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RESEARCH ARTICLE OPEN ACCESS

Bacterial Profile of Urinary Tract Infections: Evaluation of Biofilm Formation and Antibiotic Resistance Pattern of Uropathogenic *Escherichia coli*

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Abstract

Urinary tract infection (UTI) is one of the most common complaints in the outpatient clinic and a major health problem owing to the emergence of antibiotic resistance and biofilm formation. The objective of this study was to isolate and identify the causative bacterial agent of UTI and detect *in vitro* biofilm formation by *Escherichia coli* and investigate its correlation with antibiotic resistance. Urine samples from 519 patients with suspected UTIs were collected and processed by conventional microbiological procedures. Antimicrobial susceptibility testing for *E. coli* isolates was performed on Mueller Hinton agar (MHA) plates using the Kirby-Bauer disk diffusion method. Biofilm production was evaluated using the tissue culture plate method. Of 519 urine samples, 115 (22.1%) showed significant bacteriuria. The most common isolate was *E. coli* (n=57, 49.6%), followed by *Klebsiella* spp. (n=23, 20%). All *E. coli* isolates were evaluated for their ability to form biofilms *in vitro*. Of 57 isolates, 50 (87.7%) were biofilm producers and 7 (12.3%) were non-biofilm producers. Antibiogram of *E. coli* isolates revealed the highest resistance to ampicillin (96.5%) and nitrofurantoin (91.2%), followed by amoxyclav (82.5%), ceftazidime (73.7%), cefepime (71.9%), and tetracycline (71.9%). A significant association ($p<0.05$) was observed between biofilm formation and resistance to amoxyclav, ceftazidime, cefepime, imipenem, and nitrofurantoin. A significant correlation was noted between biofilm production and antibiotic resistance. Hence, screening of all isolates of uropathogenic *E. coli* for biofilm production and studying their antibiogram would allow appropriate choice of antibiotic therapy.

Keywords: UTI, Uropathogenic *Escherichia coli*, Biofilm

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INTRODUCTION

Urinary tract infection (UTI) is considered as the microbial invasion of any tissues extending from the renal cortex to the urethral meatus. The urinary system includes organs that collect, store, and release urine from the body. Accordingly, UTI is classified based on the site of infection as follows: bladder (cystitis), kidney (pyelonephritis), and urethra (bacteriuria)[1].

UTI is one of the most frequently presented complaints in outpatient clinics, and most patients are in their reproductive age (18-37 years). UTI is one of the most common hospital-acquired infections, representing as high as 35% of nosocomial infections, and accounts for the second most common cause of bacteremia in patients admitted to hospitals[2,3]. It has been estimated that about 6 million patients have UTI per year worldwide, of which around 30,000 are treated in the wards. In India, UTI is the third most common cause of hospital admission, and its prevalence varies from 21.8 to 31.3 in different parts of the country[4,5]. Uropathogenic *Escherichia coli* (UPEC) is the most common cause of UTI, accounting for approximately 90% and 50% of community-acquired and hospital-acquired UTIs, respectively. *E. coli* is as an endogenous microorganism in the human bowel and is deemed harmless under natural conditions. *E. coli* from the intestine is present in the fecal matter. The passage of trace amounts of fecal matter through the urethral opening allows entry of the microorganism into the urinary tract, wherein it thrives, multiplies, and eventually causes an infection. Some common ways involved in the migration of *E. coli* through the urethral opening are as follows:

Sexual contact

A woman's urethra is located next to the vagina and anus, making it easy for bacteria to move into the urinary tract during sexual intercourse and sexual contact.

Improper cleaning

Wiping from the back to front after excretion can drag *E. coli* directly into the urethra.

Holding urine

Frequent urination facilitates the continuous flushing of bacteria such as *E. coli* from the system. This is particularly important before and after intercourse.

Enlarged prostate gland

This exerts extra pressure on the bladder, thereby preventing it from properly emptying and flushing *E. coli* from the body[6].

About 60% to 70% of UPEC have the ability to form biofilms[7]. Relapses and chronic infections by UPEC have been associated with the ability of pathogenic strains to form biofilms. Several studies have shown that 50%-90% of isolates collected from patients with relapsed infections were biofilm producers[8]. Drug resistance among bacteria causing UTIs is increasing and considered as a major hurdle in the treatment of UTI. Biofilms protect the bacteria from the host immune response and impede the effects of antibiotics. High antimicrobial concentrations are imperative to inactivate organisms growing in a biofilm, and this may increase antibiotic resistance by 1000-fold[9]. In this context, the present study aimed to determine the correlation between biofilm production and multidrug resistance in UPEC isolates.

