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MULTI-DRUG RESISTANT *Escherichia coli* AND *Proteus mirabilis* FROM THIRUVANANTHAPURAM, KERALA (INDIA)

Pooja P. Rajan, Abhirami P. Sreekantan, Minsa Mini, Sajeeb Khan and Praveen Kumar*

Department of Zoology, Government College for Women, Thiruvananthapuram – 695 014, Kerala (India)
*e-mail: praveen@gcwtvm.ac.in

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ABSTRACT

***Escherichia coli* and *Proteus mirabilis* are Gram-negative, rod-shaped uropathogens that frequently cause urinary tract infections. They have gained importance due to the emergence of multi-drug resistance in them. This work compares the antibiotic resistance pattern of clinical isolates of *E. coli* and *P. mirabilis*, isolated from urine, emphasizing the tetracycline and β-lactam resistance mechanism. The antibiotic susceptibility of *E. coli* and *P. mirabilis* was assessed by Kirby-Bauer disc diffusion assay. Extended-spectrum β-lactamase was detected by a modified double-disc synergy test and EZY MIC™ strip test. The amplification of drug-resistant gene was done by PCR. The role of efflux pump was determined by using the efflux pump inhibitor, carbonyl cyanide 3-chlorophenylhydrazone (CCCP). The results of antibiotic susceptibility test indicated multi-drug resistant phenotypes along with tetracycline and β-lactam resistance. *E. coli* was found to be extended-spectrum β-lactamase producer; whereas *P. mirabilis* yielded negative result. PCR results showed the presence of *tetA* gene in *E. coli* and *P. mirabilis* and the absence of ESBLs (TEM, SHV, and CTX-M). Further, efflux pump assay using efflux inhibitor CCCP proved that the efflux mechanism causes tetracycline resistance. The study necessitates the continuous monitoring of antimicrobial drug resistance in uropathogens for better therapeutic approaches to curtail the risk of AMR.**

Keywords: Efflux pumps, extended-spectrum β-lactamase, *tetA*, *Escherichia coli*, *Proteus mirabilis*

INTRODUCTION

Urinary tract infections (UTIs) are caused by various pathogens, with *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis*, and *Staphylococcus saprophyticus* being the most frequently observed ones (Flores-Mireles *et al.,* 2015). *E. coli* and *P. mirabilis* are also implicated in cystitis and pneumonia of nosocomial origin (Jacobsen *et al.,* 2008). Due to the massive emergence and dissemination of antibiotic resistance mechanisms, UTIs have become extremely difficult to treat (Foxman, 2014). The rapid spread of antibiotic resistance is believed to be due to the indiscriminate use of medications and dearth of new drug development by therapeutic industry (Ventola, 2015). As per WHO, 350 million deaths are expected by 2050 due to antimicrobial activity (AMR), a severe threat to clinical therapeutics (Dadgostar, 2019). AMR mechanism of uropathogens includes limiting the drug uptake, modifying the drug target, drug inactivation via antibiotic degrading enzymes such as β-lactamases and active drug efflux (Reygaert, 2018). Of these, drug efflux and drug inactivation are the key mechanisms of drug resistance used by uropathogens (Li and Nikaido, 2009).

