



In vitro screening of multidrug resistance uropathogenic *Escherichia coli* from the urban area of Namakkal district

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ARTICLE INFO

Received 21/03/2019
Accepted 11/07/2019
Available Online: 01/09/2019

Key words:

Multidrug resistance, uropathogenic *Escherichia coli*, HiCrome UTI agar, extended-spectrum beta-lactamase, hemolytic, slime, multiplex PCR.

ABSTRACT

The aim of the study is to screen the multiple drug resistance (MDR) Uropathogenic *Escherichia coli* (UPEC) from the urban area of Namakkal district. To detect UPEC resistant by using different antibiotics and to analyze the virulent characteristics of UPEC and amplification of extended-spectrum beta-lactamases genes by multiplex polymerase chain reaction. Total 450 samples individually collected from the urinary tract infection (UTI) patients⁷ and direct streaked on to the eosin methylene blue agar plates. Significant growth indicates *E. coli*. HiCrome UTI agar was used for rapid identification of uropathogenic *E. coli*. Out of 450 samples, only 62 isolates of *E. coli* were subjected to virulence characteristics, such as slime production (34%), hemolytic activity (56%), and beta-lactamase production (43%). Antibiotic sensitivity test was performed with 13 different antibiotics. Among them, 62 isolates were *E. coli*, only five were resistant to 10 antibiotics, possess virulence characteristics. Four strains (E-12, E23, E-58, and E-97) have Temoneira, sulfhydryl variable, and cefotaxime hydrolyzing capabilities (CTX-M) antibiotic resistance genes, and E-07 have only CTX-M gene. As *E. coli* is the main infectious agent in patients with UTI and a potent pathogen, it was difficult to treat with routine antibiotics because day-by-day microbes are resisting to common drugs. Hence, they need alternative therapy.

INTRODUCTION

The urinary system of the human being helps maintain the proper balance of salt and water to the whole body and remove the urine from the body. It is a collection and vacuum process that includes the kidneys, ureters, bladder, and urethra. Sometimes, the urinary system has pathogenic microorganisms and colonization is known as urinary tract infection (UTI). The microorganisms are ascending cause of infection to urinary bladder and kidney. Around 150 million cases of UTIs are reported

every year worldwide (Källenius *et al.*, 1981; Ohieku and Magaji, 2013). Urinary tract contamination infections are acquired in the community in all ages of females and affect an estimated 11.3 million women in the United States (Jackson *et al.*, 2004), Asia 8.3 million per year. In India, it affected all age groups throughout life. It is recognized that women are more affected than men due to the associated short urethra and hormonal changes. UTIs that are untreated while pregnant include well-documented morbidity and mortality risks for the pregnant woman (Tajudeen *et al.*, 2011), widespread association with catheters (Mayon-White *et al.*, 1988), and frequent infection in outpatients (Russo and Johnson, 2003). It is due to enteric microorganisms, which are acquired from the individual gastrointestinal system. Bacteria are more prevalent and invasive. *Escherichia coli*, which lives regularly in the colon as a normal flora (Adeniyi *et al.*, 2006; Jellheden *et al.*, 1996). Not everyone who has UTI is present with symptoms but most of the patients who have at least one symptom include a frequent need to urinate, burning sensation or pain in bladder or urethra at some

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point during urination, and feel pain even when are not urinating. Often, ladies perceive uncomfortable stress in pubic bone and travel to fullness in the rectum. Urine looks milky and/or turbid, even red if the RBC is present. Usually, urinary tract infection does not cause fever if it is in the urethra or bladder, whereas it may cause fever when the infection is in kidneys. Other signs and symptoms of UTI in childhood include nausea, vomiting, and pain in the backside below the ribs.