MATERIAL AND METHODS

The study was carried out at the Department of Microbiology, Yenepoya Medical College and Hospital, after receiving ethical clearance from the Yenepoya Ethics Committee.

Inclusion criteria

Culture isolates from urine samples of patients from all age groups with a high colony count (>10^5 colony-forming units [CFU]/mL) were included.

Exclusion criteria

Colony count < 10^5 CFU/mL

Culture plates with multiple bacterial growth.

Methodology

Study design

Descriptive longitudinal study

Sampling technique

Convenience sampling

Sample collection

Freshly voided midstream urine samples were collected from patients with suspected UTI in a sterile, dry, wide-necked, leak-proof universal sterile container under aseptic conditions[10].

Culture and identification

The well-mixed and non-centrifuged

urine samples were inoculated by a wire loop to deliver 0.001 mL of the specimen onto 5% sheep blood agar, cysteine-lactose electrolyte-deficient agar, and MacConkey agar plates using the streak plate method following standard microbiological procedures. The plates were aerobically incubated at 37°C for 24 h and examined for the presence or absence of bacterial growth. Cultures that formed >10^5 CFU/mL were considered to have significant bacteriuria. All positive samples showing significant bacteriuria were further tested for physical characteristics such as colony morphology, odor, swarming, and presence of hemolysis on respective media using different biochemical reactions performed as per standard procedures. Thus, gram-negative rods were identified with the help of a series of biochemical tests such as triple-sugar iron agar, indole, Simmons citrate agar, oxidase, urease, and motility tests. Morphologically identical colonies of suspected strains were taken from agar plates, suspended in nutrient broth, and vortexed. The suspensions were inoculated into butts and slants of biochemical testing media. The inoculated media were aerobically incubated at 37°C for overnight, and bacteria were identified following the standard flow chart. Gram-positive cocci were identified based on their reactions in catalase and coagulase tests[10].

Antibiotic susceptibility test

Antimicrobial susceptibility testing of *E. coli* isolates was performed on Mueller Hinton agar (MHA) using the Kirby-Bauer disk diffusion method as per the Clinical and Laboratory Standards Institute (CLSI) guidelines.[11] The following antibiotics were used: amoxyclav (AMC), cefepime (CPM), ceftazidime (CAZ), trimethoprim-sulfamethoxazole (COT), gentamicin (GEN), imipenem (IMP), nitrofurantoin (NIT), norfloxacin (NOR), and piperacillin-tazobactam (PTZ) (Table 1).

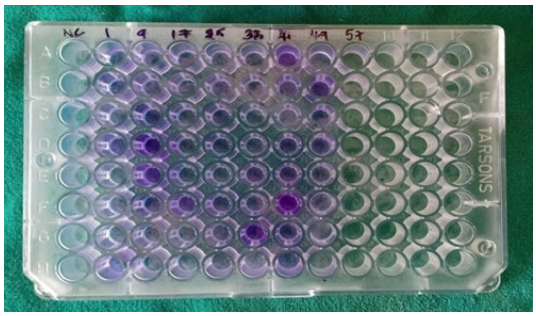

Fig. 1. Biofilm formation by tissue culture plate

Procedure

Each bacterial sample was emulsified in sterile saline in a test tube (mixed thoroughly so that no solid particles remained in the solution). The turbidity of the solution was evaluated by matching with turbidity standards (0.5 McFarland standard). The sterile swab was dipped into broth culture, and the excess fluid from the swab was removed by gently squeezing the swab against the wall of the test tube. Using the lawn culture method, the swab was streaked onto a sterile MHA plate, which was allowed to dry for a few minutes. Antibiotic disks (6 on each plate) were aseptically placed on MHA plate and the plate was incubated for 18-24 h at 37°C.

Observation and interpretation

The diameter of the zone of inhibition for each antibiotic was recorded using a metric ruler. Results were interpreted as sensitive, moderately sensitive, and resistant, as per the CLSI guidelines[11].

Biofilm production[12].

Biofilm production was determined using the tissue culture plate (TCP) method.