The extended-spectrum β-lactamases (ESBL) are plasmid-mediated enzymes that hydrolyze amide bonds of 4-membered β-lactam rings of 3rd generation cephalosporins, monobactams and carbapenems but are sensitive to β-lactamase inhibitor (clavulanic acid). The key β-lactamases are bla_{TEM} (cephalosporinase), bla_{SHV} (sulfhydryl variant) and $bla_{CTX\text{-}M}$ (cefotaximase) (Tooke *et al.*, 2019). The emergence of ESBL-producing bacteria in *Enterobacteriaceae* family is a severe global health problem. Because of the indiscriminate use of β-lactam antibiotics, its resistance is >50% of uropathogens in developing nations (Teklu *et al.*, 2019). The prevalence of ESBL-producing isolates of *E. coli* and *P. mirabilis* is increasing worldwide, posing a challenge to the public health system (Nakama *et al.*, 2016). Tetracycline is a broad-spectrum antibiotic which blocks protein synthesis by binding to 30S ribosomal subunit. The tetracycline resistance mechanism in uropathogenic bacteria is due to the energy-dependent removal of tetracycline from the cell via efflux pumps, encoded by *tetA* and *tetB* genes and ribosomal protection (EF-G like protein). There are five major families of efflux pumps, i.e. ABC (ATP binding cassette), MATE (multi-drug and toxic compound extrusion), SMR (small multi-drug resistance), MFS (major facilitator superfamily), and RND family (resistance nodulation cell division). All these systems, except ABC, use proton motive force (PMF) via ATP hydrolysis to drive the extrusion of toxic substrates (Grossman, 2016). Tetracycline efflux pumps belonging to MFS superfamily confer resistance to tetracycline only except *tetB*, which can expel tetracycline and minocycline (Guay and Rothstein, 1993). Further, *tetA* is the most prevalent form of tetracycline efflux pump gene reported in clinical isolates of *E. coli* and *P. mirabilis* (Møller *et al.*, 2016). The present study was aimed to examine the antibiotic resistance pattern in a clinical strain of *E. coli* and *P. mirabilis* and to study ESBL production and the mechanism of tetracycline resistance.

MATERIALS AND METHODS

Bacterial strains and identification

Single isolates each of *E. coli* (GC02) and *P. mirabilis* (GC10) were cultured from the urine sample of the suspected UTI patient in the State Public Health and Clinical Laboratory, Thiruvananthapuram, Kerala (India). A conventional culture-dependent protocol was used for urine culture and isolation of bacterial samples (Daoud *et al*., 2015). The strains, GC10 and GC02, were identified via VITEK 2 VERSION: 07.01 and 16SrRNA sequencing, respectively (Accession No: OK560564).

Antimicrobial susceptibility testing

The antibiotic susceptibility of *E. coli* and *P. mirabilis* was assessed by Kirby-Bauer disc diffusion assay on Muller-Hinton agar as per the guidelines of Clinical Laboratory Standards Institute (CLSI, 2013) by using antibiotic-impregnated discs (Himedia). *E. coli* strain (ATCC 25922) was used as a control for antibiotic susceptibility test. A total of 19 antibiotic impregnated discs (Himedia) belonging to 9 classes used were quinolones-ciprofloxacin (5 µg), levofloxacin (5 µg), norfloxacin (10 µg); cephalosporins-cefepime (30 µg), cefepime (30 µg), cefotaxime (30 µg), cefixime (5 µg), cefpodoxime (10 µg), ceftazidime (30 µg), ceftriaxone (30 µg); macrolides-azithromycin (15 µg), erythromycin (15 µg); aminoglycosides- gentamicin, (10 µg), streptomycin (25 µg); sulphonamides-cotrimoxazole (25 µg), monobactams-aztreonam (30 µg); carbapenems-meropenem (10 µg); tetracyclines-doxycycline hydrochloride (30 µg) and tetracycline (30 µg); protein synthesis inhibitors-chloramphenicol (30 µg). The concentration of antibiotics in discs and the breakpoints are in accordance with the CLSI guidelines. Multidrug resistance (MDR) is defined as resistance to at least one drug in three or more antimicrobial classes. The bacterial cultures were grown until the stationary phase achieved and diluted with sterile Mueller-Hinton broth to 0.5 McFarland standard. Mueller-Hinton agar (15-20 mL) was poured on glass Petri plates and allowed to solidify. Then 500 µL inoculum (0.5 McFarland standard) was poured onto the surface of solidified agar and spread by

a plate spreader. The excess inoculum was discarded, and plates dried for 10 min. The antibiotic disc was placed on the Muller Hinton agar using forceps. The antibiotic-impregnated plates were incubated at 37°C for 16 h. The inhibition zones were measured and compared with the zone size interpretation chart of Himedia to classify the strains into sensitive, intermediate and resistant. The antibiotic susceptibility test was performed in triplicate.

