0.8 % gel with marker of 1kb molecular weight was used to determine the size of plasmid DNA. Electrophoresis was carried out at 90 volts and Plasmid DNA was visualized by using UV trans-illuminator and photographs were taken for further documentation.

Detection of class 1 integron genes

Crude DNA was isolated from UPEC by colony boiling method as described elsewhere (Dallenne *et al*., 2010). Class 1 integron genes *intI1, sul1* and *qacEΔ1* were detected by multiplex PCR using primers sequences given in table 1 (Phongpaichit *et al*., 2008). Reaction volume was prepared by adding 12.5 µl of Promega green master mix, 1 µl of each forward and reverse primer, 0.5µl of molecular grade water and 10µl of extracted DNA. Amplification was performed in thermal cycler (Quanta Biotech, S-24) using cycling parameters; Initial denaturation for 3 min at 95°C then 30 cycles of 94°C denaturation, 54°C annealing and 72°C extension for 1 min each followed by final elongation at 72°C for 3 min.

STATISTICAL ANALYSIS

Association between total antibiotic resistance and presence of Class 1 integron genes in resistant isolates was analyzed by software Statistical Package for the Social Science (SPSS, Version 20.0). Statistical significance of data was determined using Fisher exact test and $p < 0.05$ was considered significance.

RESULTS

Identification of Gram negative bacteria from UTIs

Of the 74 gram negative urinary isolates, 50 isolates were *E. coli*, the rest were Klebsiella spp. (10), Pseudomonas spp. (6), Enterobacter spp. (4), Morganella spp. (3) and Proteus spp. (1).

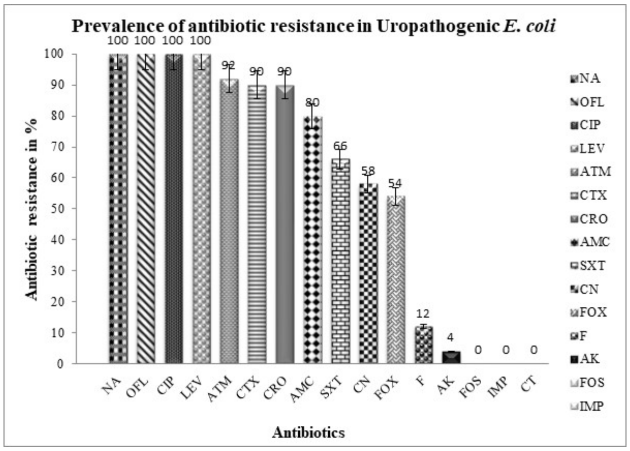

Fig. 1: Antibiotic resistance pattern of UPEC against NA, nalidixic Acid; OFL, ofloxacin; CIP, ciprofloxacin; LEV, levofloxacin; ATM, aztreonam; CTX, cefotaxime; CRO, ceftriaxone; AMC, amoxicillin/Clavulanic acid; SXT, sulfamethoxazole/trimethoprim; CN, gentamicin; FOX, cefoxitin; F, nitrofurantoin; AK, amikacin; FOS, fosfomycin; IMP, imipenem; CT, colistin

Antibiotic resistance pattern of UPEC

Disk-diffusion susceptibility testing of UPEC showed high resistance to different classes of antibiotics (fig. 1) and were found to be 100% resistant to quinolones i.e nalidixic acid, ciprofloxacin, ofloxacin and levofloxacin. However, all strains remained sensitive to fosfomycin, imipenem and colistin. *E. coli* strains also showed high resistance to aztreonam 92%, cefotaxime and ceftriaxone i.e. 90% each and co-amoxiclav 80%, while resistance to sulfamethoxazole/trimethoprim, gentamicin, cefoxitin, nitrofurantoin and amikacin were found to be 66%, 58%, 54%,12% and 4% respectively.

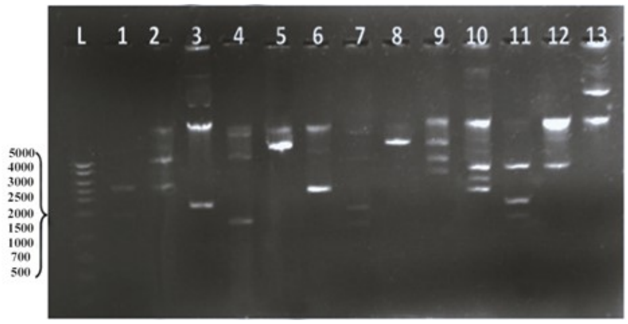

Fig. 2: Agarose gel electrophoresis of plasmid DNA. Lane L: molecular weight marker (1 Kb plus), Lane 1: Plasmid DNA of sensitive strain of *E. coli*, Lane 2-13: Plasmid DNA of MDR UPEC isolates.

