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RESEARCH ARTICLE

IN VITRO EVALUATION OF ANTIBIOTIC SUSCEPTIBILITY PATTERN OF EXTENTED SPECTRUM B LACTAMASE (ESBL) PRODUCING UROPATHOGENIC ESCHERICHIA COLI (UPEC) AND THEIR CORRELATION WITH BIOFILM FORMATION

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ABSTRACT

Extended Spectrum - Lactamase (ESBL) producing *Escherichia coli* strains is reported to be the cause of community and hospital acquired infections. *E. coli* responsible for urinary tract infection (UTI) have capability to produce ESBLs in huge amounts. Bio film are group of microorganisms encased in an exopolymer coat. The present study was undertaken over a period of one year from November 2012 to August 2013 to study the prevalence of ESBL and bio film producer *E. coli* among UTI patients attending the Raja Muthiah Medical College and Hospital (RMMCH), Annamalai University at Chidambaram, South India. Three hundred and twenty four (79.80%) isolates of *E. coli* were obtained from 478 urine samples and thereafter were subjected to susceptibility testing according to the Clinical and Laboratory Standard Institute (CLSI) guidelines using 14 different antibiotics. All the samples were confirmed by standard microbiological methods. Additionally molecular characterization of 16S rRNA sequence was carried out. They were further screened for ESBL production by double disc approximation test (62%) and CLSI combination disk method (38%) respectively. Of the 324 isolates of *E. coli*, (38%) were found to be ESBL producer. *E. coli* exhibited 57 and 53% susceptibility to levofloxacin and imipenem respectively. The resistant to amikacin, nitrofurantoin tetracycline, nalidixic acid and chloramphenicol were observed among ESBL producers. The antibiotic resistance patterns of *E. coli* and the correlation between ESBL and biofilm producing *E. coli* was also determined. There is a need for continued surveillance of antimicrobial resistance among ESBL and biofilm producing *E.coli* and concluded the resistance may be realted to β-lactamase production of the biofilm bacteria.

INTRODUCTION

Escherichia coli, the most prevalent facultative gram-negative ba¬cillus in the human faecal flora, usually inhabits the colon as an innocuous commensal (Eisenstein *et al*., 1987). Urinary tract infections (UTIs) are the most common form of the extra-intestinal Escherichia coli infec¬tions and Escherichia coli is the most common cause of UTIs. At some point of their lives, at least 12% men and 10-20% women experience an acute symptomatic UTI and even greater numbers develop asymptomatic bacteriuria (Johnson and Stamm, 1989; Anthony and Edward, 2002; Johnson *et al*., 2003).

Antimicrobial resistance is a growing threat worldwide. Resistance mechanisms have been found for every class of antibiotic agents. The predominant mechanism for resistance to the β-lactam antibiotics in gram-negative bacteria is the production of β-lactamase. The production of extended-spectrum β-lactamases (ESBLs) is an important mechanism which is responsible for the resistance to the third-generation cephalosporins. During the last 2 decades, ESBL producing gram-negative bacilli have emerged as a major problem in many settings (Paterson *et al*., 2003). The ESBLs mediate resistance to broad-spectrum cephalosporins (e.g., ceftazidime, ceftriaxone and cefotaxime) and aztreonam. The genes for the ESBL enzymes are plasmid borne and have evolved from point mutations, thus altering the configuration of the active site of the original and long known β-lactamases (Bradford, 2001).

There is a paucity of data on the molecular analysis, prevalence of ESBL and biofilm producer E.coli among UTI patients in India. The present study was undertaken to detect the 16S rRNA partial gene sequence, ESBL and biofilm producing gram negative bacilli in UTI patients and also determine their antibiogram profile, correlation between ESBL and Biofilm formation also studied, which assumes a great significance.

MATERIALS AND METHODS

Collection of urine sample

The 478 urine samples were collected from patients attending the RMMCH at Chidambaram and the specimens received during November 2012 to August 2013 were included in the analysis. Specimens were obtained using aseptic techniques to

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avoid contamination and were promptly transported to the laboratory in a sterile container in an ice-cold condition. All the E.coli isolates were confirmed by standard microbiological methods. As a molecular characterization of 16S rRNA sequence was carried out to conformed E.coli (Collee *et al*., 1996; Relman, 1993; Tamura, 2011).