The antibiotic is used for UTIs. More than 85% of the cases were cured with a single dose of an oral antibiotic. More complicated cases of kidney infection may require antibiotic injection or prolonged courses of oral antibiotics. Chloramphenicol, Ampicillin, Nalidixic acid, Kanamycin, Streptomycin, Nitrofurantoin, Norfloxacin, and Trimethoprim are used for the treatment of UTI. Evaluation of antibiotic resistance among the *E. coli* urinary tract isolates from outpatient clinics revealed greater resistance to these antibiotics (Zhanel *et al.*, 2000). In most cases, repeated cycles are needed or if prophylactic long-term is needed, the prevalence of the effects of the aspects will be problematic (Sundén *et al.*, 2006). *Escherichia coli* that produce extended-spectrum beta-lactamases (ESBL), which originates in patients with acquired infections in the community and in the hospital (Falagas *et al.*, 2010). During the last decades, the increase in the number of infections caused by *E. coli* strains resistant complicates the clinical management of UTI for multiple drugs. In recent years, the incidence of cephalosporins, fluoroquinolones, and *E. coli* resistant to trimethoprim causing UTIs is of scientific importance. UTI is the second most common clinical symptom of empirical antimicrobial remediation in essential and secondary care centers (Morgan and McKenzie, 1993).

The pathogen virulent factors such as bacterial enzymes, adhesion factors such as fimbria/pili, flagellin, urease, hemolysin, metalloprotease (Fraser *et al.*, 2002), and extended-spectrum β -lactamases (Coque *et al.*, 2008). Although resistance to antibiotics refers to the uptake of plasmids/transposons, the significant mechanisms of resistance to antibiotics are the prevention of the accumulation of antibiotics either by minimizing uptake or by increasing the outflow, inactivating antibiotics either by hydrolysis or by modification, and qualitative alteration of the antimicrobials targets (Muratani and Matsumoto, 2004). Therefore, this study is taken to determine the risk factors related to UTI acquired in the community. Distribution of bacterial strains isolated from patients and their resistance pattern were studied. A molecular virulence feature was obtained and evaluated for a greater appreciation of resistance to multiple drugs, the potential pathogens of the UPEC. Strategic control of pathogens in a geographic area requires knowledge of the etiology of the infection; the pattern of drug resistance and the subsequent formulation of an appropriate hospital therapeutic policy to overcome the associated side effects and the increase in the cost of treatment.

MATERIALS AND METHODS

Patients for the present study were those attending at private hospitals, Paramathi-Velur, Namakkal District, Tamilnadu. The sample collection period was from June to December.

Collection of sample and transport

A total of 450 samples of mid-stream urine collected in a sterile plastic container, wide mouth and screw cap.

Approximately, 3–5 ml of urine samples were collected from the individual patient. Samples were accurately labeled indicating the patient's source, date, time of collection, gender, and age. Within 4–6 hours of collection, urine samples were taken to the Microbiology Laboratory in a cooler box for bacterial analysis. Urine samples are processed by macroscopic and cultural methods (Koneman *et al.*, 1994).

Macroscopy

Visual examination of urine specimens was done. Details of samples are mentioned in the record. The color of the urine was seen on a bright background and was described as dark yellow, brown-yellow, and pinkish red. Normal urine is straw yellow.

Primary isolation of uropathogenic *Escherichia coli*

Plates of eosin methylene blue agar, MacConkey agar, Hektoein enteric agar, xylose lysine deoxycholate agar (XLD), and Hi-Chrome UTI agar were used. The collected urine samples were seeded directly on eosin methylene blue (EMB) agar plates and incubated for 24 hrs at 37°C. After incubation, the plates examined the significant growth of *E. coli*, which are colonies of greenish metallic sheen. These isolates were inoculated into the labeled plates mentioned above. Hi-Chrome agar is a differential medium recommended for early detection of microorganisms that cause UTIs. Primary identification of bacterial isolates was based on colonial aspect and pigmentation. Subcultures made on nutrient agar plates and incubated for 24 hours. Isolated organisms were recognized and confirmed by Bergey's manual of determinative bacteriology.

Microscopic examination

Simple staining, Gram staining, and Hanging drop techniques had been achieved to seem for their morphology, such as shape, Gram's nature, and motility of the isolate, respectively, among microorganisms.

