 DOI: 10.18231/2455-6807.2017.0021

Biofilm formation and Extended Spectrum Beta Lactamases (ESBL) producers among the gram negative bacteria causing Urinary tract infections

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Abstract

Emergence of antibiotic resistance and biofilm formation among the uropathogens is of serious concern due to recurrence and chronicity of infections.

The present study aims to detect the biofilm formation assay and antibiotic susceptibility profile among the gram negative isolates causing urinary tract infections.

Materials and Method: In this study, 148 urine specimens from patients with clinical history of urinary tract infection were processed and each significant isolate was identified as per standard techniques. Antibiotic susceptibility test was done by disc diffusion method.

Extended spectrum beta lactamase producers were detected by double disc method. Biofilm formation was detected by Microtitre plate method for the gram negative isolates.

Results: Out of 148 urine specimens, 113(76.35%) were culture positive. *Escherichia coli* was the predominant bacteria 34(36.1%). Overall highest susceptibility was observed for cotrimoxazole, nitrofurantoin and amikacin and 38 isolates were confirmed to be ESBL producers and 14(36.84%) of them showed biofilm formation. The Association of drug resistance (ESBL producers) and Biofilm formation was found to be statistically significant. (*p=0.023*)

Conclusion: Biofilm producing Uropathogens are of significant concern as they can cause chronic relapses and dissemination of drug resistant isolates. Hence it is necessary to detect the biofilm forming bacteria, conduct surveillance of the antibiotic susceptibility and device effective infection control measures.

Keywords: Uropathogens, ESBL, Biofilm formation, Drug resistance

Introduction

Urinary tract infections (UTI) is one of the commonly encountered clinical condition associated with significant morbidity if not treated appropriately. Studies have reported the prevalence of UTI as 3.14% to 19.87% across India.(1) Drug resistance to commonly used antibiotics among the uropathogens is on the raise.(2) Biofilms are a complex aggregation of bacteria with unique properties which facilitate them to evade the host immune response and penetration by antimicrobial agents.(3,4) Emergence of antibiotic resistance and biofilm formation among the bacterial pathogens implicated in causing urinary tract infection is of serious concern due to the high recurrence rate and chronicity of infections.(5,6) Multidrug resistance and spread of antibiotic resistance are higher among biofilm producers.(7) Biofilm producing pathogenic bacteria with high levels of resistance may make treatment options difficult. The present study aims at isolating and identifying the bacteria causing UTI, detect the biofilm producers and beta lactamase production among the gram negative bacterial isolates and to perform antibiotic susceptibility pattern.

Materials and Method

This cross sectional study was conducted in the department of microbiology between January-2013 to January 2014. The study was approved by the institutional ethical committee. All urine specimens from both the sexes with clinical symptoms suggestive of urinary tract infection were included in the study. Patients on antibiotic treatment for the past one week and Specimens which showed mixed growth were excluded from the study. Clean catch mid stream urine sample was collected in a sterile universal container and processed as per standard microbiological techniques.(8) Direct gram staining of uncentrifuged urine was performed and the presence of ≥1 bacteria per oil immersion field in at least 5 fields indicates the presence of significant bacteriuria.(8) Urine culture was performed by semi-quantitative method using a calibrated loop (0.001 ml) of 4mm diameter onto CLED(cysteine lactose electrolyte deficient media) and blood agar which were incubated aerobically at 35-37^{0}C for 24 to 48 Hours.(8) Each significant isolate was identified by colony morphology, Gram staining and biochemical reactions as per standard Microbiological techniques.(9) Antibiotic susceptibility testing was performed by Kirby Bauer disc diffusion method for the

following antibiotics Ampicillin, Cotrimoxazole, Nitrofurantoin, Norfloxacin, Amikacin, Ceftazidime, Cefotaxime, Piperacillin-tazobactum and imepenem. The results were interpreted as per CLSI guidelines.[9] Isolates showing resistance to both cefotaxime and ceftazidime were further tested by Double disc method for ESBL production.[10,11] A combination of ceftazidime/clavulanic acid (30/10 µg) and cefotaxime /clavulanic acid (30/10 µg) disc was used. Isolates those showed a five mm increase in zone size in comparison to the disc without clavulanic acid were confirmed to be ESBL producers.

Biofilm production was detected by Microtitre plated method.[12]

The isolates are inoculated into Brain heart infusion broth (BHI broth) and incubated at 37ºc for 24 hours. In the microtitre plate 200 µl BHI broth and 2 µl of each isolate was added. The plates were incubated at 37ºc for 24 hours. ATCC Pseudomonas aeruginosa 27853 was used as positive control. After 24 hours the contents of the wells were discarded and each well is washed four times with PBS (phosphate buffer saline 300 µl. Then 0.1% crystal violet was added to the wells. After 15 minutes the microtitre plate was washed repeatedly with sterile distilled water. After drying, the plates were read at 570 nm using ELISA reader.

