Antimicrobial Resistance Pattern of *Escherichia Coli* From Urinary Tract Infections in Relation to ESBL and *pap* gene Production and Fosfomycin Sensitivity

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Abstract

Introduction: Urinary Tract Infections (UTI) is becoming increasingly difficult to treat due to emergence of multi-drug resistant uropathogens. Fosfomycin can be used as an alternative due to lack of cross-resistance. We aimed to evaluate in-vitro activity of fosfomycin against uropathogenic *E.coli*.

Material and Methods: The study period was September 2017 to August 2018. Two hundred and fifty two urine samples were collected from women having uncomplicated cystitis. Out of these168 isolates was *E.coli,* Antimicrobial susceptibility testing was performed using the Kirby-baeur disk diffusion method. ESBL screening was done using the double disc synergy test. Fosfomycin susceptibility was determined by the disk diffusion method for *E.coli*. Prevalence of the *pap gene* among the isolates were checked using amplification.

Results: The study showed that among E.coli isolates frequency of ESBL producers were 30.95% while 4 % were identified as carbapenem resistant Enterobacteriaceae (CRE). Among the isolates, 84.50% were susceptible to fosfomycin. There was 92.0% fosfomycin susceptibility in ESBL producing *E.coli,* and 71.42% showed fosfomycin susceptibility in CRE. The overall *pap* gene prevalence was 18% and the expression was 22% among the ESBL isolates and 20% among the CRE.

Conclusion: The study demonstrates that, a considerable proportion (68%) of the multidrug-resistant E.coli with diverse resistance mechanisms, including ESBL and CRE. Fosfomycin resistance was low even among ESBL and CRE isolates. Pap genes were prevalent among the ESBL isolates .For the treatment of uncomplicated cystitis fosfomycin is a useful antibiotic agent.

Objective: The aim of this study was to establish *in vitro* susceptibility of uropathogenic *E.coli* to fosfomycin and to check the prevalence of the expression of pap *gene a* virulence determinant among the isolates from uncomplicated cystitis in females.

Keywords*: Escherichia coli, ESBL, fosfomycin, CRE, pap gene, antimicrobial resistance***.**

Introduction

Urinary tracrt infections is a common infection and *Escherichia coli* is the most common pathogen encountered[(1)].The high rates of resistance exhibited by the uropathogens and particularly *E.coli* is alarming which necessitate the re-evaluation of old antibiotics [(2)]. Among UTIs, cystitis is a common infection. It affects mostly women of reproductive age, while incidence declines after the age of 40[(3)].Just as in other infections there is an alarmingly high rate of multi drug resistance and extensively drug resistant bacteria causing UTI especially when patients are with co morbidities and repeated antibiotic exposures[(4)]. Since carbapenems show excellent activity against uropathogens, they are the antimicrobial agents of choice for urinary tract infections (UTIs), especially those due to extended spectrum beta-lactamase producing *Escherichia coli*[(5)].

The increase of multidrug-resistant (MDR) bacteria induced a renewed interest in old antibiotics, such as fosfomycin[6]. Because of its broad-spectrum activity,sustained urinary concentrationsand its safety profile. Fosfomycin tromethamine a stable salt of fosfomycin is licensed for the single-dose treatment of acute uncomplicated urinary tract infections (UTIs) caused by susceptible organisms[7].Fosfomycin, originally named phosphonomycin, was discovered in Spain in 1969. Fosfomycin inhibits phosphoenolpyruvate transferase, the first enzyme involved in the synthesis of peptidoglycan, inhibiting cell-wall synthesis. There are three forms of Fosfomycin : Fosfomycin tromethamine (a soluble salt) and Fosfomycin calcium for oral use, and Fosfomycin disodium for intravenous use. Fosfomycin, is a safe antibiotic with limited adverse events[8].

80%of acute urinary tract infections are attributed to *E.coli*. The ability of the bacteria to adhere to the uroepithelial cell receptors through specific fimbrial adhesins is critical for the initiation of infections [9]. *E.coli* is a common inhabitant of the gastrointestinal tract of humans and animals[10].Adhesive molecules and toxins of *E.coli* account for the most important mediators of pathogenicity[11].