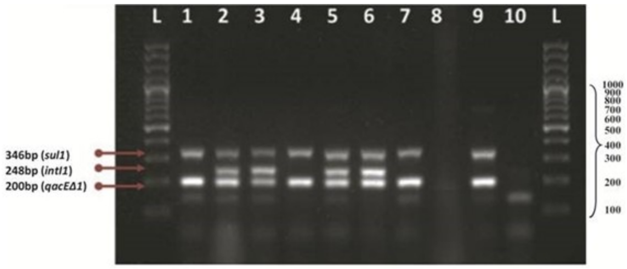

Fig. 3: Triplex PCR detection of Class 1 integron genes in UPEC isolates. L= molecular weight marker of 100 bp plus ladder, Lanes 1, 2, 3, 4, 5, 6, 7 and 9 integron genes positive UPEC while Lanes 8 and 10 showed negative results

Of the 50 *E. coli* isolates 46 (92%) were MDR showing resistance to 3 or more classes of antibiotics. More importantly among these 15(32%) were resistant to 4 classes of antibiotics and 6.52% were resistant to 5 classes of antibiotics.

Plasmid profile analysis of UPEC

Plasmid profiling analysis of *E. coli* from urine isolates was observed by agarose gel electrophoresis. Plasmid DNA of all UPEC strains was extracted and analyzed on agarose gel electrophoresis. Multiple plasmids of variable banding pattern were observed (fig. 2). Besides, most MDR strains showing resistance to more than 3 antibiotics carried large plasmids of >1 kb and showed common resistance phenotype to different antibiotics, suggesting that several resistant determinants may be encoded on the same replicon. However, no large size plasmids (> 1kb) were found in sensitive strains.

Presence of class 1 integron in UPEC and association with drug resistance

Among 50 UPEC, 41 (82%) isolates carried class 1 integron genes *intI1, sul1* and *qacEΔ1* either independently or in combination. Of 50 strains 22 (44%) carried all 3 analyzed genes (table. 2) while 9 (18%) UPEC strains did not carry class 1 integron in which 3 isolates were non MDR and 6 isolates showed resistance to other antibiotics except sulfonamide and quaternary ammonium compounds. Moreover, all UPEC showing resistance to sulfamethoxazole/trimethoprim phenotypically by disk diffusion method likewise was found positive for *sul1* gene Over all association between antibiotic resistance and presence of class 1 integron genes showed statistically significant results ($p < 0.05$) table 4. Our results also depict a strong correlation between multidrug resistance and presence of class 1 integron in UPEC isolates ($p < 0.05$) table 5.

DISCUSSION

Quinolones/fluoroquinolones, β-lactams and aminoglycosides are widely used antibiotics for UTIs caused by gram negative bacteria (S. K. Mukherjee & Mukherjee, 2019). Complete resistance against fluoroquinolones has been observed in this study. A research study conducted in 2011 from Karachi indicated resistance against ciprofloxacin was 85%, ceftriaxone 88%, gentamicin 60% and imipenem 9% (Hassan *et al.*, 2011).

Likewise, data regarding ciprofloxacin and ofloxacin antibiotics, showed high resistance in different surveys and European countries e.g. high resistance (95%) to fluoroquinolones in *Enterobacteriaceae* strains was reported by Fluit *et al.* (2000) from Europe. Various studies have reported mutations in target binding site in quinolone resistance determining region (DNA gyrase and topoisomerase IV) are involved in increased resistance although plasmid mediated quinolone resistance may also be involved (Goudarzi, 2015; Varughese *et al*., 2018). However, low levels of resistance to amikacin (4%) and no resistance against fosfomycin, imipenem and colistin was observed in the present study so these antibiotics could be used for the treatment of UTI especially against ESBL producing UPEC. In the present study resistance to Sulfamethoxazole/trimethoprim, Nalidixic Acid, Ciprofloxacin, Levofloxacin, Cefoxitin, Cefotaxime, Ceftriaxon, Gentamicin and Amikacin was found to be associated statistically with presence of class 1 integron. Most of UPEC were resistant to three or more groups of antibiotics thus showing MDR phenotypes and out of 50 UPEC isolates 46 (92%) were multi-drug resistant. This study has represented strong correlation between MDR and presence of class 1 integron (p<0.05) that is in agreement with the study of Singh *et al.* (2017).