The values were interpreted as below

Sample OD >0.12- strong biofilm producers

Sample OD values between 0.06 -0.12- moderate to weak biofilm producers

Sample OD < 0.06 – Non biofilm producers.

Statistical Analysis: The Significance of association between drug resistance phenotype like (Extended spectrum beta lactamase production) and Biofilm formation was analysed by chi square test (Graph pad Quick calcs software) and *pvalue* less than 0.05 was considered as statistically significant.

Results

A total of 148 consecutive urine specimens were obtained over a period of one year January 2013 to January2014 at the Department of Microbiology. Direct gram staining had a sensitivity and specificity of 86% and 95% respectively. Out of the 148 specimens obtained, 113(76.35%) were culture positive. Mixed growth was observed among 12(8.1%) isolates and those were excluded from the study. Female subjects (51.35%) were generally affected, and the maximum number of cases was seen among the age group 41–50years (30.76%).(Table 1)

The most common pathogen that was isolated was *Escherichia coli* 34(36.1%) followed by *Pseudomonas aeruginosa* 22(23.4%) (Table 2). Biofilm productions assessed by microtiter plate method, 79(84%) isolates were biofilm producers revealed 50 (63.2%) strong biofilm producers and 29 (36.7%) moderate biofilm producers. Biofilm producing isolates were highest among *Escherichia coli* 26(32.9%) followed by *Pseudomonas spp* 22(27.8%).(Table 3)

The antibiotic susceptibility pattern is depicted (Table 4). The most effective antibiotic was Imepenem and piperacillin tazobactum, Overall highest susceptibility was observed for cotrimoxazole 78(80.4%), nitrofurantoin 78(80.4%) and amikacin 76(78.3%). Ampicillin showed the least susceptibility of 24(24.7%). Among *Escherichiacoli* and *Pseudomonas spp* isolates which showed good sensitivity for nitrofurantoin, amikacin and cotrimoxazole.

A total of 38 ofcefotaxime and ceftazidime resistant isolates were screened for ESBL production in which 38 isolates were confirmed to be ESBL producers by double disc method. *Escherichia coli* 18(52.94%) and *Pseudomonas spp* 9(34.6%) showed the highest ESBL producers (Table 5) followed by *Klebsiella spp* 6(30%).

Out of 38 ESBL producing isolates, only 14(36.84%) showed presence of biofilm formation. The association of antibiotic resistance between the Non biofilm and biofilm producing drug resistant isolates like Extended Spectrum Beta Lactamases (ESBL) was evaluated by Chi square test (Graph pad Quick Calcs software) and the p value was found to be 0.023 (statistically significant)(Table 6).

Table 1: Age and Sex wise distribution

S. No	Sex	Number
1	Male	72(48.6%)
2	Female	76(51.35%)
S. No	**Age distribution**	**Number**
1	20-30 years	24(16.2%)
2	31-40 years	51(34.22%)
3	41-50 years	45(30.4%)
4	51- 60 years	14(9.45%)
5	61-70 years	12(8.1%)

Table 2: Distribution of the isolates

s. No	Isolate	Total number(%)
1	*Escherichia coli*	34(36.1%)
2	*Proteus spp*	17(18%)
3	*Klebsiella spp*	21(22.3%)
4	*Pseudomonas spp*	22(23.4%)
5	*Staphylcoccusaureus*	11(7.4%)
6	*Enterococcus spp*	8(5.4%)
		113(76.35%)

Table 3: Distribution of biofilm producers among the gram negative isolates

S No	Isolate	No(%) ofbiofilm producers	N(%)Strong biofilm producers isolates	N(%)Moderate Biofilm producers isolates
1	*Escherichia coli*	26(32.9%)	16(61.5%)	10(38.4%)
2	*Proteus spp*	17(21.5%)	11(64.7%)	6(35.2%)
3	*Klebsiella spp*	14(17.7%)	6(42.8%)	8(57.1%)
4	*Pseudomonas spp*	22(27.8%)	17(77.2%)	5(22.7%)
	Total	79(84%)	50(63.2%)	29(36.7%)

Table 4: Antibiotic susceptibility pattern of Gram negative isolates

Antibiotic disc	Overall Susceptibility Pattern	*Escherichia coli* N=34	*Proteus spp* N=17	*Klebsiella spp* N=20	*Pseudomonas spp* N=26
Ampicillin	24(24.7%)	16(47%)	8(47%)	-	-
Cotrimoxazole	78(80.4%)	26(76.4%)	14(82.3%)	16(80%)	22(84.6%)
Nitrofurantoin	78(80.4%)	28(82.3%)	16(94.1%)	14(70%)	20(76.9%)
Amikacin	76(78.3%)	28(82.23)	15(88.2%)	13(65%)	20(76.9%)
Ceftazidime	63(64.9%)	18(52.9%)	13(76.4%)	14(70%)	18(69.2%)
Cefotaxime	60(61.8%)	16(47%)	12(70.5%)	15(75%)	17(65.3%)
Norfloxacin	76(80.8%)	25(73.5%)	13(76.4%)	15(75%)	21(80.7%)
Piperacillin/tazobactum	94(100%)	34(100%)	17(100%)	20(100%)	26(100%)
Imepenem	94(100%)	34(100%)	17(100%)	20(100%)	26(100%)