Antibiotic susceptibility assay

The above isolates were tested for antimicrobial susceptibility test by disc diffusion technique according to CLSI guidelines (CLSI, 2006), with commercially available discs (Hi-Media, Mumbai). The following antibiotic discs (drug concentration in µg) were used: 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).
Detection of biofilm formation

All the 324 E.coli strains were subjected to biofilm production and a numbers of tests are available to identify biofilm producing UPEC by methods including Tissue Culture Plate method (Christensen *et al*., 1985), Tube method (Christensen *et al*., 1982) and Congo Red Agar method (Freeman *et al*., 1989).

Test for ESBL production

All the 324 E.coli strains were subjected to ESBL production and a numbers of tests are available to identify ESBL producing UPEC by methods including Double-disk approximation test (Jarlier *et al*., 1988),The combination-disk test using both cefotaxime and ceftazidime, alone and in combination with clavulanic acid, was performed for the detection of ESBL according to the CLSI guidelines (CLSI, 2010).

Quality control

Escherichia coli MTCC 443 was used for the quality control of the Kirby-Bauer disk diffusion method. Escherichia coli MTCC 729 were used for the quality control of the ESBL detection methods.

Statistical analysis

The statistical analysis was done by using the magnitude of ESBL and non-ESBL (Sensitive, Resistant and Intermediates) testing of all bacterial isolates. Descriptive statistics like the one-way ANOVA was performed to check the significant difference among the both groups. A difference was considered to be significant if the probability that chance would explain the results were reduced to less than 5% ($P \leq 0.05$).

RESULTS

A total of the 478 urine specimens of urinary tract infection processed 406 (84.93%) specimens showed culture positive and the rest 72 (15.06%) were negative. Among the isolates, aerobic gram negative UPEC was 324 (79.80%) and other organisms were 82 (20.19%).

The highly ESBL producing strain POONR 02 was identified by biochemical assay and 16S rRNA analysis also. POONR 02 was identified by 16S rRNA analysis, suggested that the strain belongs to E.coli cluster Fig.1a. The POONR 02 nucleotide sequence was deposited in GenBank, (NCBI) under accession number KF 963260. The strain POONR 02 shares 99% nucleotide similitude with E.coli. The PCR image result showed Fig 1b.

ESBL producing Enterobacteriaceae forms an immense need for testing several methods that appropriately identifies the enzymes in urine samples. The detection of ESBL strains is of imperative significance as they are responsible for the spread of resistant genes in hospitals settings. From 324 E.coli isolates, we detected 63% and 37% of ESBL producer by double disk approximation test and CLSI followed to detect ESBL's in isolate of E.coli.

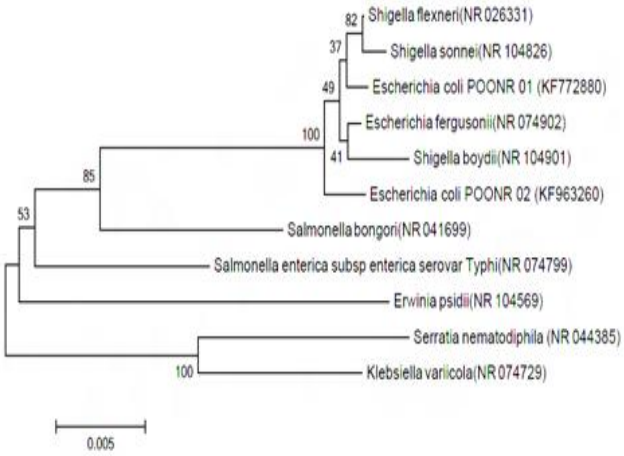

Fig 1 a Phylogenetic tree

Phylogenetic tree predicted by the neighbor joining method using 16S rRNA gene sequences. The bootstrap considered 1000 replicates. The strain POONR 02 belongs to the *Escherichia coli* cluster. Taxa are represented by type strains with GenBank accession number (KF963260). The scale bar represents the expected number of substitution average to over all the analyzed sites. Number in bracket indicates accession r

