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Lower prevalence of *hlyD*, *papC* and *cnf-1* genes in ciprofloxacin-resistant uropathogenic *Escherichia coli* than their susceptible counterparts isolated from southern India

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Summary
Objective: The study was conducted to determine the association of the *hlyD*, *papC* and *cnf-1* virulence genes with drug resistance in uropathogenic *Escherichia coli* (UPEC) isolated from cases of urinary tract infection (UTI).
Method: A total of 193 *E. coli* strains isolated from symptomatic cases of UTI in a tertiary care teaching hospital in Raichur, Northern Karnataka, India were included in the study. The antibiotic susceptibility pattern was determined by Kirby—Bauer's Disk Diffusion method, and the strains resistant to any of the third generation cephalosporins tested were further confirmed for extended-spectrum beta-lactamase (ESBL)-production by an E-strip test. Genotypic virulence markers, namely, *hlyD*, *papC* and *cnf-1*, were detected by the uniplex PCR method and the phylogenetic characterization was performed by a multiplex PCR assay.
Results: The majority of the *E. coli* isolates belonged to the B2 phylogenetic group were significantly associated with ciprofloxacin-sensitivity and non-ESBL production ($p < 0.05$). An increased prevalence of ciprofloxacin-sensitive strains over ciprofloxacin-resistant strains were observed among the UPEC isolates harboring the

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papC (72.9% vs. 40.2%; $p < 0.001$), *hlyD* (43.7% vs. 21.6%; $p < 0.001$) and *cnf-1* (30.2% vs. 12.3%; $p < 0.05$) genes. The presence of a multivirulent gene in the non-ESBL *E. coli* strains (44.5%) was significantly higher ($p < 0.05$) than in the ESBL-producing strains (21%).

Conclusions: Among the UPEC isolates, the predominant B2 phylogenetic group was significantly associated with the ciprofloxacin-sensitive strains, as well as with the non-ESBL *E. coli* strains. The genotypic virulence markers of UPEC were associated with ciprofloxacin-sensitivity, and a significant number of the non-ESBL strains harbored multivirulent genes. The relationship between the presence of the virulence genes and ESBL production was complex and warrants further intensive studies.

Introduction

Escherichia coli, a normal commensal of the human body, becomes a highly adaptive pathogen by acquiring mobile genetic elements, and they cause a wide range of diseases from intestinal to extraintestinal infections, including infections in the urinary tract, blood stream and central nervous system [1]. *E. coli* is the most common cause of urinary tract infections (UTI) in community and hospital settings. The severity of UTI depends on the virulence of the infecting organism and the susceptibility of the host. Virulence factors (VFs), such as hemolysin (*hly* gene), cytotoxic necrotizing factor type 1 (*cnf-1* gene), and pyelonephritis associated pili (*pap* gene), play important roles in the pathogenicity of uropathogenic *E. coli* (UPEC) strains by overcoming host defense mechanisms to cause disease [2]. The number of multi-drug resistant strains of *E. coli* has progressively increased, causing treatment limitations. An extremely limited range of antibacterial agents remains as a result of the emergence of fluoroquinolone-resistance and ESBL-producing isolates, ensuring that a simple case of UTI is increasingly challenging [3]. Studies have suggested that in quinolone (Q) and fluoroquinolone (FQ) resistant strains, fewer virulence genes are commonly encountered [4]. Phylogenetic group specific differences in quinolone-susceptibility have been noted, with the B2 group strains showing higher frequencies of susceptibility than those of the other phylogenetic groups [5]. The issue is complex in the case of the ESBL-producing strains because they are genetically diverse and frequently coexist with fluoroquinolone-resistance [6,7]. In most parts of India, UTI cases are treated empirically. With the increasing resistance of *E. coli* to antibiotics used for UTI treatment, it is pertinent to perform simple culture and sensitivity testing of urine and to document the prevalence of UPEC vs. non-UPEC in a region and to characterize the virulence genes and antibiotic resistance of UPEC. The complex relationships of the virulence properties and the phylogenetic background as well as the antibiotic resistance of *E. coli* resulting from their various interactions require further study. We tried to elucidate whether the lack of virulence factors (VFs) is directly associated with resistance or if resistance depends on a phylogenetic distribution or unknown factors. We analyzed antibiotic resistance, the genotypic virulence factors and phylogenetic characterization in UPEC isolates.