E.coli most often expresses P Fimbriae or mannose resistant adhesin[12].The *pap* (pyelonephritis associated pilus) operon mediates the binding of the galactosyl galactose specific P fimbriae to the epithelial surfaces of intestine ,vagina ,urinary tract and moiety of the P blood group by their tip adhesion molecul[13–15].

Materials and Methods

Patients: Female Patients between 18 and 65 years of age with the diagnosis of uncomplicated lower UTI were included in the study. Ethical approval was obtained from the institutional Ethical Committee

Bacterial strains

A total of 168 uropathogenic*E.coli* (UPEC) strains were isolated from 252 urine samples during a 12 months period (between September 2017 and August 2018) from lower UTI cases, from tertiary care hospital. All patients were females, aged above 18 and had dysuria or problems with frequency or urgency in passing urine; had >20 leukocytes/mm^3 in urine sediment and *E. coli* urine culture (>10^5 cfu/mm^3) We excluded duplicate isolates, which were defined as isolation of the same bacterial species from the same patient with the same antibiogram.

Laboratory methods

For this study, midstream urine was collected from 252 suspected cases of UTI and inoculated into Mac Conkey agar and Blood agar. A colony count of 10^5 was taken as significant bacteriuria [16]. From the samples 220 showed significant growth and 168 isolates were *E.coli* We identified the species of *E coli* by standard biochemical tests [17]. After characterization, the UPEC isolates included in the study were kept in in Luria broth medium at -20^0C.

Extended spectrum beta-lactamase production was confirmed by phenotypic testing a double disk synergy test according to Jarlier[18]. Identification of ESBL production was done based on the demonstration of synergy between clavulanic acid and broad-spectrum cephalosporins.

For genotyping bacteria was grown in Luria broth medium for 18 h at 37°C.For standardization of PCR conditions positive strains, kindly provided by Johnson lab& Brian J from Pathos pool were used. Postive control strains include J-96 Strain positive for *pap* and known negative control[19].

Antimicrobial susceptibility testings

The disk diffusion method was used to test the antimicrobial susceptibilities for the commonly used antibiotics. We used the following antimicrobial agents ampicillin, cefazolin,cefuroxime,ceftriaxone,cefotaxime,ceftazidime,cefixime,ciprofloxacin,Levofloxacin,nitrofurantoin,gentamicin,amikacin,trimethoprin-sulfa-methaxole.Testing for carbapenem susceptibility was done with imipenem and meropenem discs and the results were interpreted as susceptible (S), intermediate susceptible (I) and resistant (R) following CLSI 2016. The breakpoints of these antimicrobial agents were using CLSI criteria[20].

With regard to the antimicrobial activity of fosfomycin, the CLSI-directed disk diffusion test for *E coli*was used. *In vitro* sensitivity to fosfomycin was tested according to Clinical and Laboratory Standards

Institute(CLSI) guideline. Fosfomycin trometamol disk (200 µg fosfomycin/50 µg glucose-6-phosphate) (Hi media, Mumbai, India) was used to determine the susceptibility to fosfomycin and growth-inhibition zone was evaluated[21].

Preparation of bacterial DNA.

DNA to be amplified released from whole organisms by boiling. Bacteria were harvested from 1 ml of an overnight broth culture, centrifuged pellet suspended in 200µl of sterile water, and incubated at 100°C for10 min.

Primers: *pap,* forward and reverse primers obtained from *Eurofins Mwg*, Bangalore

Ampllification Procedure

All amplification reactions performed in Applied Biosystem thermocycler. PCR amplifications consists of 1 cycle of 94^0C for 60 s, 30 cycles of denaturation at 94^0C for 60s , annealing at 63°C for 30 sec and extension at 72°C for 90 s followed bya final cycle of 72^0C for 90s. The PCR products analyzed by electrophoresis in a 1.0% submersed agarose gel stained with ethidium bromide and visualized under UV light as described by Sambrook *et al*[22] .

Statistical Methods

Differences between non-continous variables was tested by *Chi-square* test or two tailed fischer exact test as appropriate. All results will be considered to be statistically significant at P=0.05

Results

Antimicrobial resistance of *E.coli* isolates from clinical data of 252 eligible patients was analyzed. A positive urine culture was found in 87.3%. Within the 220 pathogens, *Escherichia coli*was most frequent (76.3%).

