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Original Article

Microbiology Section

Molecular Characterization of Uropathogenic *Escherichia coli*: Nalidixic Acid and Ciprofloxacin Resistance, Virulent Factors and Phylogenetic Background

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ABSTRACT

Background and Objective: A proficient pathogen should be virulent, resistant to antibiotics, and epidemic. However, the interplay between resistance and virulence is poorly understood. Perhaps, the most commonly accepted view is that resistance to quinolones is linked to a loss of virulence factors. However, the low virulent phylogenetic groups may be more prone to acquire resistance to quinolones. The aim of this study was to identify and characterise the Nalidixic Acid (NA) and ciprofloxacin (CIP) resistant uropathogenic *Escherichia coli* (UPEC) isolates with respect to virulence and phylogenetic background, from hospital settings in Kolkata, an eastern region in India. Research based on these bacterial populations will help in understanding the molecular mechanisms underlying the association between resistance and virulence, that in turn, may help in managing the future disseminations of UTIs in their entirety.

Material and Methods: One hundred and ten *E. coli* isolates were screened against NA and CIP using Kirby-Bauer disk diffusion technique, following CLSI guidelines. Prevalence of virulent factor genes and distribution of phylogenetic groups amongst the isolates was determined by PCR, using gene specific primers against the different virulent factors and DNA markers (*chuA*, *yjaA* and DNA fragment, *TSPE4.C2)* respectively. Statistical analysis of the data was performed using SPSS software.

Results: Resistance to both NA and CIP was reported in 75.5 % of the isolates which were analysed. The virulent determinants, *papC, pap GII, papEF, afa, cnf1, hlyA* and *iroN* were significantly predominant in the drug susceptible than the resistant isolates. A significant reduction of phylogroup B2 in NA (85.7% versus 64.6%, χ^2P<0.001) and CIP (85.2 % versus 61.4%, χ^2P<0.001) resistant UPEC isolates, followed by increase in predominance of non-B2 phylotypes (group D and group B1), were observed.

Conclusion: This is the first report from India that has indicated possible evidence on horizontal gene transfer from pathogenic to commensal strains and selection of the latter, on extensive usage of this group of antimicrobials in hospital settings, where these drugs were routinely prescribed for treating urinary tract infection. Therefore, this information necessitates surveillance programs and administration of effective strategies, to put an end to random prescription policies involving this group of antimicrobials.

Keywords: Ciprofloxacin, Drug resistance, Virulence, Phylogeny

INTRODUCTION

Urinary tract infections (UTIs) are the most frequent bacterial disease in humans, affecting both inpatients and outpatients. *E. coli* is by far the most common cause of these, accounting for 80-90% of all UTIs [1]. These bacteria have evolved a multitude of virulence factors and they have developed strategies that facilitate their growth and persistence within the adverse settings of the host urinary tract. Expression of adhesive organelles like Type 1 and P pili allow uropathogenic *E. coli* (UPEC) to bind and invade host cells and tissues within the urinary tract. UPEC strains are able to produce various other types of adhesins, S fimbriae, coded by *sfa* genes and Afa adhesins/Dr adhesins (involved with diarrhoea and urinary tract infections), coded by *afa* genes. Deployment of an array of toxins, including haemolysin and cytotoxic necrotizing factor I, provide UPEC with the means for inflicting extensive tissue damage, facilitating bacterial dissemination, as well as for releasing host nutrients and disabling immune effector cells [2]. It was shown that haemolysin provoked sloughing of the uroepithelium and bladder haemorrhage. Expression of iron chelating factor, iroN, (catechole siderophore receptor) enables UPEC to pilfer host iron stores and it has been found to be more prevalent among *E. coli* isolates obtained from patients with UTIs [3].