Assessment of virulent characteristics

Virulent components are responsible for bacterial pathogens. The pathogen must possess aggressive, infectious, and reliable pathogenic properties. The availability of these dwellings is proportional to virulent properties. Surface factors, enzyme factor, genetic factors, and plasmid factors can exacerbate microbial virulence. Assessment of viral components will provide pathogenicity and help to take precautionary measures to look for strong pathogens. The isolated *E. coli* organisms were analyzed for the hemolytic activity, beta-lactamase production, slime activity followed by the below-mentioned methods (a,b,c).

a) Blood Agar Plate Assay

The hemolytic activity of *E. coli* was determined by blood agar plate assay. After 24 hours of incubation, the area of hemolysis surrounding the colonies was considered to be greatest in blood agar plates containing 5% (v/v) human blood (Brenden and Janda, 1987).

b) β -Lactamase Production Assay

The broth culture was inoculated by spots on Mueller-Hinton agar containing penicillin and 1% starch and incubated

at 37°C overnight. The plates were then flooded with freshly prepared phosphate-buffered saline containing sodium iodide and potassium. The presence of clear colorless areas around bacterial overgrowth indicates the production of beta-lactamases. Beta-lactamase converts penicillin into penicilloic acid, which, with the help of starch and iodine complexation, reduces iodine to a controlled iodide (Ahmad & Yadava, 1979).

c) Slime Activity

In the Congo red test, the medium was arranged with 37 g/l (50 g/l sucrose, 10 g/l agar and 0.8 g/l Congo red) with brain and brain infusion broth. The Congo red is used to organize into an enriched aquatic solution. The medium was autoclaved for 15 minutes at 121°C and separately from different components of the medium, then introduced when the agar was cooled to 45–55°C. The plates were incubated for 24 hours at 37°C. The resulting surface is marked by black colonies. The strains, which do not produce slime form red colonies (Freeman *et al.*, 1989).

Assessment of antibiotic sensitivity pattern

The antibiotics susceptibility test was carried out according to the CLSI guidelines and the isolates were subjected to the antibiotic susceptibility test (Bauer *et al.*, 1966). The susceptibility of isolates of *E. coli* to antimicrobial agents was examined by a Disk Diffusion Assay. Antibiotics used for the assay such as Co-trimoxazole (Co-30), Ciprofloxacin (CF-30), Nalidixic acid (Na-10), Ampicillin (A-10), Tetracycline (T-30), Erythromycin (E-15), Kanamycin (K-30), Bacitracin (B-10), Vancomycin (V-30), Carbenicillin (CB-100), Penicillin (P-10), Tobramycin (TB-10), and Nitrofurantoin (NF-10) µg/disk. All disks were purchased at Hi-Media, India. Mueller–Hinton Agar was used for the assay. A disk diffusion technique was followed to determine antibacterial activity. 0.5 McFarland standard of the test culture swabbed on Mueller–Hinton agar plates (Bauer *et al.*, 1966). Making use of the extracted template disks disbursed in the Mueller–Hinton Agar solidified with test organisms. Bacterial cultures incubated for 24 hours at 37°C. The test performed in triplicates. The zone of inhibition was measured by using the Antibiotic zone scale (Hi-media). The patterns of resistance were interpreted according to the recommendations of the CDC. These isolates of multidrug resistance continued to use molecular characterization.

Molecular characterization

The molecular method is a useful marker for the complete characterization of Uropathogenic *E. coli* strains (Yamamoto, 2007; Yamamoto *et al.*, 1995).

a) Separation of plasmid DNA

Isolation of plasmid DNA was carried out by alkaline lysis procedure with some modification. 1 ml of an overnight culture was transferred to an Eppendorf tube (Sadasivam and Manickam, 1996). The cells were pelleted by brief centrifugation (5,000 rpm) in the Eppendorf and supernatant was drained. The pellet was resuspended by adding 100 µl of lysis buffer and the contents were mixed with the vortex. Then, 100 µl of 0.1 M Tris HCl was added and mixed well by inverting the content (4–5 times). To the above viscous content, 100 µl of buffer was added

and the content was inverted 4–5 times to mix most of genomic DNA, and other cellular debris will precipitate in a viscous group. It centrifuged at 12,000 rpm and clump removed. The clear lysate (supernatant) was transferred to another Eppendorf tube. 150 µl of 100% Isopropanol was added, mixed well, and the contents were centrifuged at 12,000 rpm for 30 minutes. The supernatant drained; In addition, 150 µl of absolute alcohol was added and centrifuged at 10,000 rpm for 20 minutes. The supernatant was drained and the DNA pellets were dissolved with 20 µl TE buffer. The extracted plasmid DNA was confirmed by running of agarose gel electrophoresis.