E.coli showed the highest rate of susceptibility to imipenem (94%) followed by fosfomycin(84.5%), nitrofurantion(83%) and amikacin(82.0%).The lowest rate was found for ampicillin(45.1%).

The *pap* genes(Figure1)are associated with different forms of UTI. They are important in the pathogenesis of ascending UTI and pyelonephritis.*pap* gene coding for pap adhesin was present in 30 of the *E.coli* isolates.25 of these isolates were susceptible to fosfomycin

Table 1 Susceptibility to fosfomycin among ESBL and CRE

Fosfomycin	Escherichia coli	
	ESBL n=52	CRE n=7
Susceptible	42	5
Intermediate	08	3
Resistant	2	0
Total	52	7

ESBL: Extended spectrum beta lactmase CRE: Carbapenem resistant Enterobacteriaceae

Among the *E.coli* isolates, ESBL producers had a frequency of 30.95% (52/168) and 4%(7/168) were identified as Carbapenem resistant Enterobacteriaceae (CRE). Overall, 84.50% (142/168) isolates were susceptible to fosfomycin. There was 92.00% (48/52) fosfomycin susceptibility in ESBL producing *E.coli* and 71.42% (05/07) showed fosfomycin susceptibility in CRE.

Table 2 Demographic and clinical data of patients according to ESBL production. Fosfomycin, had good activity against gentamicin, ciprofloxacin and co-trimoxazole resistant*E.coli with* or without *pap* gene. The resistance rate of fosfomycin was significantly lower in gentamicin,ciprofloxacin, cotrimoxazole resistant *E.coli* isolates compared to isolates susceptible to antibiotics.

Characteristic Comorbidities, underlying condition	Total n=168 (%)	ESBL n=52(30.9%)	NonESBL n= 116 (69.1 %)
Previous use of antibiotics	60 (35.7)	29(56.6)	31(27.0%)
Type2 diabetes	56 (33.3)	22(41.4)	34 (29.3%)
Arterial hypertension	31 (18.4)	11(20.7)	20(17.2%)
Pregnancy	26(15.6)	4(8.3)	22 (18.9%)
ESBL, extended-spectrum beta-lactamase			

Resistance rate among *E.coli* to the trimethoprim-sulfamethoxazole was 48%, the resistance to cephalosporins ranged from 49% to 88%, ciprofloxacin 58.20%, levofloxacin 69%, imipenem 6 %. High frequency of resistance to ampicillin (72%) and moderate resistance (36.0%) to gentamicin was detected. 68% were found to be (multidrug-resistant MDR). Resistance rates lower than 20% were found for amikacin, nitrofurantoin, and fosfomycin. The overall *pap* gene prevalence was 18% and the expression was a 22% among the ESBL isolates and 20% among the CRE.

Table 3 Age Distribution of Uropathogenic *E.coli* isolates

Age distribution	female
>18-30	46
31-40	48
41-50	39
51- 60	18
above 60	17
Column Totals	168

There was significant difference between the different age groups namely (18-30), (51-60) and >60 among the patients.