Table 1: Primers for amplification of class 1 integron

Gene	Primer Detection	Primer Sequence (5’-3’)	Amlpicon size (bp)	Annealing Temp ⁰C	References
intI1	intI1 F	GGTTCGAATGTCGTAACCGC	248	54	Phongpaichit et al.,2006
	intI1 R	ACGCCCTTGAGCGGAAGTATC			
sul1	sul1 F	ATCAGACGTCGTGGATGTCG	346		
	sul1 R	CGAAGAACCGCACAATCTCG			
qacE1	qacE1 F	GAGGGCTTTACTAAGCTTGC	200		
	qacE1 R	ATACCTACAAAGCCCCACGC			

Table 2: Presence of class 1 integron in UPEC

Integron genes	No.	%
intI1	6	12
sul1+ intI1	1	2
intI1+ qacEΔ1	0	0
sul1+ qacEΔ1	12	24
sul1+ intI1+ qacEΔ1	22	44
None	9	18

Table 3: MDR strains of *E. coli* showing resistance to different classes of antibiotics

Antimicrobial resistance profiles of MDR UPEC	Number of strains showing profile (n=46)
NA, OFX, CIP, LEV, ATM, CTX, CRO, AMC, FOX, CN, SXT	7
NA, OFX, CIP, LEV, ATM, CTX, CRO, AMC, FOX, SXT	6
NA, OFX, CIP, LEV, ATM, CTX, CRO, AMC, CN, SXT	5
NA, OFX, CIP, LEV, ATM, CTX, CRO, AMC, SXT	4
NA, OFX, CIP, LEV, ATM, AMC, CTX, CRO, FOX, CN	4
NA, OFX, CIP, LEV, ATM, CTX, CRO, SXT	3
NA, OFX, CIP, LEV, ATM, CTX, CRO, AMC, CN	3
NA, OFX, CIP, LEV, ATM, CTX, CRO, CN	2
NA, OFX, CIP, LEV, ATM, CTX, CRO, AMC, FOX, CN, SXT, F	1
NA, OFX, CIP, LEV, ATM, CTX, CRO, AMC, FOX, CN, AK, SXT	1
NA, OFX, CIP, LEV, ATM, CTX, CRO, AMC, FOX, CN, AK	1
NA, OFX, CIP, LEV, ATM, CTX, CRO, FOX, SXT	1
NA, OFX, CIP, LEV, CN, SXT	1
NA, OFX, CIP, LEV, ATM, CTX, CRO, FOX, SXT, F	1
NA, OFX, CIP, LEV, ATM, AMC, CN, SXT	1
NA, OFX, CIP, LEV, CTX, CRO, FOX, CN, SXT, F	1
NA, OFX, CIP, LEV, ATM, CTX, CRO, AMC, CN, F	1
NA, OFX, CIP, LEV, ATM, CTX, CRO, AMC, FOX, CN, F	1
NA, OFX, CIP, LEV, CTX, CRO, AMC, FOX	1
NA, OFX, CIP, LEV, AMC, SXT, F	1

The study showed co-resistance among fluoroquinolones, beta-lactams, sulfamethoxazole/ trimethoprim, aminoglycosides and amoxicillin/clavulanic acid (table 3). The high prevalence of MDR UPEC isolates is comparable to the high rate of MDR isolates in other studies from Pakistan (97.46%) (Fatima *et al*., 2018), India (92.5%) (MuKherjee *et al.,* 2013) and China (78.1%) (Wang *et al.,* 2014). MDR has direct link with ESBL production which itself cause complications in UTIs management.

