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Prevalence of Integrons and Antibiotic Resistance among Uropathogenic *Escherichia Coli* from Faisalabad Region of Pakistan

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Abstract

Background: Uropathogenic *Escherichia coli* are among the major pathogens causing urinary tract infections. In the current study the uropathogenic *E. coli* were isolated from clinical samples of urine from Faisalabad region of Pakistan. The extensive use of antibiotics to treat such infections resulted in the emergence of Multiple Drug Resistant (MDR) bacteria. This study aimed to identify the prevalence of integrons and antibiotic resistance among pathogenic *E. coli* isolated from patients with urinary tract infections.

Methods: In this study, a total of 26 isolates were studied for phenotypic resistance by disc diffusion method and genotypic resistance by PCR.

Results: Most of the isolates exhibited multiple drug resistance to ampicillin (100%), tetracycline (77%), streptomycin (81%), trimethoprim sulfamethoxazole (65%), nalidixic acid (46%), ciprofloxacin (38%) and chloramphenicol (27%). The prevalence of the drug resistance genes was in following order: *tetA* (12%), *tetB* (65%), *tem* (58%), *tem β lactamase* (15%), *sulI* (4%), *sul II* (8%), *gyrA* (73%), *catp* (35%). The gene *aadA1* was absent among the isolates. It was also found that class 1 integrons were present in 69% of multidrug resistant (MDR) isolates and the isolates were more resistant to ampicillin, tetracycline, streptomycin, trimethoprim sulfamethoxazole, nalidixic acid, ciprofloxacin and chloramphenicol than isolates with no class 1 integrons. An instance of class 2 integrons was detected only in one isolate, but there was no instance of class 3 integrons in any of the isolates.

Conclusions: This study revealed that class I integrons are widely present in pathogenic *E. coli* isolated from clinical samples of urine. Antibiotic resistance to multiple drugs is a serious emerging threat in developing countries like Pakistan.

Keywords: Integrons, antibiotic resistance, pathogenic *Escherichia coli*

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Background

Urinary Tract Infections (UTIs) are most important among extra-intestinal infections caused by uropathogenic *Escherichia coli,* responsible for up to 90% of UTIs in humans [1,2]. The extensive use of antibiotics in veterinary and human medicine results in the emergence of Multiple Drug Resistant (MDR) bacteria [3,4]. These MDR bacteria rapidly spread resistance by horizontal gene transfer through transposones (integrons) or the processes of conjugation, transformation and transduction [5].

Integrons are basically assembly platforms or DNA elements that contain open reading frames as gene cassettes and convert them to functional genes. These were first identified conferring their important role in the spread of MDR genes [6]. The fundamental integron structure consists of a 5'-Conserved Segment (CS) of 1.4-kbp which contained integrase gene and 3'-CS of 2-kbp consists of *qac E delta* and *sulI* genes. DNA sequences of variable length and molecular complexity found between these conserved regions are known as gene cassettes and several have now been characterized. More than 70 different antibiotic resistance genes which represent major classes of antimicrobials are present as gene cassettes in integrons [7]. The higher level of resistance

have been observed to commonly used antibiotics like ampicillin, gentamicin and trimethoprim [8,9]. The economic burden of these infections greatly increased due to increased antimicrobial resistance and high recurrence rate among uropathgens [10].

UTIs caused by the pathogenic *E. coli* are very common in Pakistan and the rapid emergence of MDR strains is a major health problem. The purpose of present work was to determine the presence of integrons and antibiotic resistance in *E. coli* that would help to understand the spread of MDR in local region of Pakistan.

Material and Methods

Collection of bacteria

The bacterial isolates investigated in this study were isolated from urine samples collected from different clinical laboratories in Faisalabad. Midstream urine samples from patients with suspected urinary infection were collected in sterile containers with standard precautions. The samples were inoculated on nutrient agar slants placed at 37°C for 24 hrs and stored at 4°C before transfer to National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan.

Selection and biochemical identification of bacterial isolates

Each sample was inoculated in trypticase soya broth. After overnight incubation at 37°C each sample was shifted to MacConkey agar (selective and differential media) which inhibits the growth of gram positive bacteria. For biochemical identification a single colony was inoculated on triple sugar iron slants.