Materials and methods

Case definition

Urinary tract infection is defined as the presentation of a combination of the following symptoms: (i) bacteria with $\geq 10^4$ CFU/ml count in midstream urine, (ii) the presence of ≥ 5 white blood cells (WBCs) per high power field, and (iii) the presence of clinical signs and symptoms of UTI in the host including dysuria, frequency or urgency of urination.

Study setting

A total of 193 non-repetitive *E. coli* strains were isolated from symptomatic UTI cases in a tertiary care teaching hospital in Raichur, Northern Karnataka, India from December 2010 to February 2013. Among the 193 patients (out-patents, 148 and in-patients, 45) diagnosed as having symptomatic UTI, 132 (68.3%) were females and 61 (31.6%) were males, with a mean age of 35.5 ± 18.3 (mean $\pm$ SD). The study was conducted with approval from the Institutional Ethical Committee, and informed consent

was obtained from the subjects willing to participate.

Clean-catch midstream urine samples were collected and processed employing the semi-quantitative calibrated loop technique, and the significant isolates (colony count $\geq 10^5$ CFU/ml of urine) were identified by standard biochemical reactions [8].

Antimicrobial susceptibility testing

The antibiotic susceptibility testing was performed by the Kirby—Bauer's Disk Diffusion method in accordance with the CLSI guidelines [9] using antibiotic discs as follows: ampicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), ciprofloxacin (5 μg), nitrofurantoin, levofloxacin (5 μg), netilmicin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), and gentamicin (30 μg). *E. coli* ATCC 25922 was used as the control.

Detection of ESBL producing *E. coli* strains

All the isolates showing resistance to any of the third generation cephalosporins, namely ceftazidime (30 μg) and cefotaxime (30 μg), by the disk diffusion method were further confirmed for ESBL production using ESBL E-strip tests (AB BioMerieux, Solna, Sweden), as we described previously [10].

Detection of virulence genes

The presence of three types of virulence-associated genes was examined by PCR amplification using the primers described by Johnson and Stell [11]. The virulence genes consisted of *hlyD* (hemolysin) and *cnf-1* (cytotoxic necrotizing factor 1) coding for toxin secretion in UPEC and *papC* (pyelonephritis associated pili) coding for bacterial adhesion.

UPEC phylogenetic grouping

The *E. coli* strains were categorized into the four major phylogenetic groups (A, B1, B2 and D) by multiplex PCR following the protocol proposed by Clermont et al., using two virulence genes (*chu A*, encoding the heme transporter protein in *E. coli* O157:H7 and *yja A*, initially identified in the genome of *E. coli* K-12) and one DNA fragment TspE4.C2 [12].

Statistical analysis

The results were analyzed with descriptive statistics where appropriate. The Chi-square and Fisher's exact tests were used to evaluate the statistical significance of the differences in the results. A *p*-value of <0.05 was considered statistically significant. The statistical analysis was performed using Statistical package for the social sciences (SPSS) v 16.0 software.

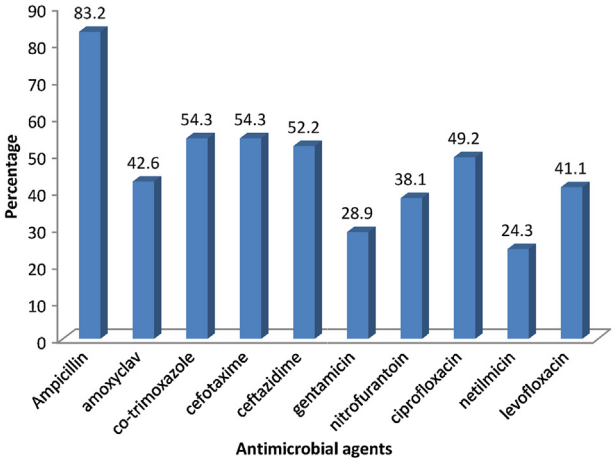

Figure 1 Antibiotic resistant pattern of the 193 UPEC clinical isolates.