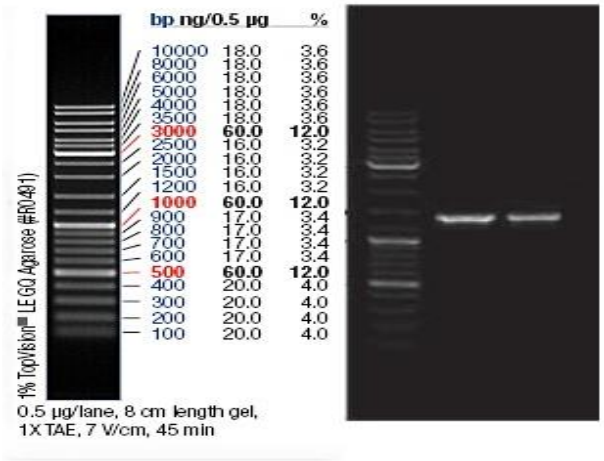

Fig 1b16S rRNA gene sequences for PCR image

The antibiotic susceptibility patterns in ESBL producer isolated from UTI infected patients were found to be as follows: the high percentage of susceptibility to levofloxacin (57%), imipenem (53%) and piperacillin/tazobactam (29%) were observed among ESBL producers. ESBL and non ESBL producing *E.coli* intermediate values were 39% vs 41% for imipenem. The results were showed in Table 1.

Table 1 Antibiotic Susceptibility results of the ESBL and non ESBL producing uropathogenic *E.coli*

Antibiotics	ESBL producer (38%)			Non ESBL producer (62%)		
	R %	I %	S %	R %	I %	S %
Amikacin	56	29	15	40	25	35
Ampicillin	49	24	27	44	33	23
Chloramphenicol	45	27	28	42	26	32
Co – Trimoxazole	49	32	19	46	30	24
Gentamicin	47	34	19	45	31	24
Imipenem	08	39	53	06	41	53
Levofloxacin	17	31	57	11	39	50
Meropenam	42	41	17	30	26	44
Nalidixic acid	49	31	20	48	32	20
Nitrofurantoin	53	42	05	38	30	32
Norfloxacin	37	40	23	35	30	35
Piperacillin/Tazobactam	35	36	29	35	38	30
Tetracycline	50	24	26	43	28	29
Tobramycin	43	45	20	42	30	28

R= Resistance, I=Intermediate, S= Sensitive

The antibiotic resistance patterns of the ESBL producing *E.coli* isolates were showed maximum resistance to nitrofurantoin (53%), tetracycline (50%), co trimoxazole (49%) and gentamicin (47%). Both ESBL producing and non - ESBL producing *E.coli* were resistant to other four antibiotics such as amikacin (56% Vs 40%), ampicillin (49% Vs 44%), chloramphenicol (45% Vs 42%) and gentamicin (47% Vs 45%) was comparatively higher among ESBL producer than non ESBL producer. Resistance among ESBL producer to nalidixic acid was also high (49% Vs 48%) when compared with non-ESBL producers. 57% sensitive was noticed only for levofloxacin. In the present study, markedly moderate resistances to amino glycosides were observed among clinical isolates of *E.coli*. Table 2

Table 2 Antibiotic resistance percentage of ESBL producing uropathogenic *E.coli*

Antibiotics	ESBL producer (%)	Non-ESBL producer (%)	Resistance (%)
Amikacin	56	40	55
Ampicillin	49	44	48
Chloramphenicol	45	45	44
Co – Trimoxazole	49	46	47
Gentamicin	47	42	46
Imipenem	08	06	07
Levofloxacin	17	11	12
Meropenam	42	30	32
Nalidixic acid	49	48	49
Nitrofurantoin	53	38	38
Norfloxacin	37	35	39
Piperacillin/Tazobactam	35	32	33
Tetracycline	50	43	47
Tobramycin	43	42	45

One hundred twenty two isolates of *E.coli* producing ESBL and two hundred two non- ESBL producing isolates were compared for their ability to form biofilm. Striking difference was observed among ESBL positive and ESBL negative isolates with regard to the biofilm formation ability based on the TCP method results. The isolates were classified according to three groups as follows: 12.29%, 46.72%, and 40.98%. Grouping the biofilm formation to strong, moderate, and weak showed that lower number of isolates with ESBL positive group had weak reaction (40.98%), in comparison with the ESBL negative group (43.56%) and the difference in the biofilm formation in all three group was significant. When comparing the potential of ESBL and non ESBL producer among UPEC with regard to the production of biofilm formation observed by CLSM. We followed that 12.29% Vs 09.90% and ESBL and non ESBL producing UPEC exhibited moderate biofilm formations. This shows that the ESBL producer have greater ability is producing biofilm among UPEC their by increase the antibiotic resistance. Table 3

