

A study on the *aac*-(6¹)-*lb*-*cr* gene prevalence among ciprofloxacin-resistant strains of uropathogenic *Enterobacteriaceae*

Nirupa Soundararajan, Priyadarshini Shanmugam, Caroline Devanbu, Shameem Banu Abdul Sattar

Department of Microbiology, Chettinad Hospital and Research Institute, Kelambakkam, Chennai, Tamil Nadu, India

ABSTRACT

Introduction: Urinary tract infections (UTIs) are very common. Indiscriminate use of antibiotics has led to the development of resistance to most of the commonly used antibiotics including quinolones. **Aim:** This study aimed to find out the prevalence of ciprofloxacin resistance among the uropathogenic *Enterobacteriaceae*, to determine the virulence factors of these isolates, and to detect the *aac*-(6¹)-*lb*-*cr* gene among those isolates with minimal inhibitory concentrations (MICs) of ciprofloxacin >256 mcg/ml. **Materials and Methods:** Urine samples reaching the microbiology laboratory were processed, pathogens belonging to the *Enterobacteriaceae* family were isolated from those with significant bacteriuria, and antibiotic sensitivity testing was performed according to the CLSI guidelines. MIC of ciprofloxacin for the isolates resistant to ciprofloxacin was determined by using the E-test, and virulence factors such as hemagglutination, hemolysis, and mucoid colonies were analyzed. *aac*-(6¹)-*lb*-*cr* gene was analyzed by polymerase chain reaction for those isolates with MIC > 256 mcg/ml. **Results:** *Escherichia coli* was the most common isolate (62%) with the highest ciprofloxacin resistance (68%). Fourteen percent of them had MIC > 256 mcg/ml and all of these isolates harbored the *aac*-(6¹)-*lb*-*cr* gene. **Conclusion:** Plasmid-mediated drug resistance can rapidly spread and lead to selection of drug-resistant mutants if not controlled.

Key words: *Aac*-(6¹)-*lb*-*cr* gene, ciprofloxacin resistance, plasmid-mediated quinolone resistance, urinary tract infections

Submission: 04-02-2016 **Accepted:** 04-04-2016

INTRODUCTION

Urinary tract infection (UTI) is one of the most common infections seen in the community. Worldwide, it has been estimated that symptomatic UTIs result in seven million visits to outpatient clinics and 100,000 hospitalizations, annually.^[1] The treatment for UTIs is empirically started even before urine is sent for culture and sensitivity. Drugs

commonly recommended for simple UTIs include sulfamethoxazole - trimethoprim (co-trimoxazole), amoxicillin, nitrofurantoin, ampicillin, and ciprofloxacin. According to a study, patients with uncomplicated UTI are started on oral fluoroquinolones and cephalosporins.^[2] This is actually not recommended as co-trimoxazole is still considered the first-line therapy for uncomplicated UTIs in areas where resistance to co-trimoxazole in the community is <10–20%. Fluoroquinolones should not be used as the first-line drug therapy except in communities

Address for correspondence: Dr. Nirupa Soundararajan, 92, 5th Cross Road, M.K.B. Nagar, Chennai - 600 039, Tamil Nadu, India. E-mail: drnirups@gmail.com

Access this article online	
Quick Response Code:	Website: www.ijabmr.org
	DOI: 10.4103/2229-516X.192603

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

How to cite this article: Soundararajan N, Shanmugam P, Devanbu C, Sattar SB. A study on the *aac*-(6¹)-*lb*-*cr* gene prevalence among ciprofloxacin-resistant strains of uropathogenic *Enterobacteriaceae*. Int J App Basic Med Res 2016;6:258-61.

wherein resistance to trimethoprim is >10–20% or in patients with risk factors for resistance.^[3] Hence, the indiscriminate use of fluoroquinolones has led to the evolution of increased resistance to fluoroquinolones. This study was carried out to study the prevalence of ciprofloxacin resistance among Gram-negative bacteria, to identify virulence factors associated with ciprofloxacin resistance, and to detect aac-(6¹)-lb-cr gene among these isolates.