Screening for ESBL production

Modified double-disc synergy test: A lawn culture of the test organism (0.5 McFarland standard) was prepared on Mueller-Hinton agar. The ESBL production was estimated by placing the discs of third-generation cephalosporins, 3GC-cefotaxime (30 µg), ceftriaxone (30 µg), cefpodoxime (30 µg), and fourth-generation cephalosporins, 4GC- cefepime (10 µg) at distances ranging from 15 to 20 mm from the centrally placed amoxyclav (30 µg). When the zone of inhibition surrounding cephalosporin discs exhibited an apparent increase towards the amoxyclav disc, the organisms were judged to be producing ESBL (Kaur *et al.,* 2013). The experiment was performed in triplicate.

Confirmation of ESBL detection by EZY MIC™ strip test: The ESBL strip (Himedia) containing a two-fold minimum inhibitory concentration (MIC) gradient of ceftazidime and cefotaxime at one end (MIX = 0.125-16 µg mL^{-1}) and same antibiotics with clavulanic acid at the opposite end (MIX^+ = 0.032-4 µg mL^{-1}) was used for ESBL confirmation. The strip was then placed on a Muller-Hinton agar plate inoculated with the suspension of 0.5 Mcfarland standard and incubated overnight at room temperature. A ratio of MIX/MIX^+ > 8 or a zone obtained in the MIX^+ side indicates the presence of ESBLs (Rawat and Nair, 2010). The test was performed twice.

Molecular identification of drug resistance genes

The PCR was carried out in a thermal cycler (T100 Thermal cycler, Bio-Rad) in a volume of 25 µL containing 1X reaction buffer (SRL), 10 pmoles of each primer100 µM of each dNTPs and 1 unit of Taq polymerase (SRL). The sequence of primers used in the study were as under:

Primers	Nucleotide sequence (5' to 3')	Amplicon size (bp)	References
CTX-M-F	SCSATGTGCAGYACCAGTAA	585	Dallenne *et al.* (2010)
CTX-M-R	CCGCRATATGRTTGGTGGTG		
TetA-F	GCTGCAAGCAATGTTGTCCA	190	Present study
TetA-R	CAGGCAGAGCAAGTAGAGGG		
SHV-F	AGCCGCTTGAGCAAATTAAAC	786	Sundsfjord *et al.* (2004)
SHV-R	GTTGCCAGTGCTCGATCAGC		
TEM-F	CATTTCCGTGTCGCCCTTATTC	846	Sundsfjord *et al.* (2004)
TEM-R	CCAATGCTTAATCAGTGAGGC		

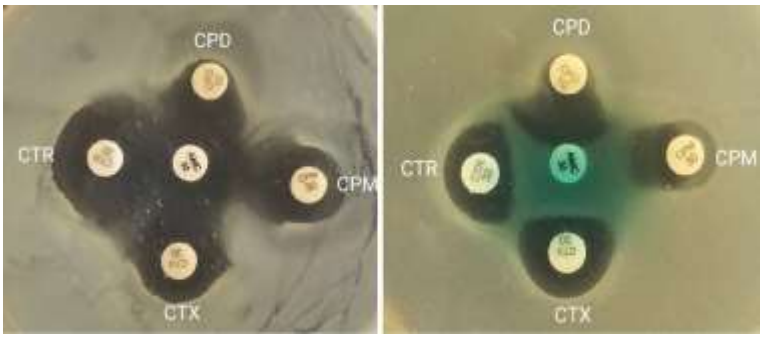
The PCR was programmed for 30 cycles consisting of denaturation of template DNA for 30 sec at 94°C, primer annealing for 30 sec at 53°C, and extension of primers for 30 sec at 72°C for the amplification of *tetA*. Initial denaturation was carried out for 3 min at 94°C and final extension for 10 min at 72°C. The amplification of bla_{CTX-M}, bla_{TEM} and bla_{SHV} genes was done as per Sundsfjord *et al.* (2004) and Dallenne *et al.* (2010). The PCR products were separated by 1% agarose gel stained with 0.5 µg mL^{-1} ethidium bromide. A 100 base pair ladder (SRL) was used as a molecular size standard. The agarose gel was visualized under EZ gel documentation system (Bio-Rad).