Result

In this study, the UPEC isolates showed a high degree of resistance to ampicillin (83.2%), followed by cefotaxime (54.3%), trimethoprim/sulphamethoxazole (54.3%), ceftazidime (53.3%) and ciprofloxacin (49.2%); the least resistance was shown to netilmicin (24.3%), gentamicin (28.9%) and nitrofurantoin (38.1%). In the study, more than 50% of the *E. coli* strains were resistant to the third generation cephalosporins used, and 49.2% were resistant to ciprofloxacin (Fig. 1).

Increased prevalence of ciprofloxacin-sensitive strains over ciprofloxacin-resistant strains were observed among the UPEC isolates with the *papC* (72.9% vs. 40.2%; $p < 0.001$), *hlyD* (43.7% vs. 21.6%; $p < 0.001$) and *cnf-1* (30.2% vs. 12.3%; $p < 0.05$) genes. A highly significant number of ciprofloxacin-sensitive *E. coli* strains ($n = 78$, 81.2%) harbored multivirulent genes compared to the ciprofloxacin-resistance strains. Among the UPEC isolates, the predominant phylogenetic group was B2, with a prevalence of 50.7%, and the association with the ciprofloxacin-sensitive *E. coli* strains was statistically significant ($p = 0004$). The second leading phylogenetic group A (27.9%) was significantly associated with the ciprofloxacin-resistance strains ($p < 0.001$) of the UPEC isolates, as shown in Table 1.

Table 1 Distribution of virulence genes and phylogenetic groups among UPEC in relation to ciprofloxacin susceptibility.

Virulence genes & phylogenetic group	Total number of *Escherichia coli* strains tested ($n = 193$)				
	Ciprofloxacin-sensitive		Ciprofloxacin-resistant		Chi-square, *p*-value*
	$n = 96$	%	$n = 97$	%	
papC gene	70	72.9	39	40.2	<0.0001
hly gene	42	43.7	21	21.6	<0.001
cnf-1 gene	29	30.2	12	12.3	0.002
Multivirulent gene (≥2 genes)	78	81.2	17	18.8	<0.0001
Phylogenetic group					
A	16	16.7	38	39.2	0.0007
B1	18	18.7	20	20.6	0.74
B2	59	61.4	39	40.2	0.004
D	3	3.1	0	0	NA

* *p*-value, ciprofloxacin-sensitive vs. ciprofloxacin resistance.

Table 2 Distribution of virulence genes and phylogenetic groups among UPEC in relation to ESBL production.

VF & phylogenetic groups	Total number of UPEC strains tested ($n = 193$)				
	Non-ESBL strains[a]		ESBL strains		Chi-square, *p*-value*
	$n = 155$	%	$n = 38$	%	
hly gene	52	33.5	11	28.9	0.7
papC gene	87	56.1	19	50	0.5
cnf-1 gene	35	22.5	7	18.4	0.001
Absence of virulence gene	34	21.9	14	36.8	0.06
Presence of any one virulence gene	52	33.5	16	42.1	0.3
Multivirulent gene (≥2 genes)	69	44.5	08	21.0	0.009
Phylogenetic group					
A	24	15.4	12	31.5	0.03
B1	18	11.6	5	13.1	0.7
B2	113	72.9	21	55.2	0.04
D	0	0	0	0	0

[a] Non-ESBL strains include third generation cephalosporin sensitive and ESBL E-strip negative strains.
* *p*-value, non-ESBL producing UPEC vs. ESBL producing UPEC.