MATERIALS AND METHODS

This study was carried out in the Department of Microbiology in our hospital, from June 2014 to November 2014. All the urine samples reaching the laboratory were processed - Gram-staining was done followed by inoculation onto cysteine lactose electrolyte-deficient medium using a calibrated loop and incubated for 18–24 h at 37°C. All the isolates yielding a significant growth of pathogenic Gram-negative bacteria belonging to the *Enterobacteriaceae* family were identified using the appropriate biochemical tests and subjected to antibiotic susceptibility testing by Kirby Bauer’s disc diffusion technique using Mueller–Hinton agar according to the CLSI guidelines, 2014.^[4] Minimal inhibitory concentration (MIC) testing for ciprofloxacin was done for the ciprofloxacin-resistant strains using ciprofloxacin Ezy MIC strip (paper strip which is coated with ciprofloxacin in a concentration gradient manner). Virulence factors such as hemolysis on 5% sheep blood agar, capsule formation (detected by mucoid colonies on culture plates), and fimbriae formation (detected by hemagglutination of 3% suspension of O blood group red blood cells by the isolate grown in nutrient broth in the presence of 2% mannose) were analyzed for those ciprofloxacin-resistant isolates with MIC >256 mcg/ml, and polymerase chain reaction (PCR) was performed for the detection of aac-(6¹)-lb-cr gene among these isolates showing high level of resistance. The DNA was extracted using DNA purification kit (PureFast® Bacterial Genomic DNA purification kit). The aac-(6¹)-lb-cr gene was detected using primers F: 5'-CCCGCTTTCTCGTAGCA-3' R: 5'-TTAGGCATCACTGCGTCTTC-3' obtained from Helini Biomolecules, Chennai, India. The reaction mixture contained Master Mix (25 µl of Master Mix contains: 10XTaq buffer, 2 mM MgCl₂, 0.4 mM dNTPs mix, and 2U proofreading Taq DNA polymerase) – 10 µl, primer-forward (10 pmoles/µl) – 5 µl, primer-reverse (10 pmoles/µl) – 5 µl, and genomic DNA – 5 µl. The reaction was performed in a thermal cycler programmed for initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. Final extension was performed at 72°C for 5 min. The amplified products were then analyzed by agarose gel electrophoresis using gel loading dye along with 10 µl Helini 100 bp DNA ladder. The

gel was then viewed in ultraviolet transilluminator and the band pattern was observed.

RESULTS

During the period of study, a total of 4161 urine samples were received in the microbiology laboratory for culture and sensitivity. Among these samples, 1124 (27%) samples showed significant growth of pathogenic bacteria. Of the 1124 culture-positive samples, 831 (74%) samples yielded members belonging to the *Enterobacteriaceae* family on culture, the rest were other Gram-negative *Bacilli* such as *Pseudomonas* species, *Acinetobacter* species, and Gram-positive cocci such as *Staphylococcus aureus*, *Enterococcus* species, and *Staphylococcus saprophyticus*. Distribution of the organisms is shown in Table I.

The most common organism to be isolated was *Escherichia coli*, followed by *Klebsiella* species, and the rest as shown in Table I. The ciprofloxacin resistance pattern was as follows.

According to Figure 1, the highest percentage of ciprofloxacin resistance was noted in *E. coli* followed by *Citrobacter* species. In this study, ciprofloxacin resistance was not observed in *Enterobacter* species and *Proteus mirabilis*. Most of these ciprofloxacin-resistant isolates were also resistant to other drugs such as beta-lactams, aminoglycosides, and carbapenems, and 32 (4%) of them were sensitive only to polymyxin B and colistin. Of the 831 *Enterobacteriaceae* isolated, 441 were

Table I: Distribution of uropathogenic *Enterobacteriaceae*

Organisms isolated	Number of isolates	Percentage (n=831)
<i>Escherichia coli</i>	515	62
<i>Klebsiella</i> species	183	22
<i>Citrobacter</i> species	75	9
<i>Enterobacter</i> species	25	3
<i>Proteus mirabilis</i>	17	2
<i>Proteus vulgaris</i>	16	2