Phylogenetic analyses have shown that *E. coli* strains fell into four main phylogenetic groups (A, B1, B2, and D) and that virulent extra-intestinal strains belonged mainly to Group B2 and, to a lesser extent, to Group D and that most of the commensal strains belonged to Group A and Group B1. These studies have also given us a better understanding on acquisition of virulence genes amongst these strains belonging to different phylotypes [4]. Quinolones and fluoroquinolones are drugs of paramount importance in the treatment of UTIs and also of several other infectious diseases. Renal excretion of these molecules and the availability of oral and parenteral formulations have allowed them to compete with aminoglycosides and beta-lactams commonly used in the therapy of UTIs, especially in hospital settings. Fluoroquinolones such as ciprofloxacin (CIP) have been suggested as an effective empirical treatment for uncomplicated urinary tract infections with high levels (~10%) of resistance among uropathogens, to trimethoprim-sulfamethoxazole or trimethoprim, in both community and hospital settings in north America [5]. However, in recent years, emergence of resistance to CIP was reported amongst these uropathogens due to frequent use of this antibiotic [6]. Reports from different parts of India have revealed high incidence of resistance to nalidixic acid (92.6%) [7] and CIP (73%- 92.7%) [8,9] amongst the UPEC isolates. A study conducted on UPEC isolates from hospital

settings in Kolkata, an eastern region in India, also indicated high prevalence of nalidixic acid (95%) and ciprofloxacin (80%) resistant bacteria [10].

Several investigations have shown that quinolone and fluoroquinolone resistant UPEC strains displayed overall reduced virulence and that they invaded compromised patients. Vila et al., [11] reported that resistance of pathogenic *E. coli* to nalidixic acid (NA) may be associated with the loss of beta-haemolysin and P fimbria expression. However, evidence has suggested that the relationship among virulence properties of *E. coli*, phylogenetic background, and antibiotic resistance is a complex phenomenon [12]. Moreover, it is known that the expressions of virulent genes and different phylotypes vary between nearby or remote countries. Therefore, geographic source of isolates represents an important additional element which has to be taken into consideration [13, 14]. However, inspite of high incidence of NA and CIP resistant UPEC isolates in India, there is no report on the characterisation of these isolates with respect to the prevalence of VFs and phylogenetic background.

Therefore, the present study was undertaken, to characterize the NA and CIP responsive and un-responsive UPEC isolates obtained from hospital settings in Kolkata, an eastern region in India, based on the distribution of virulence determinants and phylogenetic background and to compare the results with the reports obtained from different parts of the world.

MATERIAL AND METHODS

Bacterial Isolates

One hundred ten non-duplicate *E. coli* isolates collected over a period of one year were selected for the study. All these isolates were obtained from urine samples consecutively collected from hospital settings in Kolkata. The *E. coli* isolates were biochemically identified, based on their colony morphologies seen on Mac Conkey's agar plates and they were speciated by standard biochemical tests [15]. The study protocol was approved by the institutional ethical committee.

Antibiotic Susceptibility

Antimicrobial susceptibility testing of the uropathogenic isolates was performed by the disk diffusion method, as was described by Clinical Laboratory Standard Institute [16]. Antimicrobial disks of nalidixic acid (NA; 30 µg) and ciprofloxacin (CIP; 10 µg) tested were obtained from Hi-Media labs, Mumbai, India. *E. coli* ATCC 25922 was used as a negative control strain.

Isolation of Bacterial DNA

DNA for amplification was released from whole cells by boiling. Single colonies were harvested from the LB plates, suspended in 100 µl of sterile water, incubated at 100°C for 10 minutes, and centrifuged [17]. The supernatant was used in subsequent PCR, to amplify the different virulence genes.

Determination of Virulence Genes

Ten virulent genes were detected by individual PCR. All PCR assays in this study were carried out in a 20 µl reaction volume containing 1 µl bacterial lysate, 250 µM dNTPs, 1.5 mM $MgCl_2$, 80 pmole of each primer (Sigma Chemicals, USA), 1 U Taq DNA polymerase and 2.0 µl 10 X PCR buffer (Fermentas). Sequences, amplification conditions and predicted sizes of amplicons for the specific target genes have been shown in [Table/Fig-1]. DNA sequencing was used to confirm the identity of the amplified PCR products and to establish positive controls. Initially, the nucleotide sequence of an amplified PCR product of each virulence gene, representing a single isolate, was determined using ABI 3100 automated genetic analyzer (Xceleris, Hyderabad, India). Once a PCR product for an individual virulence determinant was confirmed, the DNA from this isolate was used as a positive control for all subsequent PCRs. A reaction mixture containing DNA template from DH5α was used as a negative control in each PCR assay.