DNA extraction and estimation

A single colony was again inoculated into freshly prepared and autoclaved trypticase broth and after overnight incubation at 37°C the DNA was extracted from bacterial cells conventional phenol-chloroform-isoamyl alcohol method, followed by *RNase* treatment for the removal of contaminating RNA [11]. The quantitative estimation of the isolated DNA was done spectrophotometrically (Lambda 5UV/Vis, Perkin Elmer, USA; Bio projects Gmbh, Germany) at 260 nm.

PCR for the confirmation of *E. coli* isolates

All the isolates were confirmed by PCR using specific primers by targeting conserved regions of *uidA* gene encodes *ß-glucuronidase* of *E. coli* genome **(Table 1)**. Each 50 µL PCR reaction mixture contained 1X PCR buffer (50 mM KCl, 10 mM Tris HCl; pH 8.3); 1.25 mM $MgCl_2$; dNTP's (dATP, dCTP, dGTP, dTTP) 0.2 mM each; 10 pmol of each primer; 5 U of recombinant *Taq polymerase* (Fermentas) and 0.1 µg/µL of DNA template. The PCR was done by following the thermal cycler (MasterCycler; Eppendorf, Hamburg, Germany) conditions as, denaturation for 5 min at 94°C; 30 cycles of amplification at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min; and finally extension at 72°C for 7 min.

Antibiotic sensitivity testing

Seven antibiotics were selected in this study. Antibiotic sensitivity was determined by following standard disc diffusion method according to the recommendations of the National Committee for Clinical Laboratory Standards [12]. The antibiotic discs used were Ampicillin (10 µg) belong to β lactam group, Streptomycin (10 µg) belong to aminoglycosides; Nalidixic acid (30 µg), Ciprofloxacin (5 µg) belong to quinolones; while others were Chloramphenicol (30 µg), Trimethoprim sulfomethoxazole (25 µg) and Tetracycline (30 µg). The disc diffusion breakpoints for each antimicrobial agent are given in **Table 2**.

PCR for integrons

Three classes of integrons were identified by using primers given in **Table 1**. PCR reaction mixture conditions were the same as mentioned above for *uidA* gene, whereas thermal cycler conditions for class 1 integron was 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min and final extension of 5 min at 72°C. PCR for class 2 integron was performed at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 49°C for I min and 72°C for 1 min and final extension of 3 min at 72°C.

PCR for antibiotic resistant genes

PCR was performed for the detection of nine different antibiotic resistant genes; primers are given in **Table 1**. Most commonly reported primers were selected from the database which targets antibiotic resistance genes associated with commonly used antibiotics. Thermal cycler conditions for *tem*, *tem ß lactamase, catP,* and *tetB* genes were as follows: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 1 min. PCR for *gyrA* gene was conducted at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The conditions for amplification of *aadA1* genes were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min whereas for *tetA* gene the conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 1.5 min. A final extension for 7 min at 72°C was performed at the end of each PCR.

Results

Confirmation of *E. coli* isolates

All the 26 isolates were confirmed through PCR by targeting *uidA* gene with an amplification product of 486 bp as shown is **Figure 1**.

Antibiotic resistance

It was found that among 26 pathogenic *E. coli* isolates resistance to tetracycline, ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin, chloramphenicol and streptomycin was 20 (77%), 26 (100%), 17 (65%), 10 (38%), 7 (27%) and 21 (81%) observed respectively as shown in **Table 3**. All twenty-six isolates were resistant to at least two antibiotics. Four isolates showed resistance to all seven drugs. All the isolates were 100% resistant to ampicillin.

Genotypic resistance

Genotypic resistance against each antibiotic was identified by targeting few selected antibiotic resistance genes. Relevance

Table 1 Oligonucleotide primers used for the identification of *E. coli*, antibiotic resistance genes and integrons.

