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Original Article

IN VITRO STUDY ON ANTIBIOTIC SUSCEPTIBILITY PATTERN OF BIOFILM PRODUCING UROPATHOGENIC *ESCHERICHIA COLI* ISOLATES AND THEIR MOLECULAR CHARACTERIZATION

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ABSTRACT

Objective: Urinary tract infection (UTI) is among the most common infectious diseases of humans in developed countries. Approximately 150 million cases are reported every year. UTI's caused in particular by biofilm producing *Escherichia coli* strains are related to recurrence of infections and the treatment is quite difficult. The present study is undertaken to determine the antibiotic susceptibility pattern of biofilm producing Uropathogenic *Escherichia coli* (UPEC) and molecular characterization by 16S rRNA sequencing.

Methods: The present study comprised of 478 urine samples collected from Raja Muthiah Medical College and Hospital (RMMCH) at Chidambaram, India. All the samples were processed by standard microbiological methods and *E.coli* was confirmed by 16S rRNA analysis. *E. coli* isolates were screened for biofilm formation using Tube Method (TM), Congo Red Agar (CRA) and Tissue Culture Plate method (TCP). Subsequently, the antibiotic susceptibility test was performed using 14 different antibiotics and Minimum Inhibitory Concentration (MIC) and Minimum Biofilm Eradicate Concentration (MBEC) was determined by microtiter broth dilution method was done. Confocal Laser Scanning Microscopy (CLSM) was conducted for biofilm structured analysis.

Results: Out of 478 urine sample processed, 324 (79.80 %) were found to be *E. coli* isolates, with respect to biofilm formation of TCP method classified the isolates as highly positive 40 (12.34%), moderate positive 152 (46.91%), and weakly positive 132 (40.74%). Among the antibiotics tested 56% and 51% of UPEC isolates were sensitive to levofloxacin and imipenem respectively. The MIC values (3-6 µg/ml; 6-25 µg/ml) and MBEC values (24-48 µg/ml; 48-200 µg/ml) were obtained for levofloxacin and norfloxacin against biofilm producing UPEC.

Conclusions: Among all the antibiotics tested, the present result shows imipenem and levofloxacin were found to be very effective against biofilm producing UPEC.

Keywords: *Urinary tract Infection (UTI), Uropathogenic Escherichia coli (UPEC), Biofilm, MIC, MBEC, Antibiotic resistance*

INTRODUCTION

Urinary Tract Infection (UTI) is defined as the presence of multiplying microorganisms in the tract through which urine flows from the kidneys, bladder and urethra to the outside world [1]. *Escherichia coli* are the most frequently isolated microorganism in UTIs causing more than 80% of infections. An extra intestinal pathogenic *E.coli*, UPEC are most etiologic agent that constitutes a major target for antimicrobial therapy [2, 3].

Antimicrobial resistance has been recognized as an emerging worldwide problem. The effect could be severe in heavily populated developing country such as India where there is no strict monitoring program regarding the use of antibiotics. In *Enterobacteriaceae* antimicrobial resistance in *E.coli* is of particular concern because it is the most common Gram negative pathogen causing to UTIs in humans. Antimicrobial drug resistance is on the rise worldwide with regional differences in the frequency of occurrence [4, 5]. Many bacteria are eligible to form of biofilms, which are defined as matrix-enclosed microbial population adherent to each other and to surfaces or interfaces [6]. The microbes have evolved other mechanisms to evade antimicrobial therapy and probably the most important among them is the ability to either form or live within a biofilm [7]. The present study was undertaken to determine the antibiotic susceptibility pattern and biofilm producing Uropathogenic *Escherichia coli* (UPEC).

MATERIALS AND METHODS

Collection of urine samples and demographic profile of UTI patients

Among the UTI (both male and female) suspected cases (age group 0-100) attending the Raja Muthiah Medical College and Hospital (RMMCH) at Chidambaram, 478 urine samples were collected and during the period of November 2012 to August 2013. Along with the samples, demographic profiles (only age and sex) of the patients were also collected. All the *E.coli* isolates were confirmed by standard microbiological methods. As a molecular approach, 16S rRNA sequence was carried out to conform *E.coli* [8, 9, 10].