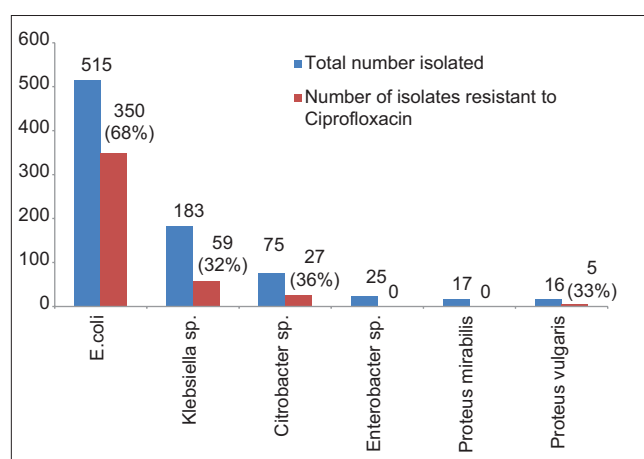


Figure 1: Ciprofloxacin resistance pattern of uropathogenic *Enterobacteriaceae*

ciprofloxacin-resistant, which is about 53%. According to Table 2, all of the isolates have MIC values above 16 mcg/ml.

Virulence factors associated with these 53 isolates which had MIC levels of >256 mcg/ml were studied. Forty-nine of these isolates were *E. coli* and four were *Klebsiella* species. The results of virulence factor analysis are shown in Table 3.

It was observed that 28 of these isolates possessed one or more of the virulence factors analyzed and 25 isolates did not possess any of the three virulence factors studied. Finally, the analysis of aac-(6')-Ib-cr gene by PCR revealed that except for one isolate (*Klebsiella* species), all the other 52 isolates possessed the gene of interest. Figure 2 shows the gel documentation image of the PCR products.

DISCUSSION

E. coli is the most common cause of UTIs, accounting for the majority of community- and hospital-acquired infections.^[5] Many studies have been carried out on the etiological profile of UTIs and their antibiotic sensitivity pattern. Knowledge of this is essential for management and treatment. Bacteria are becoming extremely drug-resistant by many mechanisms. Most bacteria nowadays show an increased resistance to quinolones compared to other drugs. A study in South India by Eshwarappa et al. has shown that 42% of the uropathogens isolated from patients with community-acquired UTIs showed extended spectrum beta lactamase production whereas 74% showed resistance to fluoroquinolones.^[6] Detection of fluoroquinolone resistance is important for patient care. Indiscriminate usage of antibiotics is the main reason for the present condition where there is almost 53% resistance to ciprofloxacin. The most common isolate *E. coli* showed 68% resistance to ciprofloxacin, which is in concordance with a study conducted by Jharna Mandal et al. where 73% showed resistance to the drug.^[7]

Table 2: Mechanisms in ciprofloxacin ranges among the ciprofloxacin-resistant isolates

MIC values (mcg/ml)	Number of isolates (441)	Percentage
>256	53	12
128	207	47
64	142	32
16	39	9

MIC: Minimal inhibitory concentration

Table 3: Virulence factor distribution among the isolates with ciprofloxacin mechanisms in ciprofloxacin >256 mg/ml

Virulence factor	Number of isolates
Hemolysis on blood agar	12
Hemagglutination (suggestive of fimbriation)	14
Mucoid colonies on culture plates (suggestive of capsule formation)	8

Bacteria, especially uropathogens have many virulence factors which help them to tide over unfavorable conditions such as host defense mechanisms and to persist and establish infections. This has been explored in many studies.^[8-10] In this study, some of the virulence factors such as capsule formation, presence of fimbriae and hemolysis were analyzed only for those bacteria which showed high MICs of ciprofloxacin >256 mcg/ml, and it was found that only 53% of them showed one or more of these virulence factors. Studies have shown that there is a decrease in the expression of virulence factors associated with fluoroquinolone resistance.^[11,12] The reason for this is not known, but it has been proposed that in these resistant isolates, the impaired function of DNA gyrase and topoisomerase may render them less fit and that quinolone resistance itself is a virulence factor which helps them to survive in the urinary tract of those treated with fluoroquinolones. However, in this study, comparison of the prevalence of virulence factors among those which are ciprofloxacin susceptible has not been done.