Procedure

A colony from an overnight grown culture of isolates on MacConkey agar plate was inoculated into a 3 mL brain heart infusion (BHI) broth with

Table1. List of antibiotics used for antimicrobial susceptibility testing

Antibiotics	Potency (µg)
Amikacin (AK)	30
Ampicillin (AMP)	10
Amoxyclav (AMC)	20 /10
cefepime (CPM)	30
Ceftazidime (CAZ)	30
Trimethoprim sulfamethoxazole (COT)	1.25/23.75
Gentamicin (GEN)	10
Imipenem (IMP)	10
Nitrofurantoin (NIT)	300mcg
Norfloxacin (NOR)	10mcg
Piperacillin-tazobactam (PTZ)	100/10
Tetracycline (TE)	30mcg

1% glucose prepared in different dilutions (1:20, 1:40, 1:80, 1:100). Then, 0.2 mL of inoculated broth was loaded into a 96-well flat bottom microtiter plate. Plates were covered and incubated for 24 h at 37°C under aerobic conditions. The contents of the wells were removed after incubation; the wells were washed four times with 0.2 mL phosphate-buffered saline, treated with sodium acetate (2%) for 30 min, and then stained with crystal violet for 1 min. The wells were treated with 0.2 mL ethanol and their optical density was measured at 570 nm wavelength using an enzyme-linked immunosorbent assay (ELISA) plate reader. The test was performed with appropriate controls in duplicates (Fig. 1).

Statistical analysis

Statistical analysis was carried out using InStat software. A chi-square (χ^2) test was performed and a value of $p<0.05$ was considered statistically significant.

RESULTS

A total of 519 urine samples were processed during the study period of 1 year, of which 115 (22.1%) samples showed significant bacterial growth (>10^5 CFU/mL). There were more female patients (n=75; 65.2%) than male patients (n=40; 34.7%). Patients were divided into nine age groups. The incidence of UTI was the highest among women from the 21-30 year age group followed by women from the 31-40 year age group and was the lowest for women 70 years or older. Among men, the incidence of UTI was the highest among those from the 41-50 year age group, followed by men from the 51-60 year age group (Table 2).

Bacteriology of UTI

Among the 115 samples, 101 (87.82%) were detected with gram-negative bacilli and 14 (12.17%) were positive for gram-positive cocci. *E. coli* (n=57; 49.6%) was the most common species isolated during the study, followed by *Klebsiella* spp. (n=23; 20%), *Pseudomonas aeruginosa* (n=08; 7%), *Enterococcus* spp. (n=07; 6.1%), *Enterobacter aerogenes* (n=05; 4.3%), *Staphylococcus aureus (*n=04; 3.5%), *Acinetobacter species* (n=03; 3.5%), CoNS (n=03;3.5%), *Providencia* species (n=03; 3.5%), and *Citrobacter koseri* (n=02; 1.7%) (Fig. 2).

Biofilm formation

Among the 57 isolates tested for *in vitro* biofilm formation ability, 50 were deemed to be biofilm producers. Of them, 08 (14%) strains

Table 2. Age wise distribution among male and female patients

Age group	Females (n 75)	Males (n 40)
0-10	5	3
11-20	4	2
21-30	17	2
31-40	15	6
41-50	6	12
51-60	11	9
61-70	9	4
71-80	4	1
Above 81	4	1

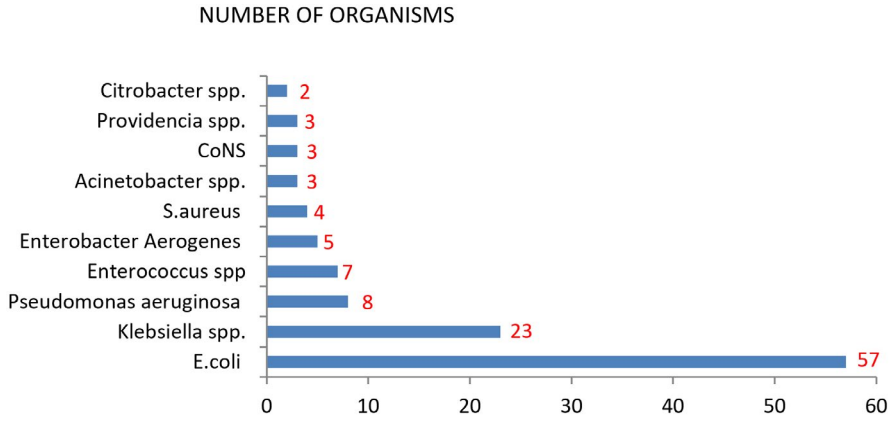

Fig. 2. Showing frequency of isolated organisms

were strong biofilm producers, 42 (73.7%) were moderate biofilm producers, and 07 (12.3%) strains were non-biofilm producers (Table 3).