Antibiotic Susceptibility Assay

All the 324 *E.coli* isolates were subjected to antimicrobial susceptibility test by disc diffusion technique according to Clinical and Laboratory Standards Institute (CLSI) guidelines [11], using commercially available antibiotics (Hi-Media, Mumbai). Antibiotic discs (drug concentration in µg): amikacin (30), ampicillin (10), co-trimoxazole (25), chloramphenicol (30), tetracycline (30), tobramycin (10), gentamicin (10), imipenem (10), norfloxacin (10), piperacillin/ tazobactam (100/10), meropenem (10), nitrofurantoin (300), nalidixic acid (30) and levofloxacin (5). *E.coli* MTCC 443 was used as reference strain.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Biofilm Eradicate Concentration (MBEC)

The minimum inhibitory concentration (MIC) was performed by microtiter broth dilution method CLSI [12], and interpreted using CLSI for levofloxacin, norfloxacin, amoxycillin/clavulanic acid, cefotaxime and ampicillin [12], in the concentration range from 3.125 to 400 µg/ml against 15 strong biofilm producing UPEC. Similarly the MBEC assay was performed by pervious discussed procedures [13, 14].

Detection of biofilm formation

All the 324 *E.coli* isolates were subjected to biofilm production and a numbers of tests are available to identify biofilm producing *E.coli* by methods including Tissue Culture Plate method [15], Tube method [16] and Congo Red Agar method [17]. Using CLSM, the structure of biofilm matrix was studied [18, 19].

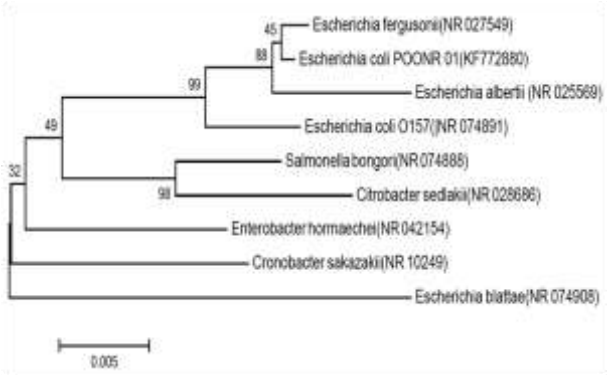

Fig.1: Phylogenetic tree predicted by the neighbor joining method using 16S rRNA gene sequences. The bootstrap considered 1000 replicates. The strain POONR 01 belongs to the *Escherichia coli* cluster. Taxa are represented by type strains with GenBank accession number (KF772880). The scale bar represents the expected number of substitution average to over all the analyzed sites. Number in bracket indicates accession number.

Statistical analysis

One-way ANOVA ($p \leq 0.05$) was performed to compare the significance differences of resistance, intermediate and sensitivity among biofilm and non-biofilm producing uropathogenic *E.coli*.

RESULTS

The demographic profiles of 324 UTI patients are presented in Table 1.Among the patient ratio of female to male were found to be 56:44. The male and female UTI patients are subdivided into in-patient (IP) and out-patient (OP). Higher percentages of *E.coli* were isolated from patients in the age group of 51-60 years, followed by 50% (age group) 0-10 and 42% (11-20 years). When comparing the *E.coli* isolates obtained from OP and IP samples processed for IP (both male and female) recorded highly percentage of *E.coli*. Out of the 478 urine samples 406 (86.93%) sample showed positive and the rest 72 (15.06%) showed negative (Table 2). Among the isolates, 324 (79.80%) were found to be *E.coli* and remaining 82 (20.19%) sample were harboring other microorganisms. (data not shown)

The strong biofilm producing isolates POONR 01 was identified and confirmed both by biochemical test and 16S rRNA analysis. The strain POONR 01 was identified by16S rRNA analysis, suggested that the strain belongs to *E.coli* cluster with phylogenetic tree is shown in (Figure 1).The POONR 01 nucleotide sequence was deposited in GenBank National Centre for Biotechnology Information (NCBI) under accession number (KF772880).