An increased association has been noted with increase in quinolone prescriptions and bacterial resistance to quinolones from several countries.^[13-16] Quinolone resistance was first seen in *S. aureus*, *Pseudomonas* species, and it was because of mutations. But, nowadays, it is seen in almost all the isolates including *Enterobacteriaceae* and this cannot be explained for by mutations because of the high MICs. Plasmids responsible for quinolone resistance - qnr have been discovered only after 1998, and it was only after 2002 that qep plasmid coding for efflux pump and other plasmids was discovered.^[17] Since then, several studies have been conducted to study the prevalence of these plasmids and their association with resistance to other drugs, especially beta lactams.^[18-21] It has been found that these plasmids co-exist with other genes responsible for resistance to beta lactams, aminoglycosides, etc. This study shows that there is a high occurrence of aac-(6')-Ib-cr gene. This is responsible for acetylation of ciprofloxacin and norfloxacin, thus inactivating it.^[22] This is very common among the members of the *Enterobacteriaceae* family. In a study conducted in Italy by Frasson et al., 13% of isolates were positive for the aac(6')-Ib gene.^[23] A study in Tunisia and another study in China revealed that aac(6')-Ib-cr gene was the most common among the quinolone-resistant plasmids,

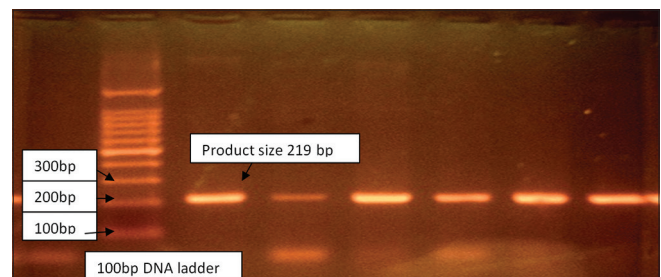


Figure 2: Gel electrophoresis picture analyzing aac-(6')-Ib-cr gene

similar to this study.^[24,25] This study shows that the prevalence of aac(6)-Ib-cr gene is increasing over the years, especially in Gram-negative bacteria. This might lead to the selection of chromosomal mutations that might lead to high levels of resistance to the fluoroquinolones.^[26]

CONCLUSION

Since quinolone-resistant genes are plasmid-mediated, dissemination of these antibiotic resistance determinants can easily occur between different bacterial genera and thus result in selection of drug-resistant mutants, which makes treatment of common infections very difficult. This can be avoided by antibiotic stewardship. The hospital infection control committee plays a major role in formulating and enforcing the antibiotic policy of the hospital and thus preventing the development of drug-resistant mutants.