b) Polymerase chain reaction for amplification of ESBL genes

All sequences of partial or total resistance genes available from the resistance gene were determined according to the protocol with some modification (Maya *et al.*, 2011). The primers were obtained from Sigma, India, and were used in the polymerase chain reaction (PCR) comprised Primer. PCR mixture was prepared in a thin-walled PCR tube in a sterile laminar flow hood. Add the following reagents as follows. Each PCR reaction mixture (20 µl) contained 2 µl of template DNA (plasmid DNA), 2 µl of 10X PCR buffer, 0.5 µl of (0.5µM) each of the primers, 1 µl of 0.2mM of each deoxynucleotide triphosphate, and 1 µl of Taq DNA polymerase (Con 5U/µl) and 8.0 µl of molecular grade water. A short spin was given to establish the materials that the tubes kept in a thermocycler (Genei). After initial denaturation at 95°C for 15 min, the samples were subjected to 30 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 1.30 minutes and extended at 72°C for 1 minute. A remaining extension was performed at 72°C for 10 minutes. Following PCR, aliquots (20 µl) of the response mixtures were analyzed by electrophoresis on a 1.5% Agarose gel, containing ethidium bromide (0.2 mg/ml), in the presence of a gorgeous DNA molecular weight marker. Then observe the amplification bands below the UV Transilluminator and detection of resistance gene with the use of the marker.

RESULTS AND DISCUSSION

Urinary tract infections are the most frequent bacterial infection. The UTI is responsible for the huge morbidity and high costs of medical illnesses around the world (Kudinha *et al.*, 2012). A total of 450 urine samples were collected from male and female patients with UTI during the study period. 65% ($n = 292$) of urine samples were from females and 35% ($n = 158$) were from males. The top UTI women with this study are largely supported by different writers around the world. In Belgian peoples affected that 63% of UTI cases were women and 37% were men (Ismaili *et al.*, 2011), common problem in primary health care (Mwaka *et al.*, 2011). In India, most UTI was assessed using urine in the middle of the stream. It is a simple and easy method and it refers to the true state of the infection of the system (Acharya *et al.*, 2011).

Generally, females have shortness of urethra in the urinary tract, which is 1.5 inches compared to 8 inches in males. Bacteria from fecal matter depend on anal opening and may turn into urinary tract opening without complications. Frequent sexual commitment is the most essential threat feature for urinary tract infection in young women. Almost 80% of postmenopausal women have UTIs that appear within 24 hours after intercourse. Early in pregnancy, common urination is the most common

symptom of UTI, mainly due to pressure on the bladder. Women are at increased risk for UTI after menopause. This is mainly caused by the loss of estrogen, which slows down the walls of the urinary tract and reduces its ability to resist bacteria. Loss of estrogen can reduce certain immune factors in the vagina, which can help prevent *E. coli* from joining the vaginal cells. Women who have skin pores and hypersensitive reactions to substances such as soaps, vaginal creams, bubble baths, or other chemicals used in the genital area are at a multiple risk for UTIs. In such cases, hypersensitivity reactions can also cause small lesions that can introduce bacteria. Age distribution of aggregated urine samples between the target group is shown in Figure 1.

The most variety of urine samples amassed from the age group 41–50 years. In all age groups, females have been at the top. This study confirmed that the occurrence of UTI was high among female than the male of different age groups. The female UTI incidences were high in all age groups. A slight variation noted with the age above 50 as only less number of female sample was collected during the study period (Sharma et al., 2007). Collected urine samples were visually examined the color. The maximum number of urine samples collected from UTI cases was brownish-yellow ($n = 298$); 66%, followed by dark yellow ($n = 89$); 20%, and pinkish-red in color ($n = 63$); 14%.

Generally, the color of the urine samples depends on the inflammatory and pathological conditions associated with the urinary tract. Cystitis and pyelonephritis are major inflammatory diseases caused by bacterial infections. Brownish-yellow and pinkish-red color of the urine was due to the inflammatory response. Identification of pure isolates of *E. coli* was carried out using the standard bacteriologic method. EMB agar, MacConkey Agar, XLD agar, Hektoein Enteric agar, and Hi-Chrome UTI agar were used for the selective cum differential isolation of Uropathogenic *E. coli* (Fig. 2, Table 1).