Of the 193 UPEC strains studied, 103 (53.3%) were resistant to any of the third generation cephalosporin, of which 38 (19.7%) strains were positive for ESBL by the quantitative E-strip method. The B2 phylogenetic group was predominant in the non-ESBL (72.9%) and ESBL (55.2%) producing UPEC strains, and the association of group B2 with the non-ESBL strains was statistically significant ($p < 0.05$). The UPEC isolates that belonged to phylogenetic group A were more significantly associated with the ESBL-producing strains (31.5%) than with the non-ESBL strains (15.4%) ($p = 0.03$). Although, the presence of any one virulence marker was higher in the ESBL-producing strains (42.1%) than in the non-ESBL strains (33.5%), the presence of a multivirulent gene was significantly higher ($p < 0.05$) in the non-ESBL strains (44.5%) than in the ESBL-producing strains (21%). Of the 3 virulence genes tested, only *cnf-1* showed a significant difference ($p = 0.001$) between the non-ESBL (22.5%) and the ESBL-producing (18.4%) strains, as depicted in Table 2.

Discussion

In this study, we observed that the B2 phylogenetic group was significantly associated with the ciprofloxacin-sensitive *E. coli* strains as well as with the non-ESBL strains. Phylogenetic group A was significantly associated with the ciprofloxacin-resistant and ESBL-producing *E. coli* strains. The

ciprofloxacin-sensitive strains harbored virulence genes at a higher frequency; however, a complex association was noticed between ESBL-production and the presence of virulence genes in the UPEC isolates.

The initial step in the pathogenesis of UPEC is adhesion. The expression of adhesive organelles such as Type 1 and P pili allow UPEC to bind and invade host cells. The subsequent step is the development of an array of toxins, including hemolysin and cytotoxic necrotizing factor 1 that provide UPEC with the ability to cause tissue damage, facilitate bacterial dissemination, release host nutrients and disable immune effector cells [13].

The virulence factors of extraintestinal pathogenic *E. coli* (ExPEC) such as hemolysin (*hly* gene), cytotoxic necrotizing factor 1 (*cnf-1*), P pili F13 (*pap* gene), and S family adhesins (Sfa gene) form clusters, named 'pathogenicity islands' (PAI). They are typically located in chromosomes and also in plasmid, and they comprise a large segment of the DNA associated with the tRNA gene. Additionally, the PAI contribute a remarkable benefit to bacterial fitness by allowing the transmission of genes that could provide a survival advantage to bacteria or to its ability to cause disease. In this study, we selected three representative PAI virulence genes, *hlyD*, *papC* and *cnf-1*.

In our study, the UPEC strains showed a higher prevalence of resistance to the antibiotics commonly used for the treatment of UTI, which was in agreement with the reports of recent studies [14,15]. The UPEC strains showed the least resistance to the aminoglycosides, and among them, netilmicin (24.3%) was more effective than gentamicin (28.8%) in sensitivity. Similar to our results, Chitnis et al. observed that the highest number of Gram-negative bacilli were resistant to ampicillin (83.2%), and the lowest number were resistant to netilmicin (24.3%) [16].

In our study, a lower prevalence of the three virulence genes studied (*hly* gene, *cnf-1* gene and *papC* gene) was significantly associated ($p < 0.05$) with the ciprofloxacin-resistant *E. coli* strains. Similar to our findings, Horcajada et al. reported that quinolone resistance was associated with a significantly decreased prevalence of three virulence factors including *sfa*, *hly* and *cnf-1* [17]. Johnson et al. showed that the presence of P fimbriae and hemolysin were significantly associated with a lack of antimicrobial resistance [18]. The lower incidence of *papC*, *hly* and *cnf-1* among the ciprofloxacin-resistant B2 strains appears to result from the loss of the corresponding PAI that appeared without *gyrA* mutation and without any of the antibiotics in the medium indicating the instability of PAI. This PAI instability could be exacerbated by several stimuli, of which the presence of ciprofloxacin might be only one [19].

Soto et al. investigated the capacity of the quinolones to induce the loss of virulence factors such as hemolysin (*hly* gene), cytotoxic necrotizing factor 1 (*cnf-1* gene), P fimbriae (*pap* gene), and the autotransporter (*sat* gene) in uropathogenic *E. coli* strains. They found that all the strains lost hemolytic capacity in subinhibitory concentrations of ciprofloxacin, showing a partial or total loss of the PAI containing the *hly* and *cnf-1* genes. They observed no spontaneous loss of PAIs on incubation in the absence of quinolones in the wild type or mutant *E. coli* strains [4].