Efflux inhibition assay

For efflux inhibition assay, 20 mL Muller Hinton agar was poured on Petri plates and allowed to solidify. Then 100 µL inoculum (0.5 McFarland standard) was poured on its surface and spread uniformly. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) [15 µg], an efflux inhibitor, was added to tetracycline and doxycycline discs and allowed to dry for 30 min. The tetracycline (TE: 30 µg), doxycycline (DO: 30 µg), TE (30 µg) + CCCP (15 µg), DO (30 µg) + CCCP (15 µg) and control disc without CCCP were used. A difference of ≥ 5 mm in zone diameter between TE and DO disc alone, and TE and DO + CCCP was taken as positive for efflux pump mediated tetracycline resistance.

RESULTS AND DISCUSSION

In the present study, except for chloramphenicol, streptomycin and norfloxacin, *E. coli* showed resistance to all the remaining 16 antibiotics. Nevertheless, except for aztreonam, cefpodoxime, chloramphenicol, streptomycin, and norfloxacin, *P. mirabilis* showed resistance to the remaining 14 antibiotics. The ESBL production, analyzed by MDDST, revealed that only *E. coli* showed a synergism for all four cephalosporins, including cefotaxime, cefpodoxime, ceftriaxone, and cefepime with amoxyclav disc. The organisms were identified as ESBL positive when the inhibition zone surrounding cephalosporin discs increases toward the amoxyclav disc **(**Fig. 1**)**. Further, the ESBL Ezy MIC™ strip test was conducted for the phenotypic confirmation of ESBL detection in *E. coli*. The strip test showed an inhibition zone on ceftazidime, cefotaxime + clavulanate (MIX$^+$) and the point of intersection ellipse with the MIC was 0.25 µg mL^{-1}. On the other end, marked as 'MIX', the MIC of ceftazidime and cefotaxime was observed as 16 µg mL^{-1}. Since the ratio of MIX/MIX$^+$ was greater than 8, it confirms the presence of ESBL. of ESBL **(**Fig. 2**)**. The results showed that ≥ 3 two-fold dilutions reduced MIC of ceftazidime and cefotaxime in the presence of clavulanic acid.



A **B**

Fig. 1: Modified double-disc synergy test: A) *E. coli*, GC02; B) *P. mirabilis* GC10. Cefotaxime (CTX), cefpodoxime (CPD), ceftriaxone (CTR), and cefepime (CPM) with amoxyclav disc (AMC)

Fig. 2: Phenotypic confirmation of ESBL in GC02 via ESBL Ezy MIC™ strip test. Point of intersection of inhibition zone ellipse with MIC of MIX$^+$ (0.25 µg mL^{-1}). No zone was obtained for MIX, and the zone obtained with MIX + (at 0.25 µg mL^{-1}) is interpreted as ESBL positive.