It produced specific colonies on these media. All the viable microorganisms were identified by biochemical experiments through the development of selective and differential media. Identification of *E. coli* in terms of stable culture and biochemical properties was also supported (Fig. 3) (Daoud and Afif, 2011).

The bacterial etiology of UTIs is properly established, *Escherichia coli* being the predominant pathogen (Daza et al.,

2001; Hooton, 1991; Raz et al., 2000; Zhanel et al., 2006). In this study, a total of 105 *E. coli* was recovered from 450 of urine sample. Sixty two *E. coli* were isolated as a pure isolate and 43 isolates were isolated as mixed isolates. Age-wise analysis, that the higher incidences of *E. coli* were noted in the age group 21–30 followed by the 31–40 age group. Lowest incidence noted among 1–10 age group followed by 11–20 (Fig. 4).

Out of the six types of age groups, females showed the highest prevalence in 21–30 age groups ($n = 14$). Male showed the highest prevalence in 31–40 age groups ($n = 11$). The higher rate of *E. coli* was recovered from the female urine samples (68%;

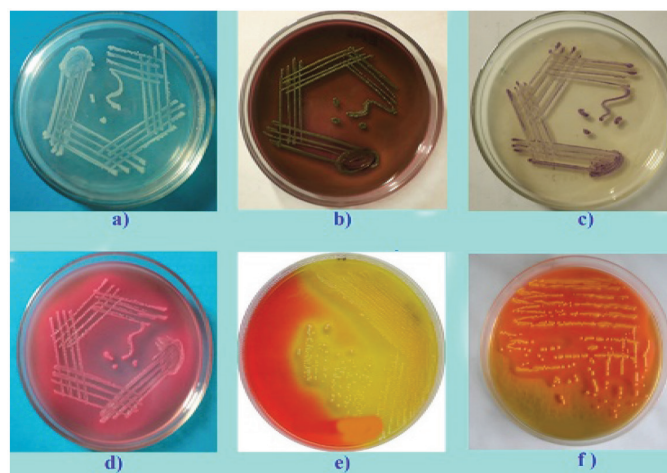


Figure 2. Growth characteristics of Uropathogenic *Escherichia coli* on Different media. (a) Nutrient agar, (b) EMB agar, (c) HiCrome UTI agar, (d) MacConkey agar, (e) XLD agar and (f) Hektoen enteric Agar.

Table 1. Growth characteristics of uropathogenic *E. coli* on different media.

S. No.	Media	Growth Pattern
1	Nutrient Agar	Small, circular, entire colony
2	EMB Agar	Greenish Metallic sheen colonies
3	HiCrome UTI Agar	Pinkish Purple color colonies
4	Mac Conkey Agar	Lactose Fermenting pink colonies
5	XLD Agar	Yellow color colonies
6	Hektoen enteric Agar	Salmon color colonies

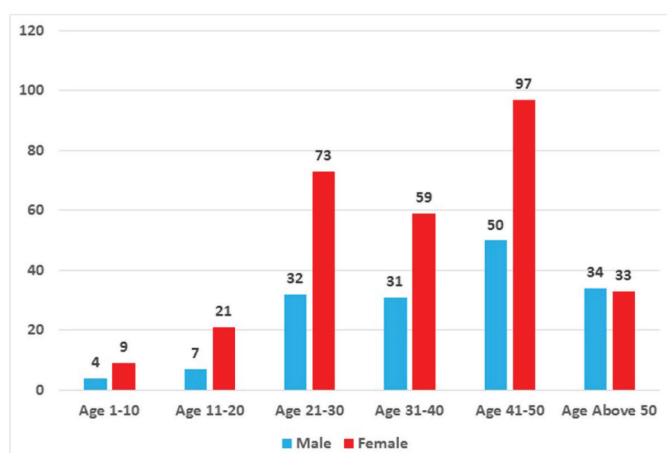


Figure 1. Age-wise distribution of UTI among the study population.