All the 324 *E.coli* isolates were subjected to antibiotic sensitivity tests. The resistant pattern of amikacin, nalidixic acid, ampicillin, co-trimoxazole, tetracycline, gentamicin, tobramycin, chloramphenicol, norfloxacin, nitrofurantoin, piperacillin/tazobactam and meropenem were found to be in the order of 55, 49, 48, 47, 47, 46, 45, 44, 39, 38, 33 and 32%, respectively. The intermediate resistance imipenem were observed for (42%) and high sensitivity was observed for levofloxacin (56%). The results are shown in Figure 2.

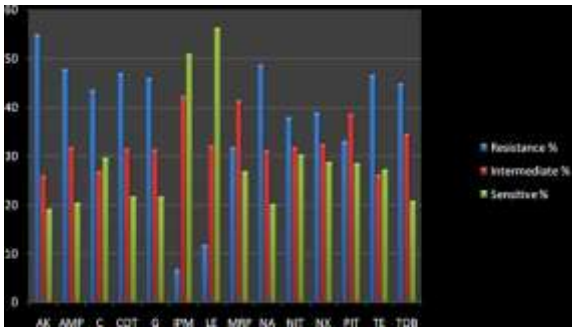

Fig.2: Antibiotic susceptibility patterns

AK: Amikacin, AMP: Ampicillin, C: Chloramphenicol, COT: Co – trimoxazole, G: Gentamicin, IPM: Imipenem, LE: Levofloxacin, MRP: Meropenam, NA: Nalidixic acid, NIT: Nitrofurantoin, NX: Norfloxacin, PIT: Piperacillin/Tazobactam, TE: Tetracycline, TOB: Tobramycin.

Fifteen strong biofilm producing UPEC *E.coli* isolates were subjected to MIC and MBEC, we followed that, levofloxacin was most effective against all isolates, with a MIC and MBEC values of about 3-6 and 24-48 µg/ ml, respectively. Norfloxacin was effective with a MIC and MBEC values of about 6-25 and 48-200 µg/ml, also ampicillin showed the MIC value to be > 400 µg/ ml. In MIC and MBEC amoxycillin/clavulanic acid was found to be less effective against biofilm producing UPEC (Table 3).

Table 1: Distribution of Urinary tract infected patient by age and sex vice (n =324)

Microorganism	Age (Year)	Male = 142 (44%)		Female = 182 (56%)		Total
		IP 108 (76.05%)	OP 34 (23.94%)	IP 96 (52.74%)	OP 86 (47.25%)	324
E.coli	0 – 10	24	9	13	4	50
	11 – 20	17	2	14	9	42
	21 – 30	12	4	15	10	41
	31 – 40	10	1	10	16	37
	41 – 50	5	4	14	17	40
	51 – 60	15	5	23	15	58
	61 – 70	11	3	4	8	26
	71 – 80	11	6	2	5	24
	81 – 90	1	0	1	2	4
	91 – 100	2	0	0	0	2

IP: In Patients, OP: Out Patients

Table 2: The overall status of urine specimens processed

Urine samples collected and processed	
No. of urine specimens processed	478
No. of positive specimen	406 (84.93%)
No. of negative specimen	72 (15.06%)
No. of *E.coli* specimen	324 (79.80%)
No. of other organism	82 (20.19%)

Table 3: Determination of Minimum Inhibitory Concentration (MIC) and Minimum Biofilm Eradication Concentration (MBEC)

Name of Drug	MIC (µg/ml)	MBEC (µg/ml)
Levofloxacin	3 - 6	24 - 28
Norfloxacin	6 - 25	48 - 200
Amoxycillin/Clavulanic acid	25 - 200	200 - 1600
Cefotaxime	12 - 25	96 - 200
Ampicillin	> 400	> 3200