The co-existence of ESBL-production and ciprofloxacin-resistance was observed in 49.2% of the *E. coli* isolates in the study. The association of ESBL-producing strains with resistance to other classes of antibiotics such as the fluoroquinolones (FQ) was reported by Paterson et al. [20] and Lautenbach et al. [21].

We found that, among the urinary *E. coli* isolates, ciprofloxacin-resistance was associated with significant shifts in the phylogenetic distribution and virulence genotypes. The shifts observed among the ciprofloxacin-resistant *E. coli* isolates were toward the non-B2 phylogenetic groups (notably, group A). Our results are in complete agreement with the findings of Johnson et al., who observed an association of ciprofloxacin resistance with phylogenetic group A and ciprofloxacin sensitivity with phylogenetic group B2 [22]. Piatti et al. also observed that B2 being the frequent phylogenetic group, was significantly higher among susceptible strains than the resistant strains [19]. Houdouin et al. proposed that the group A strains in the fecal flora could have developed from a greater exposure to antibiotics. The quinolone-resistant isolates might be mutants of quinolone-sensitive strains, and the strains might have lost their PAI in exchange for resistance [23].

The shift in phylogenetic distribution was consistent with the hypothesis of Johnson et al. that, although VFs and antibiotic resistance each might confer increased fitness in extraintestinal infections, they might do so via mutually exclusive pathways and in distinct populations. In healthy hosts with little antibiotic exposure, a robust VF repertoire might be essential to a pathogen overcoming intact host defenses, whether antibiotic resistance is present or not. Conversely, in compromised hosts, who might have been exposed to many antibiotics as well as having weakened defenses, antibiotic resistance might provide a substantial advantage to the pathogen, whereas a

more robust arsenal of VFs might be unnecessary [23].

This hypothesis might not be true for all the isolates, especially for the ST131 *E. coli* isolate. The *E. coli* ST131 isolates significantly exceeded the non-ST131 isolates in the extent of the resistance and/or the virulence profile. Colpan et al. reported that, in the FQ-Sensitive *E. coli* isolates, the virulence factor scores were high regardless of the ST131 genotype; in the FQ-resistant and ESBL isolates, the virulence factor scores were much higher in the ST131 than in the non-ST131 isolates [24]. The ST131 *E. coli* isolate appears to combine resistance and virulence, which to some extent are mutually exclusive. This combination of resistance and virulence might give ST131 a competitive advantage over other *E. coli*, promoting its clonal expansion and dominance over less virulent and/or more susceptible clones [25].

In our study, the ESBL-producing UPEC isolates were predominantly observed in the B2 phylogenetic group, and this finding is in agreement with a previous report by Demirel et al. [26]. We observed an increased prevalence of the *hlyD* and *papC* genes in the non-ESBL strains compared to the ESBL-producing strains; however, the difference was not statistically significant, and the *cnf-1* gene showed a significant association with the non-ESBL strains. In previous studies, hemolysin, cytotoxic necrotizing factor 1 (CNF1) and secreted autotransporter toxin (SAT) have been shown to be less prevalent in ESBL-producing *E. coli* strains than in susceptible isolates [13,27]. In a recent study, Qin et al. showed that the adhesion genes were more prevalent in non-ESBL producing strains than in ESBL-producing strains [28]. Pathogens acquire resistance determinants and express multi-resistant phenotypes at the expense of their virulence properties [22]. Contrary to our findings, Jadhav et al. reported that the ESBL-producing isolates were frequently associated with hemolytic phenotypes at a higher rate (65.6%) than were the non-ESBL *E. coli* strains (58.5%) [29].

A major part of our observation strengthens the argument that ciprofloxacin-resistant UPEC strains are less virulent than ciprofloxacin-susceptible strains. Our observation is consistent with earlier reports and suggests that antibiotic resistant strains have less virulent properties. The relationship between the presence of virulence genes and ESBL production is complex and requires further intensive studies.

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Competing interests

None declared.

Ethical approval

Not required.

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