Phylogenetic Analysis

The distribution of phylogenetic group amongst the isolates was determined by individual PCR using gene specific primers [4] against the three DNA markers (*chuA*, *yjaA* and the DNA fragment *TSPE4.C2*).The size of amplicons obtained as PCR products allowed the *E. coli* isolates to be classified into one of the four major *E. coli* phylogenetic lineages: A, B1, B2 and D [4].

STATISTICAL ANALYSIS

The data were analysed by using SPSS, version 17.0. The Chi-square test or the Fisher exact test was used to compare categorical variables. χ^2P value of <0.05 was considered to be statistically significant. We declare no conflicting or dual interests.

RESULTS

A 110 *E. coli* strains were isolated from 900 urine samples collected during 2010-2011 from hospitalized patients. The antimicrobial susceptibility patterns against NA and CIP revealed that out of 110 isolates examined, 14 isolates were susceptible to both drugs, 13 were susceptible to CIP only and that 83 were resistant to both NA and CIP respectively. Furthermore, all the 110 isolates were characterised with respect to virulence and phylogenetic background. Susceptible isolates exhibited marked differences from the drug resistant isolates with regards to the distribution of the different genes responsible for virulence. Prevalences of *papC, papEF, papGII, afa, hlyA* and *iroN* (χ^2p≤0.001) were significantly reduced in the both NA and CIP unresponsive isolates in comparison to the drug responsive isolates respectively [Table /Fig. 2]. Reduced predominance of *sfa* gene was observed in both drug resistant isolates as compared to their susceptible counterparts. However, significant reduction in *sfa* gene was observed amongst the CIP susceptible and resistant isolates (χ^2P=0.001) than amongst the NA susceptible and resistant isolates respectively. Result of distribution of *cnf1* gene was inconsistent when NA and CIP susceptible versus resistant isolates were compared. However, a consistent distribution of *fimH* was observed in both drug responsive and un-responsive isolates. [Table/Fig-2]. Moreover, it was observed that all the virulent factors analyzed were positively correlated with both NA and CIP resistances and that the correlations were significant, χ^2p<0.001 [Table/Fig–3]. Predominance of phylogenetic Group B2 (67.3%) was apparent as compared to those of the other biotypes (A, B1, and D) amongst the UPEC isolates, irrespective of their drug sensitivities [Table/Fig-4]. However, on further analysis, isolates belonging to B2 biotype were found to be significantly more prevalent among the drug sensitive isolates than the drug unresponsive isolates (χ^2p<0.001). Among NA and CIP susceptible isolates, in fact, the frequencies of groups A, B1, and D were 0%, 7.1%, 7.1% and 3.7%, 3.7% and 7.4%, while amongst NA and CIP resistant isolates, these frequencies were 4.2%, 14.6%, 16.7% and 3.6%, 16.9%, 18.1% respectively [Table/Fig-4]. Moreover, prevalences of isolates belonging to group B1 and D were significantly higher amongst NA and CIP resistant isolates as compared to those of the susceptible isolates respectively.

DISCUSSION

In the clinical management of infectious diseases, with respect to multidrug-resistant pathogens, it has been frequently assumed that more antimicrobial drug resistance equates with greater virulence. Interestingly, controversy arises, as evidence has indicated that there existed an inverse relationship between quinolone and fluoroquinolone resistance and distribution of virulence factors