Table 4: The demographic profile of biofilm and antibiotic resistance pattern

			Antibiotic resistance												
Patients 79.80%	**Type**	**Biofilm 12.34%**	**AK %**	**AMP %**	**C %**	**COT %**	**G %**	**IPM %**	**LE %**	**MRP %**	**NA %**	**NIT %**	**NX %**	**PIT %**	**TE %**	**TOB %**
Male	IP	18(16%)	67	47	47	28	39	6	28	56	34	56	39	50	34	47
44%	OP	1(3%)	1	0	1	0	0	0	0	0	1	0	0	1	0	1
Female	IP	9(9.37%)	67	47	55	34	34	0	22	34	44	34	44	11	55	44
56%	OP	12(14%)	50	50	33	42	42	17	8	33	42	50	33	33	58	58

AK: Amikacin, AMP: Ampicillin, C: Chloramphenicol, COT: Co - trimoxazole, G: Gentamicin, IPM: Imipenem, LE: Levofloxacin, MRP: Meropenam, NA: Nalidixic acid, NIT: Nitrofurantoin, NX: Norfloxacin, PIT: Piperacillin/Tazobactam, TE: Tetracycline, TOB: Tobramycin.

Table 5: Antibiotic susceptibility result of the biofilm and non biofilm producing uropathogenic *E. coli* by TCP method

Antibiotics	**Biofilm producer (192) 59 %**			**Non biofilm producer (132) 41 %**			**p value**
	R %	**I %**	**S %**	**R %**	**I %**	**S %**	
Amikacin	57	16	27	48	26	26	**p ≤ 0.05**
Ampicillin	52	23	25	42	27	31	
Chloramphenicol	49	17	34	36	30	34	
Co – Trimoxazole	49	21	30	45	19	36	
Gentamicin	48	22	30	45	21	34	
Imipenem	09	41	50	04	44	52	
Levofloxacin	13	41	46	07	43	50	
Meropenam	36	18	46	29	34	37	
Nalidixic acid	50	20	30	44	23	33	
Nitrofurantoin	36	26	38	29	34	37	
Norfloxacin	41	21	38	36	30	34	
Piperacillin/Tazobactam	40	23	37	37	22	41	
Tetracycline	46	19	35	47	25	28	
Tobramycin	54	16	30	43	20	37	

R = Resistance, I = Intermediate, S = Sensitive

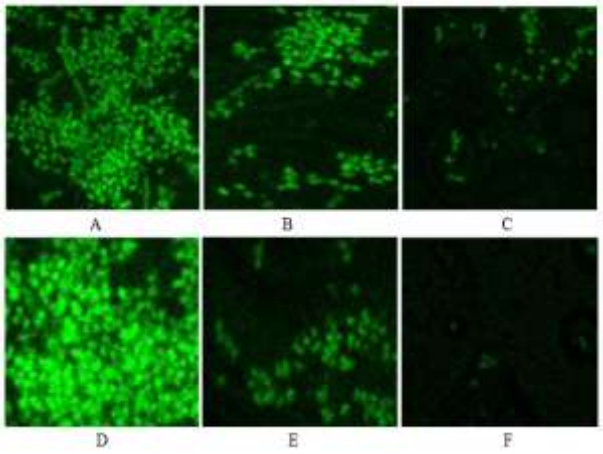

Fig. 3: The confirmation of biofilm formation on glass slide surfaces by acridine orange staining and confocal laser scanning microscopy of biofilm producing strain *Escherichia coli* POONR 01. Confocal laser scanning microscopy image: A, D: Strong positive, B, E: Moderate positive, C, F: Non/Weak Positive.

Among 324 *E. coli* isolates subjected to biofilm production, 66(20.37%) strains showed strong positive, 73 strains (22.53%) showed moderate positive, 107 strains (33.02%) showed weakly positive and 78 strains (24.07%) showed negative in tube method. Similarly, in Congo Red Agar method (CRA), 72 strains (22.22%) showed highly positive, 112 strains (34.56%) showed moderate positive and 140 strains (43.20%) were weakly positive, whereas in Tissue Culture Plate Method (TCP), 40 (12.34%) strains showed highly positive, 152 strains (46.91%) showed moderate positive and 132 strains (40.74%) showed weakly positive.