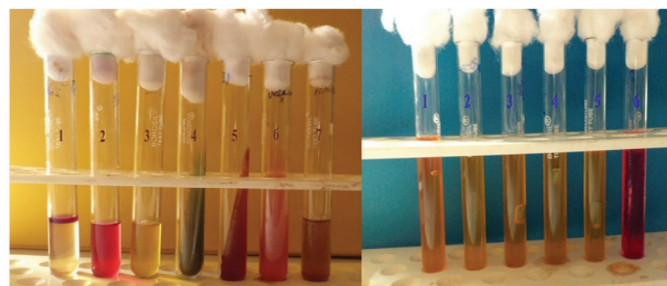


Figure 3. *Escherichia coli* biochemical features: (a) 1. Indole +ve, 2. Methyl Red +ve, 3. Voges Proskauer -Ve, 4. Citrate -ve, 5. TSI -A/A Gas +ve, 6. Urease, 7. Nitrate, (b) Carbohydrates fermentation 1. Glucose—AG, 2. Sucrose—A, 3. Maltose—AG, 4. Lactose—AG, 5. Xylose—AG, 6. Sorbitol—Negative, A—Acid, AG—Acid and Gas.

$n = 42$), whereas male patients derived specimens showed only (32%; $n = 20$). The present study revealed *E. coli* as the major pathogen of UTI— supported different authors in different research from different countries (Chlabicz *et al.*, 2011; Gower and Wardener 1971; Sharma *et al.*, 2011; Siedelman *et al.*, 2012; Walters *et al.*, 2012; Yamamichi *et al.*, 2012). In the present study, 62 pure isolates of *E. coli* selected for further characterization.

Assessment of virulent factors

The production of slime, β -lactamase, and hemolytic activity is the major virulence determinant of uropathogens. Sixty two isolates of *E. coli* from the study were subjected to virulence factor determination and showed the presence of one of the tested

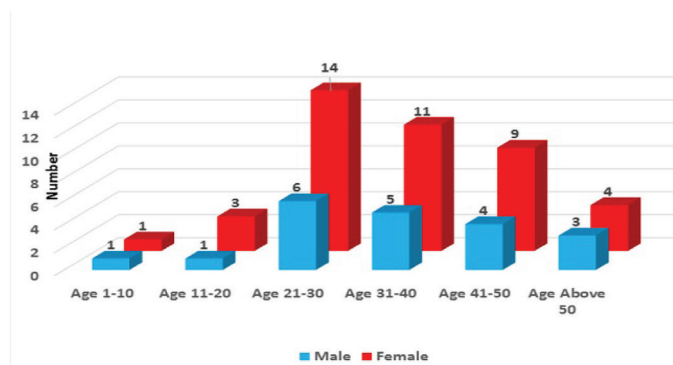


Figure 4. Age-wise distribution of *E. coli* positive among male and female.

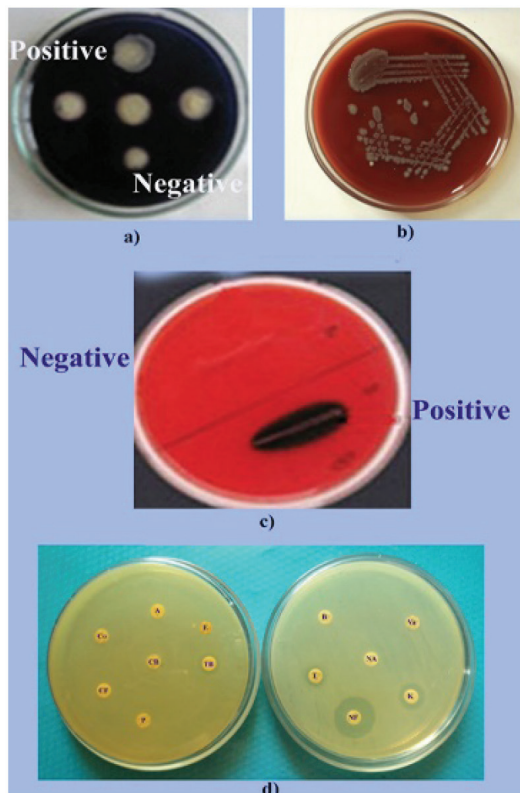


Figure 5. *Escherichia coli* with their virulent factors: (a) Beta-lactamase, (b) Hemolysis, (c) Slime activity, and (d) Antibiogram.

virulent factors. Of those tested, *E. coli* isolation showed 34% production of slime factors, 43% had β -lactamase activity, and 56% of the strains represented hemolytic activity (Fig. 5). UPEC has been exposed to a number of virulence factors and helps colonize *E. coli* in the bladder. 27 (35.1%) and 50 (64.9%) ESBLs produce neonates and UPEC strains, respectively (Hilbert *et al.*, 2012). This study has led to a less similar finding and can highlight that many environmental conditions play an important role in the expression of the respective gene product that determines the virulence.