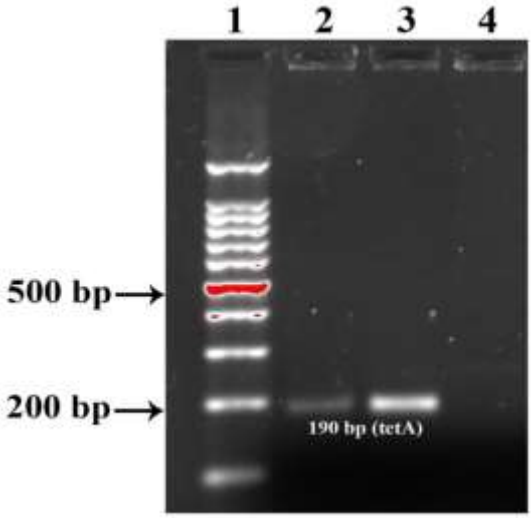
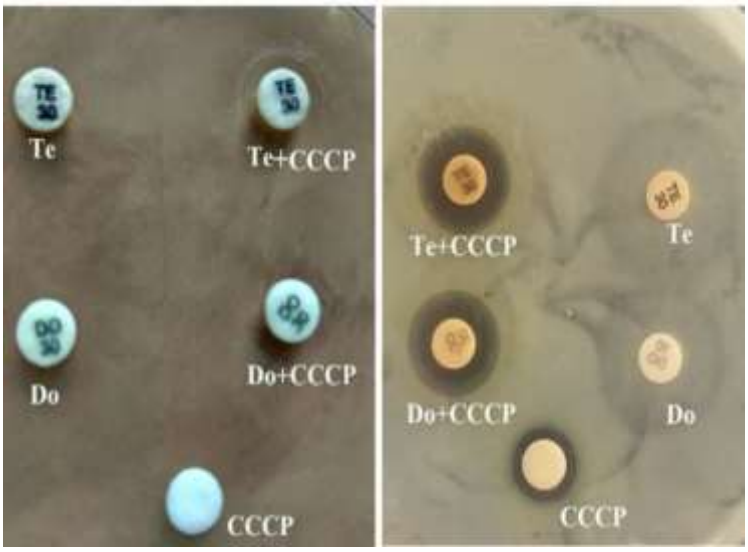


Fig. 3: Amplification of *tetA* gene: Agarose gel showing PCR product amplified from tetA-F and tetA-R primers. Lane 1 (100 bp ladder, SRL); Lane 2 (GC02); Lane 3 (GC10); Lane 4 (negative control). Bands at 190 bp confirm the presence of *tetA* gene.

The molecular detection of the genes encoding tetracycline resistance (*tetA*) and ESBL (*TEM, SHV,* and *CTX-M* genes) was carried out in *E. coli* and *P. mirabilis* using PCR. The presence of *tetA* gene with 190 bp amplicon was confirmed in both strains, whereas the ESBL encoding genes were not amplified, implying the absence of ESBL genes **(**Fig. 3**)**. The results of the efflux assay showed increased susceptibility of *E. coli* and *P. mirabilis* towards tetracycline and doxycycline in the presence of efflux inhibitor, CCCP. The tetracycline + CCCP and doxycycline + CCCP zone sizes were 14 and 12 mm for *P. mirabilis* and 15 mm each for *E. coli* (Fig. 4). However, for the CCCP alone disc (15 µg), no zones were obtained in *P. mirabilis*, indicating no detrimental effect on the bacterial growth, but contrarily in the case of *E. coli* the zone size of the CCCP alone disc was 9 mm.

Urinary tract infection is one of the most common bacterial infections, responsible for 40% of nosocomial infections, and globally about 150 million people are diagnosed with UTI annually (Khoshnood *et al.,* 2017). Drug resistance in Gram-negative uropathogens such as *E. coli* and *P. mirabilis* is a major clinical concern leading to significant morbidity and high medical costs. In present study, *E. coli* and *P. mirabilis* were found to be multi-drug resistant due to their resistance to >3 classes of antibiotics. Previous studies have reported widespread antibiotic resistance in both *E. coli* and *P. mirabilis* derived from UTI's (Adamus-Bialek *et al.,* 2013). In present study, resistance to meropenem, a carbapenem, was observed. The recent emergence of carbapenem-resistant Enterobacteriaceae cast doubt on the efficacy of therapy with carbapenems, because they are frequently employed as a final alternative for treating MDR *E. coli* and *Klebsiella* infections (Sekar *et al.,* 2016).