Genes	Primer sequences (5'-3')	PCR condition (time)	No. of cycles	Amplicon size (bp)	Primer Reference
fimH	F-TGCAGAACGGATAAGCCGTGG R-GCAGTCACCTGCCCTCCGGTA	95 ºC (30 sec) 60 ºC (30 sec) 72 ºC (1min)	30	508	[23]
papC	F-GACGGCTGTACTGCAGGGTGTGGC R-ATATCCTTTCTGCAGGGATGCAATA	95 ºC (30 sec) 63 ºC (30 sec) 72 ºC (1min)	30	328	[23]
papEF	F-GCAACAGCAACGCTGGTTGCATCAT R-AGAGAGAGCCACTCTTATACGGACA	95 ºC (30 sec) 55 ºC (30 sec) 72 ºC (1min)	30	336	[23]
papGII	F-GGAATGTGGTGATTACTCAAAGG R-TCCAGAGACTGTTCAAGAAGGAC	95 ºC (30 sec) 52 ºC (30 sec) 72 ºC (1min)	30	562	[23]
papGIII	F-CATGGCTGGTTGTTCCTAAACAT R-TCCAGAGACTGTGCAGAAGGAC	95 ºC (30 sec) 52 ºC (30 sec) 72 ºC (1min)	30	421	[23]
afa	F-GCTGGGCAGCAAACTGATAACTCTC R-CATCAAGCTGTTTGTTCGTCCGCCG	95 ºC (30 sec) 60 ºC (30 sec) 72 ºC (1min)	30	559	[23]
sfa	F-CGGAGGAGTAATTACAAACCTGGCA R-CTCCGGAGAACTGGGTGCATCTTAC	95 ºC (30 sec) 58 ºC (30 sec) 72 ºC (30 sec)	30	407	[23]
cnf1	F-AAGATGGAGTTTCCTATGCAG R-TCAGAGTCCTGCCCTCATTAT	95 ºC (30 sec) 54 ºC (30 sec) 72 ºC (30 sec)	30	498	[24]
hlyA	F-AACAAGGATAAGCACTGTTCTGGCT R-ACCATATAAGCGGTCATTCCCGTCA	95 ºC (30 sec) 63 ºC (30 sec) 72 ºC (2min)	30	1,177	[23]
iroN	F-AAGTCAAAGCAGGGGTTGCC R-GACGCCGACATTAAGACGCAG	95 ºC (30 sec) 63 ºC (30 sec) 72 ºC (1min)	30	665	[3]

[Table /Fig-1]: Primers sequences for the virulence genotyping

Virulent Factors	No. (%) of isolates						
	Total n=110(%)	Nalidixic acid (NA)		χ^2p-value	Ciprofloxacin (CIP)		χ^2p-value
		Susceptible n=14(%)	Resistant n=96(%)	Susceptible vs Resistant	Susceptible n=27(%)	Resistant n= 83(%)	Susceptible vs Resistant
fimH	104(94.5)	13(92.9)	91(94.8)	<0.001	24(88.9)	80(96.4)	<0.001
papC	43(39.1)	9(64.3)	34(35.4)	<0.001	15(55.6)	28(33.7)	<0.001
papGII	36(32.7)	5(35.7)	31(32.3)	0.001	11(40.7)	25(30.1)	<0.001
papEF	50(45.5)	8(57.1)	42(43.8)	<0.001	15(55.6)	35(42.2)	<0.001
afa	21(19.1)	6(42.9)	15(15.6)	0.001	8(29.6)	13(15.7)	<0.001
sfa	12(10.9)	3(21.4)	9(9.4)	NS	7(25.9)	5(6.0)	0.001
cnf1	17(15.5)	2(14.3)	15(15.6)	0.007	5(18.5)	12(14.5)	0.001
hlyA	65(59.1)	11(78.6)	54(56.3)	<0.001	19(70.4)	46(55.4)	<0.001
iroN	43(39.1)	7(50.0)	36((37.5)	<0.001	17(63.0)	26(31.3)	0.001

[Table/Fig-2]: Prevalence of virulent factors in the antibiotic susceptible and resistant isolates
NS; indicates statistically not significant result

Virulent genes	No. of isolates (n=110)			
	Nalidixic acid resistant (n=96)	χ^2 p-value	Ciprofloxacin resistant (n=83)	χ^2 p-value
fimH	91	<0.001	80	0.005
papC	34	<0.001	28	<0.001
papGII	31	<0.001	25	<0.001
papEF	42	<0.001	35	<0.001
afa	15	<0.001	13	<0.001
sfa	9	<0.001	5	<0.001
cnf1	15	<0.001	12	<0.001
hlyA	54	<0.001	46	<0.001
iroN	36	<0.001	26	<0.001

[Table/Fig-3]: Correlation amongst antibiotic resistance and virulence genes in the uropathogenic *E. coli* isolates