Genes	Primer sequences (5′ to 3′)	Amplicon size (bp)	References
uidA F *uidA* R	ATCACCGTGGTGACGCATGTCGC CACCACGATGCCATGTTCATCTGC	486	[26]
tetA F *tetA* R	GTAATTCTGAGCACTGTCGC CTGCCTGGACAACATTGCTT	1000	[27]
tetB F *tetB* R	CTCAGTATTCCAAGCCTTTG CTAAGCACTTGTCTCCTGTT	440	[28]
tem F *tem* R	GCACGAGTGGGTTACATCGA GGTCCTCCGATCGTTGTCAG	311	[29]
tem β lactamase F *tem β lactamase* R	ATGAGTATTCAACATTTCCGTGT TTACCAATGCTTAATCAGTGACG	876	[30]
sul1 F *sul1* R	CTTCGATGAGAGCCGGCGGC GCAAGGCGGAAACCCGCGCC	437	[31]
sul2 F *sul2* R	TCAACATAACCTCGGACAGT GATGAAGTCAGCTCCACT	707	[30]
gyrA F *gyrA* R	TACCGTCATAGTTATCCACGA GTACTTTACGCCATGAACGT	342	[32]
catp F *catp* R	CCTGCCACTCATCGCAGT CACCGTTGATATATCCC	639	[33]
aadA1 F *aadA1* R	CGGTGACCATCGAAATTTCG CTATAGCGCGGAGCGTCTCGC	250	[34]
int1 F *int1* R	ATCATCGTCGTAGAGACGTCGG GTCAAGGTTCTGGACCAGTTG	550	[35]
int2 F *int2* R	GCAAATGAAGTGCAACGC ACACGCTTGCTAACGATG	467	[36]
int3 F *int3* R	GCAGGGTGTGGACGAATACG ACAGACCGAGAAGGCTTATG	760	[37]

F = forward; R = reverse.

Table 2 Zone diameter breakpoints for Enterobacteriaceae for different antimicrobial agents (NCCLS, 2004).

Antimicrobial Agent	Disc potency (µg)	Resistant (R) (mm)	Intermediate (I) (mm)	Susceptible (S) (mm)
Ampicillin	10	≤13	14-16	≥17
Chloramphenicol	30	≤12	13-17	≥18
Ciprofloxacin	5	≤15	16-20	≥21
Nalidixic acid	30	≤13	14-18	≥19
Streptomycin	10	≤11	12-14	≥15
Tetracyclin	30	≤14	15-18	≥19
Trimethoprim/ Sulfamethoxazole	1.25/ 23.75	≤10	11-15	≥16

of genotypic and phenotypic resistance is given in Table 3. Two ampicillin resistance genes *tem* and *tem β lactamase* were observed in 15 (58%) and 4 (15%) isolates respectively. No isolates was positive for both ampicillin resistant genes. Twenty (77%) isolates showed resistance to tetracycline, whereas *tetA* and *tetB* genes which are tetracycline resistance genes were present in 3 (12%) and 17 (65%) isolates respectively. Two (8%) isolates harbored both *tetA* and *tetB* genes. Resistance to chloramphenicol was observed in just 7 (27%) isolates while chloramphenicol resistance gene *catp* was detected in 9 (35%) of isolates. Antibiotic resistance to ciprofloxacin, nalidixic acid, trimethoprim-sulfamethoxazole and streptomycin was observed in 10 (38%), 12 (46%), 17 (65%), and 21 (81%) isolates respectively. The *gyrA* gene confers the resistance to ciprofloxacin was observed in 19 (73%) isolates whereas *sul1* and *sul2* genes responsible for trimethoprim-sulfamethoxazole resistance were observed in 1 (4%) and 2 (8%) isolates, respectively. No isolate was positive for both genes. Streptomycin resistance gene, *aadA1,* was observed in 3 (12%) isolates. Chloramphenicol is the antibiotic against which least resistance was observed.

Identification of integrons

Out of 26 isolates class 1 integron was detected in 17 (65%) of isolates showing amplification product of 550 bp **(Figure 2)**, whereas class 2 integron was detected in only 1 (4%) of clinical isolate of extraintestinal *E. coli* **(Figure 3)**. All other extraintestinal *E. coli* isolates were negative for class 3 integrase. In 8 (31%) isolates no integron gene was detected.