For the biofilm structured analysis of UPEC study employed of *E.coli* isolates. (Each two weak, moderate and strong). The biofilm structure observed through CLSM with correlates well with TCP method (Figure 3).

Comparing within the *E.coli* isolates 79.80% were obtained from male 44% and female56% respectively. The patients profile was comparing with biofilm producing *E.coli* isolates based on TCP method 12.34%. In antibiotic resistance profile highly resistances male IP 16% were found to amikacin 67% and meropenam 56%, the highest susceptibility were found to be imipenem 6%, levofloxacin and chloramphenicol 28% respectively. About 3% male OP was highly resistance to biofilm producing *E.coli* isolates. Among the female IP 9.37% highest resistance to amikacin 67% and chloramphenicol 55% respectively, lowest was piperacillin/tazobactam 11%, OP 14% was highest resistance to tetracycline and tobramycin 58%, lowest levofloxacin 8% of resistance. The results were showed Table 4.

Antibiotic susceptibility result of the biofilm and non biofilm producing uropathogenic *E.coli* is given in (Table 5). Among 324 *E.coli* isolates 192 and 132.The biofilm formation strains showed high percentage of 59% and non biofilm results were found in 41%. All biofilm producing strains showed maximum resistance to amikacin 57% followed by tobramycin, ampicillin with 54% and 52%, respectively. The biofilm producing strains showed intermediate to levofloxacin, nitrofurantoin 41% and 26%. Along with the tested 50% sensitivity was observed only imipenem. The non biofilms were high resistant value was amikacin and

tetracycline 48% and 47%. Non biofilms were showed high intermediate and sensitive value to imipenem 44% and 50% respectively.

Furthermore, One-way ANOVA analysis indicated that the difference in the biofilm and non-biofilm which was observed among the 324 isolates against the 14 different antibiotics which were tested was statistically significant ($p < 0.05$).

DISCUSSION

In spite of the availability and use of the antibiotic drugs and community bacterial infection of the urinary tract is one of the common causes for seeking medical attention. In the present study, the total number of *E.coli* isolated from in-patients was 62.96% and it is compared to 37.03% from out-patients. *E.coli* was isolated in higher proportion in-patients 83.3% than in out-patients (16.7%) [20]. Similar results were obtained using 125 *E.coli* strains with 75 (60%) inpatients and 50 (40%) outpatients [21].

The total number of male samples was 142 (43.82%), which included 108 in-patients and 34 outpatients. The total number of female samples was 182 (56.17%), which included 96 in-patients and 86 out-patients. In previous studies, the clinical sample size was similar with female 227 (60%) and male 151 (40%) [22]. Therefore, the infection male and female UPEC were commonly observed in all age groups and high percentage *E.coli* was observed in the age group of 15-50 years [22-24]. Where us in the present study, *E. coli* was isolated frequently in the age group between 21 -60 years then similarly it is confirmed that UPEC is commonly observed in all age groups which was consistent with previous studies.

The prevalence of *E.coli* in UTI was found to be double when compared to the previous reports. Various organisms have been reported to be isolated from patients with UTI, among which *E.coli* is the most common [25-27]. Even in the present study, out of 406 uropathogens, 324 (79.80%) isolates of *E.coli* were obtained and confirmed as the most common organism. We conclude that *E.coli* is the major etiological agent in causing UTI, which accounts for up to 90% of cases [28]. This is the highest values compared to previous studies, which reported *E.coli* isolates from urine samples as 68.5% [29], 71% [30] and 24.4% [31]. Furthermore, the results of the current study show that the isolates are predominantly gram negative bacteria *E.coli*.