Financial support and sponsorship
Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Foxman B. Epidemiology of urinary tract infections: Incidence, morbidity, and economic costs. *Am J Med* 2002;113 Suppl 1A: 5S-13S.
2. Gonzalez CM, Schaeffer AJ. Treatment of urinary tract infection: What's old, what's new, and what works. *World J Urol* 1999;17:372-82.
3. Jancel T, Dudas V. Management of uncomplicated urinary tract infections. *West J Med* 2002;176:51-5.
4. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing M 100 S 24, Twenty fourth informational supplement. Vol. 34. Wayne, PA, USA: Clinical and Laboratory Standards Institute; 2014.
5. Nerurkar A, Solanky P, Naik SS. Bacterial pathogens in urinary tract infection and antibiotic susceptibility pattern. *J Pharm Biomed Sci* 2012;21:1-3.
6. Eshwarappa M, Dosegowda R, Aprameya IV, Khan MW, Kumar PS, Kempegowda P. Clinico-microbiological profile of urinary tract infection in South India. *Indian J Nephrol* 2011;21:30-6.
7. Mandal J, Acharya NS, Buddhapriya D, Parija SC. Antibiotic resistance pattern among common bacterial uropathogens with a special reference to ciprofloxacin resistant *Escherichia coli*. *Indian J Med Res* 2012;136:842-9.
8. Wiles TJ, Kulesus RR, Mulvey MA. Origins and virulence mechanisms of uropathogenic *Escherichia coli*. *Exp Mol Pathol* 2008;85:11-9.
9. Lloyd AL, Henderson TA, Vigil PD, Mobley HL. Genomic islands of uropathogenic *Escherichia coli* contribute to virulence. *J Bacteriol* 2009;191:3469-81.
10. Mittal S, Sharma M, Chaudhary U. Study of virulence factors of uropathogenic *Escherichia coli* and its antibiotic susceptibility pattern. *Indian J Pathol Microbiol* 2014;57:61-4.
11. Drews SJ, Poutanen SM, Mazzulli T, McGeer AJ, Sarabia A, Pong-Porter S, et al. Decreased prevalence of virulence factors among ciprofloxacin-resistant uropathogenic *Escherichia coli* isolates. *J Clin Microbiol* 2005;43:4218-20.
12. Horcajada JP, Soto S, Gajewski A, Smithson A, Jiménez de Anta MT, Mensa J, et al. Quinolone-resistant uropathogenic *Escherichia coli* strains from phylogenetic group B2 have fewer virulence factors than their susceptible counterparts. *J Clin Microbiol* 2005;43:2962-4.
13. Zervos MJ, Hershberger E, Nicolau DP, Ritchie DJ, Blackner LK, Coyle EA, et al. Relationship between fluoroquinolone use and changes in susceptibility to fluoroquinolones of selected pathogens in 10 United States teaching hospitals, 1991-2000. *Clin Infect Dis* 2003;37:1643-8.
14. Arslan H, Azap OK, Ergönül O, Timurkaynak F; Urinary Tract Infection Study Group. Risk factors for ciprofloxacin resistance among *Escherichia coli* strains isolated from community-acquired urinary tract infections in Turkey. *J Antimicrob Chemother* 2005;56:914-8.
15. Karlowsky JA, Kelly LJ, Thornsberry C, Jones ME, Sahn DF. Trends in antimicrobial resistance among urinary tract infection isolates of *Escherichia coli* from female outpatients in the United States. *Antimicrob Agents Chemother* 2002;46:2540-5.
16. Kahlmeter G; ECO.SENS. An international survey of the antimicrobial susceptibility of pathogens from uncomplicated urinary tract infections: The ECO.SENS Project. *J Antimicrob Chemother* 2003;51:69-76.
17. Guan X, Xue X, Liu Y, Wang J, Wang Y, Wang J, et al. Plasmid-mediated quinolone resistance – Current knowledge and future perspectives. *J Int Med Res* 2013;41:20-30.
18. Fihman V, Lartigue MF, Jacquier H, Meunier F, Schnepf N, Raskine L, et al. Appearance of aac(6)-Ib-cr gene among extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in a French hospital. *J Infect* 2008;56:454-9.
19. Tausova D, Dolejska M, Cizek A, Hanusova L, Hrusakova J, Svoboda O, et al. *Escherichia coli* with extended-spectrum β -lactamase and plasmid-mediated quinolone resistance genes in great cormorants and mallards in Central Europe. *J Antimicrob Chemother* 2012;67:1103-7.
20. Silva-Sánchez J, Cruz-Trujillo E, Barrios H, Reyna-Flores F, Sánchez-Pérez A; Bacterial Resistance Consortium, Garza-Ramos U. Characterization of plasmid-mediated quinolone resistance (PMQR) genes in extended-spectrum β -lactamase-producing *Enterobacteriaceae* pediatric clinical isolates in Mexico. *PLoS One* 2013;8:e77968.
21. García-Fulgueiras V, Bado I, Mota MI, Robino L, Cordeiro NF, Varela A, et al. Extended-spectrum β -lactamases and plasmid-mediated quinolone resistance in enterobacterial clinical isolates in the paediatric hospital of Uruguay. *J Antimicrob Chemother* 2011;66:1725-9.
22. Hooper DC. Emerging mechanisms of fluoroquinolone resistance. *Emerg Infect Dis* 2001;7:337-41.
23. Frasson I, Cavallaro A, Bergo C, Richter SN, Palù G. Prevalence of aac(6)-Ib-cr plasmid-mediated and chromosome-encoded fluoroquinolone resistance in *Enterobacteriaceae* in Italy. *Gut Pathog* 2011;3:12.
24. Jouini A, Ben Slama K, Vinué L, Ruiz E, Saenz Y, Somalo S, et al. Detection of unrelated *Escherichia coli* strains harboring genes of CTX-M-15, OXA-1, and AAC(6)-Ib-cr enzymes in a Tunisian hospital and characterization of their integrons and virulence factors. *J Chemother* 2010;22:318-23.
25. Xia LN, Li L, Wu CM, Liu YQ, Tao XQ, Dai L, et al. A survey of plasmid-mediated fluoroquinolone resistance genes from *Escherichia coli* isolates and their dissemination in Shandong, China. *Foodborne Pathog Dis* 2010;7:207-15.
26. Warburg G, Korem M, Robicsek A, Engelstein D, Moses AE, Block C, et al. Changes in aac(6)-Ib-cr prevalence and fluoroquinolone resistance in nosocomial isolates of *Escherichia coli* collected from 1991 through 2005. *Antimicrob Agents Chemother* 2009;53:1268-70.