The overall resistance pattern of UPEC was evaluated and the highest resistance was confirmed toward ampicillin (96.5%) and nitrofurantoin (91.2%), followed by amoxyclav (82.5%). Medium resistance was observed for ceftazidime (73.7%), cefepime (71.9%), tetracycline (71.9%), co-trimoxazole (66.7%), piperacillin/tazobactam (49.1%), and gentamicin (45.6%) and minimum resistance was observed for norfloxacin (17.5%), followed by amikacin (22.8%) and imipenem (33.3%) (Table 4).

Association between antimicrobial resistance and biofilm formation

In comparison with non-biofilm producers, biofilm-producing isolates showed stronger resistance to antibiotics. The highest level of resistance was reported for ampicillin (82%) followed by nitrofurantoin (78%) and amoxyclav (72%), while the least resistance was conferred toward norfloxacin (6%) (Table 5). There was a significant association between resistance to amoxyclav, ceftazidime, cefepime, imipenem, and nitrofurantoin and biofilm formation (p<0.05). *E. coli* isolates resistant to three or more classes of antibiotics were categorized as multidrug-resistant (MDR) strains.

In the present study, among the 50 biofilm producers, approximately 10%, 8%, 32%, 14%, 18%, 12%, and 6%, were resistant to 12, 11, 10, 8, 9, and 7 drugs, respectively. Among seven non-biofilm producers, only one isolate was MDR that showed resistance to 8 of 12 antibiotics. The other six isolates were sensitive to most of antibiotics tested.

Table 3. Biofilm producers and non biofilm producers

Mean OD values	Adherence	Biofilm formation	N=57, (%)
<0.120	None	None/weak	07 (12.3)
0.120-0.240	Moderate	Moderate	42 (73.7)
≥0.240	Strong	High	8 (14)

Table 4. Antibiogram of *E.coli*

Antibiotics	Sensitive n, (%)	Resistant n, (%)
AK	44(77.1)	13(22.8)
AMC	10(17.5)	47(82.4)
AMP	2(3.5)	55(96.4)
CPM	16(28)	41(71.9)
CAZ	15(26.3)	42(73.6)
COT	19(33.3)	38(66.6)
GEN	31(54.3)	26(45.6)
IMP	38(66.6)	19(33.3)
NIT	5(8.7)	52(91.2)
NOR	47(82.4)	10(17.5)
PTZ	29(50.8)	28(49.1)
TE	16(28)	41(71.9)

Table 5. Comparison of antibiotic resistance with biofilm production

Antibiotics	Biofilm formation				P value
	Biofilm Producers (n=50), n(%)		Non biofilm producer (n=7), n(%)		
	Resistant	Sensitive	Resistant	Sensitive	
AK	12 (24)	38 (76)	1 (14)	6 (86)	0.5662
AMC	44 (88)	6 (12)	3 (43)	4 (57)	0.0032
AMP	49 (85)	1(15)	6 (86)	1 (14)	0.0980
CPM	39 (68)	11 (22)	2 (28)	5 (72)	0.0064
CAZ	39 (68)	11 (22)	3 (43)	4 (57)	0.0480
COT	35 (70)	15 (30)	3 (43)	4(57)	0.1536
GEN	23 (46)	27 (54)	3 (43)	4 (57)	0.8768
IMP	19 (38)	31 (62)	0 (0)	7 (100)	0.0457
NIT	47 (94)	3 (6)	5 (72)	2 (28)	0.0482
NOR	9 (18)	41(82)	1 (14)	6 (86)	0.8088
PTZ	26 (52)	24 (48)	2 (28)	5 (72)	0.0612
TE	37 (74)	13 (26)	4 (57)	3 (43)	0.3526

DISCUSSION

UTI is one of the most common health problems affecting millions of people worldwide and is a leading cause of morbidity and high healthcare expenditures in people of all ages[5]. In the present study, the incidence of UTI was higher in female patients than in male patients. Our results are in line with those by Momoh et al.[13] and Ahmed et al,[14]. who reported UTIs in 60·2% and 73% women and 39·8% and 23% men, respectively. The difference in the female:male ratio may be related to different clinical components. Women remain at a much higher risk of UTI (compared to men) owing to shorter urethra, which permits bacterial entry and infection in the bladder**.** In addition, hormonal changes may influence the beneficial bacteria that are responsible for competing with harmful microorganisms in the urinary tract.