A **B**

Fig. 4: Efflux pump assay with CCCP: A) *P. mirabilis* GC10; B) *E. coli* GC02. Upon addition of efflux inhibitor, CCCP, the sensitivity towards tetracycline and doxycycline is increased (14 and 12 mm for *P. mirabilis* and 15 mm each for *E. coli,* respectively)

The rapid emergence of β-lactamase has resulted in a surge of β-lactam resistance in Gram-negative bacteria (Bush, 2018). The bacterial strains producing ESBL show resistance to a range of β-lactams (Poole, 2004). Since β-lactamase inhibitors such as clavulanate can cause a detrimental effect on the bacteria having beta-lactamase, it can be suggested as a better treatment option. New β-lactam/β-lactamase inhibitors such as cefepime-enmetazobactam, ceftaroline fosamil-avibactam, aztreonam-avibactam, and cefepime-zidebactam can be a breakthrough for the treatment of ESBL infections (Bassetti *et al.,* 2020). Recent reports from Kenya and Nepal showed that 24 and 40.3% uropathogenic *E. coli* isolates were ESBL producers (Pandit *et al.,* 2020; Muriuki *et al.,* 2021). Previous studies from India have reported the emergence of ESBL-producing *E. coli* (Bharara *et al.,* 2018; Mirkalantari *et al.,* 2020). This is the first report confirming the presence of ESBL in *E. coli* isolated from Kerala. Therefore, continuous monitoring of ESBL-producing strains in controlling serious infections is vital. Since *E. coli* is an ESBL-positive strain, the lack of amplification of ESBL genes such as SHV, TEM, and CTX-M may be due to its absence or variation in nucleotide sequences or the presence of other *β*-lactamases like VIM or IMP (Swaminathan *et al.,* 2016; Shams *et al.,* 2018). However, *P. mirabilis* was found to be a non-ESBL producer, which is in line with Kanayama *et al* (2010) and Pal *et al.* (2016).

Tetracycline resistance in Gram-negative bacteria such as *E. coli* and *P. mirabilis* is due to the presence of *tet* genes. Most tet genes encode energy-dependent efflux pumps; for example, *tetA*, *tetB*, *tetC*, *tetD*, and *tetE* (Roberts, 2005). The key tetracycline resistance mechanism in Gram-negative bacteria is tetracycline efflux pumps, which expel tetracycline from the bacterial cell via ATP hydrolysis. Efflux pump assays can change susceptibility on addition of CCCP, which disrupts the energy levels of bacteria and inhibits drug efflux (Ardebili *et al.,* 2014). Efflux pump inhibitors (EPI) can be a future alternative to tackle the issue of emerging AMR. According to previous reports, *tetA* and *tetB* efflux pump genes are responsible for tetracycline resistance in clinical strains of *E. coli* and *Proteus mirabilis* (Karami *et al.,* 2006; Saeb *et al.,* 2017). Our analysis of the presence of tetracycline resistance genes in *E. coli* and *P. mirabilis* agrees with their findings. This is the first report confirming the presence of *tetA* gene from *P. mirabilis* and *E. coli* in Kerala, India.

Conclusion: The work reports the presence of *tetA* gene in *P. mirabilis* and *E. coli* with efflux as a possible mechanism of tetracycline resistance and ESBL producing *E. coli* from South Kerala. Continuous monitoring and studying the molecular mechanism leading to antibiotic resistance patterns may help identify the alternative drugs for these pathogens and thereby control the infection. The study emphasizes the need to monitor multidrug-resistant *E. coli* and *P. mirabilis* to impede the emergence and dissemination of AMR. The study may prove effective in designing innovative strategies to curtail the imperil of AMR.

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