Table 4 *pap* gene distribuition

pap gene	absent	present	Total
case	138	30	168
Total			168

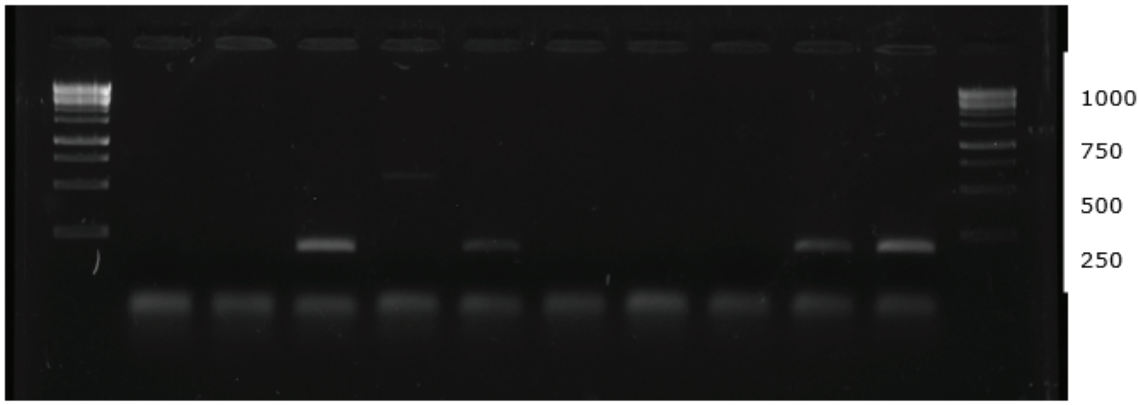

Figure 1 pap gene(210bp)

Lane 1&12: 1kb ladder; Lanes 2-11:*E.coli* isolates. Lane 4,6,10&11 positive for *pap* gene.

Discussion

The ability to adhere to epithelial surface has been shown to be a prerequisite for *E.coli* strains to colonise the urinary tract and cause UTI in the absence of urological abnormalities. *pap* is the pyelonephritis-associated pili. In this study we have looked at the sensitivity of the antibiotics with relation to *pap* genes and found its prevalence to be 17.8%. There is whole lot of difference in the frequency of *pap* gene among UPEC strains isolated from different region[23–28]. This could be because UPEC strains utilize a variety of adhesins to bind to the urinary epithelial cells and start the infection.

Ciprofloxacin has commonly been used as oral therapeutic option for ESBL producing isolates[29]. The resistance rate of ESBL *E.coli* to ciprofloxacin was 58.2% in this study.ESBL-producing enterobacteriaceae have emerged in both the community and hospital settings in most countries, including India. Carbapenems are the drugs of choice for treating severe infections caused by the ESBLproducing isolates[30]. In our study, imipenem and meropenem were most actively against ESBL.

Trimethoprim-sulfamethoxazole is one of few oral therapeutic options for ESBL-producing isolates. But in this study, it was the not an active antimicrobial agent against ESBL producing *E.coli.*Ko et al showed that the resistance rates for trimethoprim-sulfamethoxazole among *E coli* are rising accompanied with ciprofloxacin resistance and ESBL production[31].In our study, a high percentage of strains susceptible to amikacin and nitrofurantoin were found 234 (99.5% and 92.1%, respectively). Nitrofurantoin is an old drug used for uncomplicated UTI, but its use is limited because of its nephrotoxicity. the potential role of nitrofurantoin for uncomplicated UTI in the growing resistance era has been mentioned by Kashanian et al[32].In our study, a high percentage of strains susceptible to amikacin and nitrofurantoin were found, amikacin(82.0%) and nitrofurantion(83%) respectively).

Fosfomycin has been reported to have good potential in treating UTI caused by multidrug resistant *E.coli*.

Fosfomycin is well tolerated in humans and causes little nephrotoxicity. Fosfomycin-tromethamine, an oral form of fosfomycin, is also indicated for uncomplicated UTI[33,34]. In a systematic review, fosfomycin is found actively against Enterobacteriaceae producing ESBL, particularly *E coli*[35,36]. In Switzerland and Italy, fosfomycin has been found to be the most active agent against *E. coli*, presenting 100 and 98% efficacy, respectively [37,38]. Fosfomycin susceptibility rates in Sweden are high in *E. coli*, particularly in ESBL-producing strains (97–99 %), supporting the replacement of antibiotics exhibiting reduced activity by fosfomycin [39]. Our study showed that the activity of fosfomycin against ESBL isolates remained reliable even in

ciprofloxacin-non susceptible isolates. Ko et al has shown that fosfomycin does not have cross-resistance with other antimicrobial agents[40]. This finding may be because of the unique antibacterial mechanism of fosfomycin.

This study has few limitations. First, only disk diffusion method was used to determine the fosfomycin susceptibility. Only women candidates from outpatient setting was evaluated.

Conclusion

Fosfomycin remains a viable option for the treatment of *E.coli* in uncomplicated UTIs; different susceptibility testing platforms can give very different results regarding the prevalence of fosfomycin resistance, with false positives being a potential problem that may unnecessarily limit the use of this agent.

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Conflicts of Interest: There are no conflicts of interest.

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