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IN VITRO BIOFILM PRODUCTION AND VIRULENCE FACTORS OF UROPATHOGENIC *ESCHERICHIA COLI*

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ABSTRACT

The aim of the study is to assess *in vitro* biofilm production and other virulence factors in uropathogenic *E.coli* (UPEC) and correlate with drug susceptibility. Materials and methods The study include 300 uropathogenic *E.coli* strains collected from patients with significant bacteriuria. Biofilm production was quantified by modified Christensen's method. All the strains were tested for virulence factors like proteinase production, extended spectrum beta lactamase production, haemolysin production, mannose resistant haemagglutination and antibiotic sensitivity. Results: Out of the 300 strains, 265(67%) were *in vitro* biofilm producers, multiple drug resistance were more common among strong biofilm producing strains of Uropathogenic *E.coli*. Among the isolates 5% were positive for proteinase, 30% of the produced haemolysin and 71% were positive for mannose resistant haemagglutination. The anti-biogram was as follows, 35% strains were sensitive to Gentamicin and Norfloxacin; 34% sensitive to Co-trimoxazole; 84% were sensitive to Amoxyclav; 87% to Nitrofurantoin; 38% were sensitive to Ceftazidime and cefotaxime; 85% sensitive to Amikacin and 100% to Imipenem respectively. 56% produced extended spectrum beta lactamase. Conclusion The study shows that the ability to produce biofilm varies among strains of uropathogenic *E.coli* and multidrug resistance is more prevalent among strong biofilm producers. Mannose resistant haemagglutinating fimbria has a role in biofilm formation. ESBL.production is prevalent among uropathogenic *E.coli.*

KEY WORDS : Biofilm, Virulence factors, Uropathogenic *E.coli.*

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INTRODUCTION

Uropathogenic *Escherichia coli* are common cause of urinary tract infections(UTI)[1]. UPEC strains of *E.coli* possess virulence factors that make them to infect and survive in highly acidic urinary tract environment[2]. The virulence genotype differs in different geographic area. Therefore, characters of isolate of different regions need to be assessed. The present study was to assess different virulence factors of uropathogenic *E.coli* with an emphasis on biofilm production and drug susceptibility[3,4].

MATERIALS AND METHODS

A total of 300 non-repeating UPEC strains isolated from hospitalized patients of different age group with clinical symptoms of UTI which yielded a significant bacterial count on semi-quantitative urine culture were included in the study. The strains were collected from three major tertiary care centers in Dakshina Kannada district of Karnataka ie Yenepoya Medical College Hospital, K.S. Hegde Medical College Hospital, Fr. Muller Hospital Mangalore respectively. The strains were identified by standard biochemical reactions and stored in glycerol broth at - 70^0C and the strains were subjected to the following virulence tests.

Biofilm production

The biofilm production was assayed by modified Christensen method[4,5]. The strains were grown overnight in brain heart infusion broth and the growth was adjusted to 0.5 Mcfarland standard. 3ml of the broth was added to polyethylene cuvette containing 1 ml of fresh BHI broth and incubated at 37^0 C for 24 hours without shaking. The contents of the cuvettes were emptied and unattached bacteria were removed by washing in normal saline. The biofilm formed on the cuvette walls were stained by 0.1% crystal violet for 20 minutes. Cuvettes were washed three times with phosphate buffer. The stained biofilm was then dissolved in 60% ethanol and biofilm was quantitated by measuring the Optical Density(O.D) (Fig.1) value of the dissolved solution[2].

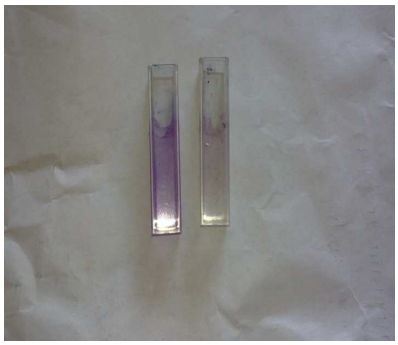


Figure 1
Cuvette contatining biofilm (Rt.postive Lt negative)stained with crystal violet

Haemolysin production

Production of haemolysin was detected by inoculating *E.coli* strains on 5% sheep blood agar plates and incubated at 37^0C for 24 hours. A complete clearing of RBC around the colony was considered as haemolysin producer[7].

Antibiotic susceptibility test

Antibiotic susceptibility test for the antimicrobial agents, Gentamicin (15µg), Amikacin (30µg), Ciprofloxacin (5µg), Norfloxacin (10µg) Co-trimoxazole (25µg), Nitrofurantoin (300µg), Cefotaxime (30µg), Ceftazidime (30µg) Amoxiclav (20/10µg), Imipenem (10µg) were

done by standard Kirby-Bauer disc diffusion method on Muller Hinton agar, the result was interpreted according to NCCLS criteria[8,9].

Proteinase production

Proteinase enzyme production among uropathogenic *E.coli* was tested by inoculating the strains into gelatin agar (Hi-Media, Mumbai) and incubated at 37^0C for 24 hours. The plates were then flooded with mercuric chloride solution, development of opacity in the medium and a zone of clearance around the colony was considered positive for proteinase enzyme production[9].

Extended spectrum beta lactamase (ESBL) production

E.coli strains resistant to ceftazidime and Cefotaxime were subjected to phenotypic confirmation test, in a lawn culture of strains, ceftazidime (30µg) Vs ceftazidime-clavulanic acid and Cefotaxime (30µg) Vs Cefotaxime-clavulanic acid (30/10µg). Discs were placed and incubated at 37^0C for overnight, ≥ 5mm increase in zone of inhibition of the cephalosporins tested in combination with clavulanic acid Vs its zone size when tested alone was taken as ESBL producer. *E.coli* strain ATCC25922 was used as the negative control in the study[10].