Table 5: Distribution of ESBL producers

S. No	Isolate	Isolates showing resistance to cefotaxime and ceftazidime Number
1	*Escherichia coli*	18(52.94%)
2	*Proteus Spp*	5(29.41%)
3	*Klebsiella spp*	6(30%)
4	*Pseudomonas spp*	9(34.61%)
		38(40.4%)

Table 6: Antibiotic resistance pattern between Biofilm forming and non-biofilm forming Extended spectrum beta lactamase producers: Statistical significance

Antibiotic	Biofilm forming ESBL producer Resistant isolates	Non biofilm forming ESBL producers Resistant isolates
Ampicillin	16	22
Cotrimoxazole	15	23
Nitrofurantoin	19	19
Amikacin	21	17
Ceftazidime	20	18
Cefotaxime	29	9

P-value = 0.023 statistically significant

Discussion

Urinary tract infections are one of the common diseases encountered in hospitals. Emergence of drug resistant and biofilm forming uropathogenic bacteria is of significance because of complications like chronicity and relapses arising out of treatment failure owing to multidrug resistance.

In the present study Female patients comprised the predominant sex 76(51.35%) similar to various cited studies.[13,14,15] This might be attributed to short urethra and proximity to anus. Predominant of the patients are in the age group of 31-40 years. Among the gram negative bacteria *Escherichia coli* was the predominant 34(36.1%) bacteria isolated followed by *Pseudomonas spp* 22(23.4%). *Escherichia coli* was the most common bacteria isolated in UTI as reported by various studies across the world.[5,16,17] Uropathogens express virulence factors that facilitate the bacteria to attach to the epithelial cells and forms biofilms thereby enhancing the virulent nature of the uropathogen. In the present study biofilm detection was observed in 79(84%) of the isolates. Strong biofilm production was observed in 50(63.2%) of the isolates. Studies have reported biofilm production to be in the range of (18% to 80%) among uropathogens.[5,18-21] In the present study maximium biofilm producers were observed among *Escherichia coli* 26(32.9%) followed by *Pseudomonas spp* 22(27.8%). Biofilm producing bacteria are usually related with drug resistance, relapses and chronic infections.[19,21] This is of important concern while initiating therapy[4]. The cells present within the biofilm can separate from one site and initiate the infection at a different site. Therefore appropriate aggressive antibiotic therapy should be instituted thereby eradicating and interfering with the formation of biofilm. Biofilm producing Uropathogens are of substantial concern owing to their association with nosocomial and hospital infection control problem since the drug resistant bacteria within the biofilm can disseminate within the hospital environment.[19,22] It is

essential to devise effective hospital infection control programmes so that the antibiotics are selected based on the resistance report. In the present study good susceptibility was observed for cotrimoxazole (80.4%) Nitrofurantoin, nitrofurantoin 78(80.4%) and Amikacin amikacin 76(78.3%) Similar to Sanchez CJ and *Alves MJ et al.*,[23,24] Cefotaxime and ceftazidime showed a resistance percentage of 36% and 39% respectively. Kaur DC and Wankhede S reported a resistance of 56.6% to ceftazidime.[7] *Alves MJ et al.,* showed 25% resistance for cefotaxime ceftazidime respectively and norfloxacin Norfloxacin showed a susceptibility of 80%.

In the present study 38(40.4%) were ESBL producers. Studies have reported ESBL producers as ranging from 36.5% to 51.78% among the isolates causing Urinary tract infection.[25,26] Among the ESBL producers 68% were Multidrug resistant and 14(36.84%) were biofilm producers. In a study by Shahidul KM, 80% of the strains producing biofilm were multidrug resistant.[13] In the present study among *Escherichia coli* and *Pseudomonas spp,* 52.9% and 34.6% were ESBL producers respectively. In a study by *Jena J et al*., *Escherichia coli* and *Pseudomonas spp* showed ESBL producers as 47(61.8%) and 1(20%) respectively.[26] Antibiotic resistance among uropathogens is on the raise leading to prolonged hospitalisation and excessive treatment costs. Drug resistant bacteria is of a therapeutic challenge. Inappropriate use of antibioitcs is an important factor contributing to the emergence of multidrug resistant bacteria. Hence its necessary to conduct surveillance of the antibiotic susceptibility pattern for commonly isolated bacteria in a community based set up and empiric therapy initiated based on it. This will help in the prevention of the emergence of drug resistant isolate.

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How to cite this article: Shanmugam K., Thyagarajan R, Katragadda R, Vajravelu L, Jayachandran AL. Biofilm formation and Extended Spectrum Beta Lactamases (ESBL) producers among the gram negative bacteria causing Urinary tract infections. International Journal of Medical Microbiology and Tropical Diseases 2017;3(3):86-90.