Table 3 Relevance of genotypic and phenotypic antibiotic resistance among uropathogenic *E. coli.*

Antibiotics	Disc diffusion method resistant isolates		Targeted Genes	PCR	
	(n=26)	(%)		(n=26)	(%)
Tetracycline	20	77	*tetA* *tetB* *tetA+tetB*	3 17 2	12 65 8
Ampicillin	26	100	*tem* *tem β lactamase* *tem+ tem β lactamase*	15 4 0	58 15 0
Trimethoprim	17	65	*sul1* *sul2* *sul1+sul2*	1 2 0	4 8 0
Ciprofloxacin	10	38	*gyrA*	19	73
Chloramphenicol	7	27	*Catp*	9	35
Streptomycin	21	81	*aadA1*	0	0

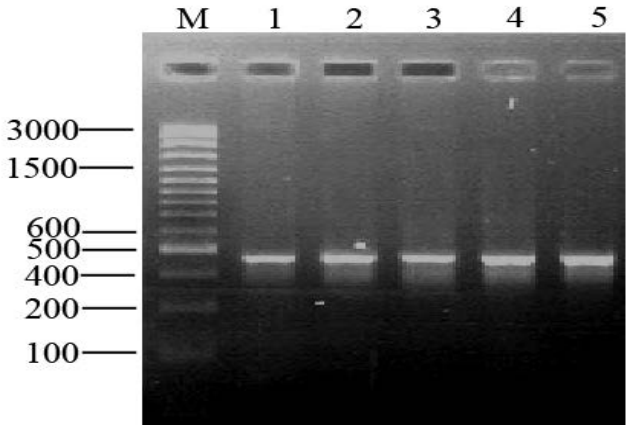

Figure 1 PCR based confirmation of pathogenic *E. coli* isolates (M = Molecular marker of DNA {GeneRuler SM0323, Fermentas}; Lanes 1-5: pathogenic *E. coli* isolates showing amplification product of 486 bp *uidA* gene).

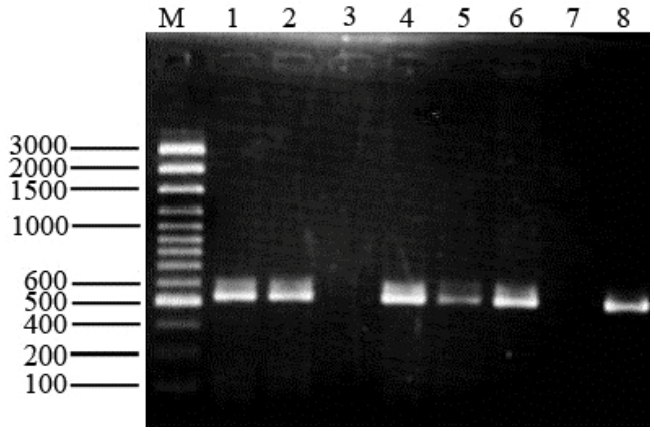

Figure 2 Identification of class 1 integron from pathogenic *E. coli* isolates (M = Molecular marker of DNA {GeneRuler SM0323, Fermentas}; Lane 1,2,4-6,8: pathogenic *E. coli* isolates showing class 1 integron of size 550 bp; Lane 3 & 7: Isolates showing no amplification for class 1 integron).

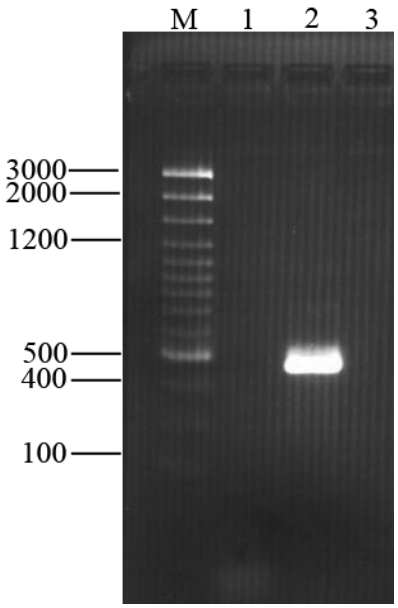

Figure 3 Identification of class 2 integrons from pathogenic *E. coli* isolates (M = Molecular marker of DNA {GeneRuler SM0323, Fermentas}; Lane 2: Pathogenic *E. coli* isolates showing class 2 integron of size 467 bp; Lane 1 & 3: Isolates showing no amplification for class 2 integron).

Discussion

Pathogenic *E. coli* are the major cause of UTIs [1]. Most of the work on this subject has been done in Western countries where climatic, social and environmental conditions are quite different from developing countries with a distinct host-pathogen interaction. In developing countries like Pakistan, UTIs are much more common, complicated and poorly documented. These infections are usually difficult to eradicate because the pathogenic bacteria have acquired resistance to most of the drugs. Emerging antibiotic resistance among these pathogens has become a big threat for treatment strategies for UTIs [4].