Table 3 Biofilm formations among ESBL and non-ESBL producing uropathogenic *E. coli* isolates

E. coli (n=324)	Biofilm formation		
	Strong	Moderate	Weak/Non
ESBL (n=122)	15 (12.29%)	57 (46.72%)	50 (40.98%)
Non ESBL (n=202)	20 (09.90%)	94 (46.53%)	88 (43.56%)

The antibiotic resistances of 324 *E. coli* isolates were studied under multidrug combination between biofilm and ESBL-producing strains. The biofilm-producing *E. coli* strains were selected using the TCP method within highly biofilm producer, weakly producer (but not including moderate positive) and ESBL positive, ESBL negative. The *E. coli* strains were classified into four different categories: B+, E+ (n= 15), B+, E- (n=25), B-, E+ (n=50) and B-, E- (n=82). The resistance values in percentage are shown in Table 4. Uropathogenic *E. coli* had high antibiotic resistance of B+, E+ for co-trimoxazole (67%) followed by nalidixic acid and ampicillin 60%. The resistance values of B+, E- for tobramycin (64%) and norfloxacin (60%) were also high. Similarly, B-, E- had high resistance values for piperacillin/tazobactam (61%) and norfloxacin (60%).Very high values of resistance was found in the case of B-, E+ for Gentamicin (78%) and Chloramphenicol (72%).The resistance for imipenem was the least in all cases: 3% (B-,E-), 7% (B+,E+), 8% (B-, E+), and 12% (B+,E-). Similarly levofloxacin was found to have also low resistance values ranging from 2% (B-, E+) to 20% (B+, E+).

B+ - Biofilm positive, E+ - ESBL positive, B- - Biofilm negative, E- - ESBL negative

Comparing within the *E.coli* isolates 79.80% were obtained from male 44% and female56% respectively. The patients profile was comparing with ESBL producing *E.coli* isolates based on combination-disk test 38%. In antibiotic resistance profile highly resistances male IP 36% were found to amikacin 59% and chloramphenicol 51%, the highest susceptibility were found to be levofloxacin 08%, imipenem 13% and nitrofurantoin 38% respectively. About 41% male OP was highly resistance to ESBL producing *E.coli* isolates. Among the female IP 27% highest resistance to ampicillin 73% and tetracycline 69% respectively, lowest was levofloxacin 07%, OP 50% was highest resistance to amikacin 51% and co-

trimoxazole 49%, lowest imipenem 04% of resistance (Table 5).

Table 4 Resistance values for antibiotics with correlation between biofilm and ESBL-producing UPEC based on TCP method

Antibiotics	B+, E+ (n=15)	B+, E- (n=25)	B-, E+ (n=50)	B-, E- (n=82)
	Resistance %	Resistance %	Resistance %	Resistance %
Amikacin	47	48	58	58
Ampicillin	60	56	64	53
Chloramphenicol	54	44	72	46
Co – Trimoxazole	67	36	68	43
Gentamicin	54	52	78	47
Imipenem	07	12	08	03
Levofloxacin	20	08	02	04
Meropenam	40	48	56	53
Nalidixic acid	60	56	60	49
Nitrofurantoin	47	52	66	57
Norfloxacin	54	60	58	60
Piperacillin/Tazobactam	34	44	64	61
Tetracycline	40	56	70	58
Tobramycin	47	64	66	55

Table 5 The demographic profile of ESBL and antibiotic resistance

Patients 79.80%	Type	ESBL 38%	Antibiotic resistance													
			AK %	AMP %	C %	COT %	G %	IPM %	LE %	MRP %	NA %	NIT %	NX %	PIT %	TE %	TOB %
Male	IP	39(36%)	59	46	51	44	49	13	08	26	44	38	41`	51	41	49
44%	OP	14(41%)	43	50	50	36	50	0	07	17	50	29	29	36	57	43
Female	IP	26(27%)	62	73	58	85	65	15	07	23	50	42	50	65	69	54
56%	OP	43(50%)	51	47	40	49	42	04	09	26	37	33	39	44	37	47

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.