(VFs). Perhaps, the most commonly accepted view is that resistance to quinolones is linked to a loss of virulence factors [11,12,14,18]. Being consistent with these observations, the results of the present study also indicated a statistically significant reduction in the distribution of urovirulence genes (*papC, papEF, papGII, afa, hlyA and iroN*) amongst the NA and CIP resistant and susceptible uropathogenic *E. coli* isolates circulated in Kolkata. Typical VFs such as haemolysin (*hly* gene), cytotoxic necrotizing factor Type 1 (*cnf-1* gene), S-family adhesins (*sfa* gene) can form clusters named pathogenicty islands (PAIs). They are usually located in the chromosome, but they can be found in plasmids as well. Therefore, the distribution of virulence factors and acquisition of resistance to this group of drugs by mutations, were actually associated with some specific genetic background of the isolates and the relationship was complex. Moreover, resistance and virulence gene correlation also supported the above fact and confirmed the complex phenomenon.

Phylogenetic groups	No. (%) of isolates						
	Total n=110(%)	Nalidixic acid (NA)		χ^2p value	Ciprofloxacin (CIP)		χ^2p value
		Susceptible n=14(%)	Resistant n=96(%)	Susceptible vs Resistant	Susceptible n=27(%)	Resistant n= 83(%)	Susceptible vs Resistant
A	4(3.6)	0(0)	4(4.2)	NS	1(3.7)	3(3.6)	NS
B1	15(13.6)	1(7.1)	14(14.6)	0.067	1(3.7)	14(16.9)	0.067
B2	74(67.3)	12(85.7)	62(64.6)	<0.001	23(85.2)	51(61.4)	<0.001
D	17(15.5)	1(7.1)	16(16.7)	0.059	2(7.4)	15(18.1)	0.007

[Table/Fig-4]: Distribution of phylogenetic groups amongst antibiotic sensitive and resistant uropathogenic *E. coli* isolates
NS; indicates statistically not significant result

Phylogenetic studies have revealed a significant association of group B2 with the quinolone and fluoroquinolone resistant pathogenic *E. coli* isolates [12,14,18-20]. However, association of the susceptible isolates with the phylogenetic group B2 and that of the resistant isolates with non-B2 group have also been reported [21-24]. The controversy existent among these reports may be interpreted as being the result of the number of samples analyzed, geographical variations, or various clinical sources. Our study confirmed a significant depletion for phylogroup B2 and an increase for group D, followed by that for group B1 in the NA and CIP resistant UPEC isolates respectively, with overall predominance of group B2 amongst both the drug resistant isolates. However, the low virulent phylogenetic groups may be more prone to acquisition of resistance to quinolones. Therefore, acquisition of virulence factors amongst the UPEC isolates belonging to group B1 and their association with NA and CIP resistances, may be an interplay of a complex phenomenon, that possibly paves the way for the bacteria to achieve new niches without particular damage to host and through avoidance of host defenses, where they colonise or cause chronic infections spreading the possible resistance. Moreover, results of this study also indicate a risk of acquisition of resistance amongst commensal *E. coli* (phylogroup B1) upon exposure to the quinolone and fluoroquinolone drugs. Therefore, implementation of strict control measures must be undertaken, to monitor empirical use of quinolones and fluoroquinolones in hospitals as well as in community settings. This is believed to be the first report on association of virulence genes, phylogenetic background and NA and CIP resistance among uropathogenic *E. coli* isolated from hospital settings in Kolkata, an eastern region in India. Similar studies must be initiated, to provide a better insight into pathogenesis and acquisition of antibiotic resistance amongst the UPEC isolates in this part of the world, so that effective control measures can be administered.

CONCLUSION

In conclusion, our study reported a detailed characterization of nalidixic acid and ciprofloxacin resistant UPEC isolates from hospital settings in Kolkata, an eastern region in India. Susceptible isolates exhibited marked differences from both NA and CIP resistant isolates with regards to the distribution of virulence determinants. The virulence determinants studied had a positive correlation with both NA and CIP resistances. A significant reduction of phylogenetic group B2 was observed, that correlated with partial loss of virulence determinants in the resistant isolates. On further analysis, resistant isolates belonging to B1 and D phylogenetic group were observed. Therefore, our findings reinforce the urgent efforts which are needed for proper clinical management using molecular tools, to monitor the nalidixic acid and ciprofloxacin resistance in UPEC isolates in Kolkata, India, against the alarming predominance of these resistant uropathogens. Empirical usage of these drugs must be avoided, to put an end to the spread of such resistant bacteria.

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