Antibiotic susceptibility assay

Antibiotic susceptibility testing of *E. coli* isolates was carried out according to the Kirby & Bauer disk diffusion method. All confirmed *E. coli* isolates with 62 cultures were tested for antimicrobial susceptibility and were noted to be resistant to a minimum of 10 various groups of antibiotics (77%) out of 13 antibiotics added in the study. Only 23% ($n = 4$) of the isolates are sensitive to all antibiotics tested. This indicates that all isolates are resistant to several levels of antibiotics. Against the aminoglycoside-gentamycin, 32% of the isolates were sensitive which was in accordance (Zaman *et al.*, 2006). 76.5% of community-acquired infections were due to *E. coli* and 60.6% of which were *E. coli* ESBL producers. (Siedelman *et al.*, 2012; Walters *et al.*, 2012).

Percentage of resistance patterns of *E. coli* of the study were found to be 100% for Ciprofloxacin (CF), Ampicillin (A), Carbenicillin (CB), Tetracycline (T), Co-trimoxazole (Co), 98% for Erythromycin (E) and Bacitracin (B), 93% for Penicillin (P), 91% for Nalidixic acid (Na), and 89% for Vancomycin (V). Additionally, most sensitivity rates were reported for Nitrofurantoin (NF) (100%), followed by Tobramycin (90%) and Kanamycin (62%) (Table.2). The present evaluation has brought out the current antibiotic and antimicrobial resistance of many antibiotics that have registered a dangerous situation. Therefore, it is becoming relevant to the new antimicrobial treatment developed with fewer side effects, such as the urinary tract leading to chronic infection in the bladder and kidney and effective treatment to reduce stress in the human mind. This problem is for the short term. Severe implications for

Table 2. Antibiotic sensitivity patterns of uropathogenic *E. coli*.

S. No.	Antibiotics	Resistant (%)	Sensitive (%)
1	Carbenicillin (CB)	100	00
2	Ciprofloxacin (CF)	100	00
3	Ampicillin (A)	100	00
4	Erythromycin (E)	98	02
5	Co-trimoxazole (Co)	100	00
6	Nalidixic acid (Na)	91	09
7	Tetracycline (T)	100	00
8	Kanamycin (K)	38	62
9	Bacitracin (B)	98	02
10	Vancomycin (Va)	89	11
11	Penicillin (P)	93	07
12	Nitrofurantoin (NF)	00	100
13	Tobramycin (TB)	10	90

should be used and hospitalized broad-spectrum cephalosporin drugs (Petrove *et al.*, 2007). The presence of CTX-M type extended the spectrum of beta-lactamases reported among pathogens from different parts of the world. The CTX-M was a significant reason for antibiotic resistance as reported (Lepeule *et al.*, 2012). The study found that all the isolates belonged to ESBL types predictably leading to complications in therapy. It also indicated that the increased prevalence of MDR-UPEC pathogens complicated the treatment of UTI (Molina-López *et al.*, 2011).

CONCLUSION

UTI prevention is always the goal of doctors. There are many proven and unproven strategies to accomplish this task. Most UTIs disappear spontaneously if left untreated, but symptoms can persist for a long time (Buonanno *et al.*, 2006). In this study concluded that *E. coli* is one of the most predominant pathogens of UTI infection in patients of Paramathi Velur, Namakkal district, Tamil Nadu, India. *E. coli* possess multiple virulent factors and were MDR-ESBL pathogens, which are difficult to treat. The emergence of Multidrug resistance uropathogens is very difficult to treat.

ACKNOWLEDGMENT

The authors express their gratitude to all the staffs of the Department of Microbiology, Kandaswami Kandar's College, Periyar University.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest in the study.

FUNDING SOURCE

None.

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How to cite this article:

Poongothai P, Gnanasekaran A, Manikandan P, Senthilkumar PK, Vigneshwari J. *In vitro* screening of multidrug resistance uropathogenic *Escherichia coli* from the urban area of Namakkal district. *J Appl Pharm Sci*, 2019; 9(09):084–091.