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

DISCUSSION

In the community, bacterial infection of the urinary tract is one of the common causes for seeking medical attention. UPEC observed in all age groups (Den Heijer *et al*., 2010). Increase in the spread of ESBL-producers is noticeably rapid globally, indicating the need in continuous monitoring systems and effective infection control measures (Metri *et al*., 2011).

Previous studies have reported the incidence of 71%, 68.5%, and 36% UPEC isolated from urine sample (Murugan *et al*., 2011; Poovendran *et al*., 2012; Madira *et al*., 2013). Since, 58% ESBL producing *E.coli* isolated from urine sample (Jigna and Pratibha, 2012). Even the current study 79.80% isolated of UPEC from urine sample.

Agrawal *et al* (2008); Metri *et al* (2011); Jigna and Pratibha (2012) have reported that, the prevalence of ESBL producer to be 22, 32.1 and 66%, respectively. Other studies from India have reported the ESBL production varying from 6 to 87% (Mathur *et al*., 2002; Machanda *et al*., 2005; Tankhiwale *et al*., 2004). In recent years increase in ESBL production was reported from several countries such as USA, Canada, China and Italy (Saurina *et al*., 2000; Cordro *et al*., 2004; Xiong *et al*., 2002; Luzzaro *et al*., 2006). Similarly, in a large survey of 1610 *E. coli* isolates from 31 centers, 10 European countries found that the prevalence of ESBL in these organism range from as low as 1.5% in Germany to high as 39-47% Russia, Poland and Turkey (Goosens, 2001). In the Arabian Gulf region, high ESBL production is 31.7% in Kuwait and 41% in the United Arab Emirates (Mokaddas *et al*., 2008; Al-Zorounie *et al*., 2008). Similarly, Husam *et al* (2009) have reported that prevalence the ESBL production is 60% in Saudi Arabia. Poovendran *et al* (2013)]; Babypadmini *et al* (2004); reported that in Coimbatore (South India) ESBL production is 34 and 41% in *E. coli*. Even in the present study 38% ESBL producing *E.coli* were isolated.

In the present study all the uropathogenic isolates of ESBL and non ESBL producers were found to be 53 and 57% sensitive to imipenem and levofloxacin respectively. ESBL and non ESBL producing UPEC were sensitive which is comparatively lower than our present study findings. *E.coli* was recorded to be sensitive to imipenem (96.8%) suggested by [38]. Similarly another author reported to be highly sensitive to imipenem (100%) which is the drug of choice against UTI infections. Additionally they have studied amikacin resistance rate was found to be 95% as compared to tobramycin resistant rate which was 67% respectively (Babypadmini *et al*., 2004). Ramesh *et al* (2008) reported the resistance rate of amikacin and tobramycin to be 59.5% and 81.31% respectively. Neelam *et al* (2008) reported ESBL producing *E. coli* with a high degree resistance to piperacillin/tazobactam and amoxyclav to be 93.1%, 93.4% as compared to non ESBL producers piperacillin/tazobactam and imipenem to be 31.06% and 11% respectively. Even in the present data is ESBL and non ESBL producing urinary strains highly resistance to ampicillin 49% Vs 44% and nitrofurantoin 53% Vs 38%.

In the current study is correlation between ESBL and biofilm formation strain POONR 02. Earlier studies have reported that correlation between biofilm producing isolates and multidrug resistance isolates of UPEC (Murugan *et al*., 2011). Similarly another author studied correlation between ESBL and biofilm production (Babypadmini *et al*., 2004).

Murugan *et al* (2011) 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 was highly antibiotic resistance 67% of amikacin. Additionally combination with patients profile and biofilm producing uropathogenic *E.coli* also studied.

In this study, we found a significant difference between the biofilm and ESBL producing *E.coli* from clinical isolates in comparison with non ESBL producing isolates and correlation between biofilm and ESBL also studied. We found that ESBL producing isolates had ability to form of biofilm formation. The use of broad spectrum antibiotics such as 1, 2, 3 and 4 generation cephalosporin's, β lactams combined with β lactamase inhibitors and hospital settings some of risk factors for the infection caused by ESBL producing enterobacteriaceae. In the end, it has been felt that there is a need to formulate strategies to detect and prevent the emergence of ESBL producing strains for the effective treatment of infection.

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