In our study, the frequency of UTI was the highest in women from 21 to 30 years of age and men between 41 and 50 years of age, consistent with the results of Santosh John thattil et al[15]. and Fatima S. et al.[16] that reported the highest incidence of UTI in women from 26 to 35 years and men from 46 to 60 years of age.

In our study, the most common isolate was *E. coli* (49.5%), consistent with the observation reported by Kaur et al.[17] (71.7%) and George et al.[18] (69.8%). Thus, the host fecal flora may be a source of *E. coli* that spreads via the perineal, vaginal, and periurethral areas to the lower urinary tract, wherein it is established. Some common ways for the migration of *E. coli* include sexual contact, improper cleaning, holding urine (especially before and after intercourse), and enlarged prostate gland[6].

We investigated the biofilm formation ability of UPEC. Among 57 isolates, 87.7% were positive for biofilm formation *in vitro*, which is in line with the results of Suman et al.[19], Poursina F et al.[20] and Yadav et al.[21], showing 92, 80, and 76% *E. coli* isolates to be biofilm producers, respectively.

The correlation between biofilm production and resistance to amoxyclav, ceftazidime, cefepime, imipenem, and nitrofurantoin was found to be statistically significant ($p<0.05$); no significant correlation was observed ($p>0.05$) with amikacin, ampicillin, tetracycline, cotrimoxazole, piperacillin/tazobactam, gentamicin, and norfloxacin. The antibiotics found to be effective against biofilm-producing *E. coli* isolates were norfloxacin, amikacin, imipenem, and piperacillin/tazobactam. A significant correlation was observed between multidrug resistance and biofilm formation. Approximately 90% biofilm producers were resistant to more than three classes of antibiotics. The results of our study are in agreement with those reported by Deotale et al.[22] and Sevanan et al.[23]. where in biofilm-producing organisms were more resistant to antibiotics than non-biofilm-producing isolates. The correlation between antibiotic resistance and biofilm formation may be associated with multiple factors such as restricted penetration of drugs through the biofilm matrix or longer time needed to penetrate the biofilm than treatment duration. The expression of efflux pumps is considered as a mechanism underlying antimicrobial resistance not only in planktonic cells but also in biofilm structures[21]. It has been demonstrated that biofilm-producing microorganisms can tolerate up to 100-1000 times higher concentrations of antibiotics and disinfectants than planktonic cells, and biofilm-producing isolates showed increased resistance against phagocytosis and other host defense mechanisms[9].

Limitations of the Study

In this study, other virulence factors such as hemagglutination, gelatinase production, and extended-spectrum beta-lactamase Amp C were not evaluated. The biofilm-producing capability of UPEC may differ *in vivo*. Further studies regarding the *in vivo* biofilm-forming capacity of uropathogens are warranted in case of treatment failure.

CONCLUSION

UTI was found to be more common in women than in men. The most common isolate was *E. coli*. Biofilm producers showed higher resistance to antibiotics than non-biofilm producers. Biofilm formation by UPEC may pose a health problem, as these bacteria are difficult to treat and increase the chances of chronic UTI. Norfloxacin, amikacin, imipenem, piperacillin, and tazobactam antimicrobials are particularly effective against biofilm-producing *E. coli*. These antibiotics may be used in the empirical therapy of UTI caused by biofilm-producing UPEC. A

significant correlation was observed between biofilm production and antibiotic resistance in our study. Hence, screening of all isolates of UPEC for biofilm production and studying their antibiogram may help in providing an appropriate antibiotic therapy.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

AUTHOR'S CONTRIBUTION

All the authors substantially contributed to the conception, design, analysis and interpretation of data, checking and approving final version of manuscript.

FUNDING

None

DATA AVAILABILITY

All the datasets analyzed during the study are included in manuscript.

ETHICS STATEMENT

The study was carried out after obtaining approval from institutional ethics committee; Protocol number 2018/091 dated 28.05.2018.

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