In the present study highest resistance (100%) was observed against ampicillin. Similar observations have been reported [13]. The prevalence of *tem* and *tem β lactamase* resistance genes

were 58 and 15%, respectively whereas point mutations in *tem β lactamase* are highly responsible for resistance [9].

Ten (38%) out of twenty-six isolates showed resistance to ciprofloxacin by disc diffusion method. When these isolates (n=26) were checked for the presence of *gyrA* (ciprofloxacin resistance gene) 19 (73%) isolates showed positive results. The detection of this gene in 9 isolates that were sensitive by disc diffusion method, indicated that in these cases, the gene was non-functional due to point mutations [14] or some other reasons. This high occurrence of ciprofloxacin resistance is in sharp contrast to some other studies which reported only one ciprofloxacin resistant strain of *E. coli* [15]. Shigemura et al. has also reported an emergence of fluoroquinolne resistant *E. coli* responsible for UTIs [16]. It was reported that ciprofloxacin is a good choice for UTIs therapy in women [17].

In this study very high tetracycline resistance (77%) was observed. An increased tetracycline resistance in human isolates was found unexpected because in humans the use of tetracycline is less as compared to animals [18]. The *tetB* gene was present more frequently than *tetA* gene. This finding is in accordance with some previous reports which showed that *tetB* gene is frequently responsible for resistance to tetracycline in the clinical settings [14]. The use of chloramphenicol is very rare in the clinical setting. Seven (27%) of isolates showed resistance to chloramphenicol, whereas c*atp* gene was observed in 9 (35%) isolates. Resistance to this drug is due to the presence of chloramphenicol acetyle transferase (*cat*), which inactivates the drug [19]. In another study prevalence of chloramphenicol resistance was 36% among uropathogenic *E. coli* [20].

Trimethoprim or trimethoprim–sulfamethoxazole or alone has been widely used as empirical therapy of UTIs from the past two decades. In US resistance to trimethoprim-sulphamethoxazole exceeds 20%, whereas in southern Europe, Bangladesh and Israel the prevalence of resistance is now 30-50% [21]. In the present study, 65% resistance was observed. Sulfonamide resistance is usually encoded by *sul1* and *sul2* genes. Strangely, we could detect *sul1* gene in only one isolate and s*ul2* gene was detected in 2 (8%) isolates, which is also an integral part of a conserved region of class 1 integron. Whereas in another study the class 1 integron (43.56%) along with *sul1* (45.5%) and *sul2* (51.48%) were observed among uropathogenic *E. coli* [22]. Streptomycin belongs to aminoglycosides and it was found that 81% of isolates were resistance to this antibiotic. The *aadA1* gene was not found among these isolates. These resistance genes can result from mutations in preexisting 'housekeeping' genes such as the sugar kinases and aminoglycoside acetyltransferases that may have evolved to modify antibiotics [14]. A study conducted in Pakistan in the year 2014 showed that urinary tract infections are caused by multiple drug resistant uropathogenic *E. coli* i.e. 81% [23].

The presence of integrons in the clinical pathogenic isolates is also highly related to antibiotic resistance. In the present study different classes of integrons were investigated. Class 1 integron was highly prevalent in these pathogenic isolates. In this study, 65% of isolates harbored class 1 integron, whereas in another study class 1 integron was found in 49% of uropathogenic isolates [24]. A high prevalence (67/89; 75%) of class 1 integrons was observed in *E. coli* isolates from UTIs. In another study class 1 integron was detected in 41% of uropathogenic isolates [25]. Only one isolate was positive for class 2 integron all were negative for class 3 integron. It was investigated in another study that the prevalence of class 1 and 2 integrons were (22%) and (8%), respectively [26-33]. In another study conducted at China the prevalence of class 1 integrons was 54.9%, three isolates were positive for class 2 integron, whereas class 3 intergon was not present in any isolate [34-39], it showed that class 1 integrons are mostly responsible for multiple drug resistance among uropathogenic *E. coli.*

Conclusion

It was concluded that multiple drug resistance is very common in local isolates of uropathogenic *E. coli* and in most cases integrons were also present. In developing countries like Pakistan proper surveillance for antibiotic use is very essential. The information provided in this work is novel in Pakistan and should serve as a guideline for clinicians for the treatment strategies of urinary tract infections.

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