The antibiotics susceptibility test related the repetition found to be 54% *E.coli* was sensitive to gentamicin, followed by tobramycin (50%), co-trimoxazole (44%) and ciprofloxacin (44%) [32]. On the other hand, the present study explains that the uropathogenic *E.coli* are less susceptible to chloramphenicol (29.62%) and nitrofurantoin (30.24%), respectively. Levofloxacin and imipenem are highly sensitive to UTI. In a previous study, *E.coli* were found to be highly reported to 53% for augmentin, 44% to amoxicillin, 49% to norfloxacin, 46% to nalidixic acid, and 41% to ciprofloxacin, respectively [33]. In the present study, the high resistance rates of uropathogenic *E.coli* is found as follows: amikacin, nalidixic acid, ampicillin and co-trimoxazole, 55, 49, 48, and 47%, respectively. The resistance rate found to be lower than the values obtained in amikacin (90%), piperacillin/tazobactam (89%), co-trimoxazole (88%), amoxyclv (86%), norfloxacin (73%), ampicillin (71%), erythromycin (64%), tobramycin (58%), tetracycline (56%), and gentamicin (54%) respectively [29].

In the present study we have used five different antibiotics and the best two drugs were levofloxacin with a MIC of 3-6 µg/ml, MBEC of 24-48 µg/ml and for norfloxacin it was observed as MIC at 6-25 µg/ml MBEC at 48-200 µg/ml respectively. Earlier studies have reported that antibiotic concentration of CSE 1034 MIC-32-34 µg/ml and MBEC-256-512 µg/ml fully eradicating ESBL producing *E.coli* [34]. Similarly another author has reported that ciprofloxacin MIC-1.0 µg/ml; MBEC- 950 µg/ml and nitrofurantoin MIC-7.2 µg/ml; MBEC- 120 µg/ml eradicating of biofilm producing uropathogenic *E.coli* [35].

Murugan *et al*, [30] have studied that, multidrug combination of biofilm producing uropathogenic *E.coli* was highly resistant combination of ampicillin, norfloxacin and tobramycin 50.25%. Similarly in the present study is highly antibiotic resistance 67% of amikacin. Additionally combination with patients profile and biofilm producing uropathogenic *E.coli* also studied.

Additionally, among 72 *E.coli* strains were reported to display a biofilm positive phenotype under optimized conditions in the tube method [36]. Similarly, in another report 81 *E.coli* strains displayed a biofilm-positive phenotype under optimized conditions in the tube method [33]. In the present study 66 *E.coli* strains are biofilm strong positive in tube method. In previous studies, all biofilm forming strains were reported to show the maximum resistance to amoxyclav (100%), followed by chloramphenicol (100%), gentamicin and cephotaxime (86%), ceftazidime (84%), co-trimoxazole (83%), and amikacin (70%) [34]. similarly, the high prevalence (63%) of biofilm formation among strains from patients with prostatitis was also reported [37].

These results suggest that UPEC employ their biofilm-forming abilities to invade and successfully occupy tissues in urogenital tract. Majority of clinical isolates of *E.coli*, which were isolated from urine samples showed strong positive result for the biofilm formation. The present study concludes that the increasing trend of antimicrobial resistance and the knowledge of local antimicrobial susceptibility patterns of common uropathogens are essential for prudent empiric therapy of community acquired UTIs. *E.coli* is still the most common uropathogenic bacteria having UTI in both male and female surrounding in hospital settings. In this report we note the very high resistance rate in UTI infected patients of both genders.

The levofloxacin and imipenem were found be very effective to UTI, so these two antibiotics are recommended for both in-patients and out-patients in the hospital setting. Other antibiotics showed very low sensitivity when compared to these two antibiotics. Biofilm formation is closely related with the resistance of *E.coli* towards the antimicrobial drugs and also it increases the chronicity of UTI. Therefore, the UTI caused by biofilm producing *E.coli*, may promote the colonization and increased the incidence rate of UTI's. Collectively, this study magnifies the view of biofilm forming UTI and multi drug resistance and will provide guidance of using different kind of antibiotics.

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