Uropathogens harboring plasmids that carry resistance genes have clinical significance due to the limited therapeutic options (Mukherjee & Mukherjee, 2019). The high frequency of conjugative R plasmid has been reported worldwide in association with resistance to more than three drugs in *E. coli* (Rozwandowicz *et al*., 2018). Especially in case of extended spectrum β-lactamase (ESBL) producing gram negative uropathogens drug resistance character is most frequently encoded on plasmids. Plasmid profiles have been reported to use as epidemiological tool and to determine the transmission pattern of antibiotic resistance genes among microorganisms (Kammili *et al*., 2020). It is usually difficult to establish the size limits of plasmids or the real size distribution in any organisms because the method of isolation of plasmid is more effective with the smaller plasmids than with the larger ones. Pathogenic strains of

Table 4: Association between antibiotic resistance and presence of class 1 integron genes in UPEC isolates

Antibiotics Tested	Total resistant UPEC (50) No. (%)	Resistant isolates with Class 1 integron genes (41) No. (%)	Association of resistance with Class 1 integron genes (*p*-value)
Nalidixic Acid	50 (100)	41 (100)	0.049*
Ofloxacin	50 (100)	41 (100)	0.049*
Ciprofloxacin	50 (100)	41 (100)	0.04*
Levofloxacin	50 (100)	41 (100)	0.041*
Imipenem	0 (0)	0 (0.00)	-
Cefotaxime	45 (90)	37 (90.24)	0.049*
Ceftriaxon	45 (90)	37 (90.24)	0.049*
Colistin	0 (0)	0 (0.00)	-
Gentamicin	29(58)	23 (56.09)	0.01*
Amikacin	2 (4)	1 (2.43)	0.00*
Cefoxitin	27 (54)	23 (56.09)	0.01*
Aztreonam	46 (92)	37 (90.24)	0.04*
Amoxicillin+Clavulanic Acid	40 (80)	34 (82.92)	0.04*
Nitrofurantoin	6 (12)	6 (14.63)	-
Sulfamethoxazole/ trimethoprim	33 (66)	33 (80.48)	0.001*
Fosfomycin	0 (0)	0 (0.00)	-

*significant *p*-value. Frequencies are given as absolute numbers with their percentages.

Table 5: Relationship between MDR and presence of class 1 Integron

Integron	Antibiotic		*p*-value
	MDR (%)	Non MDR (%)	
Integron Positive (n= 41)	40 (86.95)	01 (25)	<0.05
Integron Negative (n=9)	06 (13.04)	03 (75)	
Total	46	04	

The fisher exact test value is 0.0155. The result is significant at *p*-value < 0.05.

E. coli carried plasmids range from 2-3 kb to 6.5 kb with highest range of 26 kb (Dadi *et al*., 2018). In this study UPEC harbored plasmids ranging from 1-5 kb to >5kb. Study from India also revealed that *E. coli* isolates harbored 1.6 kb and 5 kb plasmids (Jan, Meshram, & Kulkarni, 2009). Other studies from Nigeria and Ethiopia showed that MDR clinical isolates of *E. coli* carried plasmids having diverse molecular sizes from 2 kb to 6.5-23 kb to maximum of 26 kb (Akingbade *et al*., 2014; Dadi *et al*., 2018). Genes for β-lactamases and ESBLs can be carried on plasmids as well.

Integron are remarkable genetic elements allowing bacteria to adapt by capturing, integrating and expressing antibiotic resistant genes hence facilitate horizontal transmission of antibiotic resistance genes. Class 1 integron is frequently found in gram negative bacteria. Various studies showed high prevalence of class 1 integron in MDR *E. coli* (Bashir *et al.,* 2015; Khan *et al*., 2018) and other studies also showed prevalence about 22-59% (Fluit & Schmitz, 2004; Sung & Oh, 2014). ESBLs regulating genes are also found on integrons. Integrons contain more than 70 antibiotic resistance genes as gene cassettes. The findings of this study indicate integron prevalence to be 82%. However 6 MDR strains did not carry class 1 integron probably due to alternative mechanism of drug resistance. In this study we observed the association of class 1 integron and multiple plasmids which may play a significant role in the dissemination of antibiotic resistance among clinical isolates which can result in serious consequences. Resistant plasmids and integrons are major contributors help understanding the drug resistance at global level.

CONCLUSION

This study has indicated high resistance towards quinolone and cephalosporins leaving limited therapeutic options. Plasmids and presence of integron genes predominantly play a significant role in the dissemination of antibiotic resistance. This study will help us in understanding the mechanism of drug resistance in UPEC, which may help designing new policies in preventing the spread of antibiotic resistance genes in hospital as well in community settings.

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