Mannose resistant haemagglutination

This is to demonstrate fimbriae which are important virulence factor in uroepithelial cell attachment, which specifically bind with O positive RBC antigen. *E.coli* were grown on colonization factor antigen agar. 100µl of bacterial suspension in buffer was added to the wells of haemagglutination plates, equal volume of 1% O positive human RBC and 1% D-mannose was also added to each well. The wells containing only erythrocytes suspension were used as negative control. An even sheet of erythrocytes at the bottom of the wells after one hour at 4^0C was considered as positive and a small pellet of erythrocytes was considered as negative for haemagglutination[11-13].

RESULT

Biofilm production

Out of the 300 strains tested for biofilm production, 203 (67%) were strong biofilm producers and remaining 97(33.4%) strains were weak or non-biofilm producers. On analysis of antibiotic resistance pattern of strong biofilm producers and weak or non producers, there was correlation between biofilm production and multiple drug resistance[14,15]. The drug resistant pattern of biofilm producers and non biofilm producers is shown in table1.

Table 1

The drug resistant pattern of biofilm producers and non biofilm producers

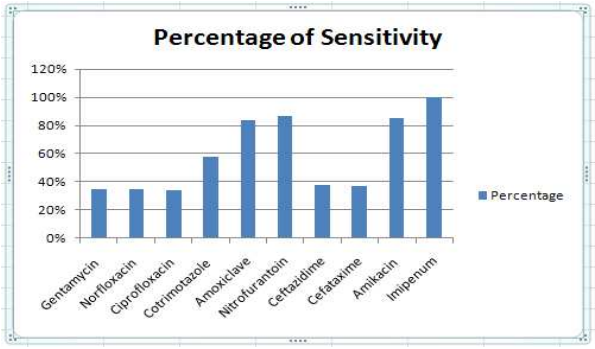
Antibiotics	Strong biofilm producers n=203	Non biofilm producers n=97
Gentamicin	139(68%)	55(56%)
Norfloxacin	141(69%)	58(59%)
Amikacin	45(22%)	13(13.4%)
Co-trimoxazole	106(52%)	43(44%)
Amoxiclav	45(22%)	16(15%)
Ceftazidime	137(67%)	50(51%)
Cefotaxime	139 (68%)	47(48%)
Nitrofurantoin	25(15%)	9(12%)
Imipenem	None(0%)	None (0%)

Antibiotic susceptibility test

Out of the 300 UPEC strains, 105(35%) were sensitive to Gentamicin and Norfloxacin,102 strains(34%) were sensitive to Co-trimoxazole,252 strains (84%) were sensitive to Amoxiclav, 261 strains (87%) were sensitive to Nitrofurantoin, 114 strains (38%) were sensitive to Ceftazidime, 111 strains(38%) were sensitive to Cefotaxime, 255 (85%) were sensitive to Amikacin,300(100%) of the strains were sensitive to Imipenem respectively.

Haemolysin production

90 Strains(30%) were positive for haemolysin production.



Proteinase production

Out of 300 strains, 15 strains (5%) were producing proteinase enzyme. 168 strains (56%) of the UPEC were ESBL producers.

Mannose resistant haemagglutination

Out of the 300 strains of uropathogenic *E. coli,* 213 (71%) were positive for mannose resistant haemagglutination.

DISCUSSION

Urinary tract infections (UTI) are very common infectious diseases where re-occurrence is very high and often become chronic*. E.coli* is the leading cause of urinary tract infections and constitutes 80-85% all UTI. Biofilm formation by uropathogenic *E.coli* is a known virulence factor. Biofilm is microbiological phenomenon where bacteria undergo transition from a planktonic existence to community based existence . Biofilm may form on variety of surface, including living tissue, indwelling medical devices, water pipes, etc. Biofilm protects the bacterium from host defense mechanisms and antibiotic action[16,17]. Bacterial biofilms are often associated with long term persistence of bacteria in various environments[17]. Bacteria in biofilm exhibit increased resistance to antibiotic[18]. According to Coserton *et al,* 50% of all bacterial infections involve biofilm formation[16,18]. According to the present study, 67% UPEC strains were *in vitro* biofilm producers. Increased rate of biofilm production was observed in UPEC strains which were positive for mannose resistant haemagglutination .73%. of the mannose resistant haemagglutinating strains were strong biofilm producers. Fimbriae were probably one of the factor which contribute to form biofilm on the surface. Various structures such as flagella, fimbriae, outer membrane proteins (OMP), curli and extracellular polymeric matrix (EPS) are involved in biofilm formation[19,20]. However, the study results show that biofilm production cannot be directly correlated with any single virulence factor. Haemolysin production is another important virulence factor of uropathogenic *E.coli*. In the present study 30% of the strains were haemolysin producers. There was no correlation between haemolysin production and biofilm formation. The study results show proteinase production is not an important virulence factor of in urinary tract infections; only 5% of the strains produced proteinase. Treatment of urinary tract infections is becoming difficult because of multidrug resistance. The study result reveals antibiotic resistance pattern of UPEC in this geographical area. Antimicrobial resistance pattern varies with time and place. Production of beta lactamase is a major means by which Gram negative bacteria acquire resistance to beta lactam antibiotics. Extended

spectrum beta lactamase are a group of enzymes that can hydrolyze a variety of beta lactams including cephalosporins, monobactams and penicillins. The global spread of ESBL producing *E.coli* has potential to cause major problem in treatment[21,22,23] .The present study showed increased prevalence of ESBL production among uropathogenic *E.coli*. Multidrug resistance is common among strong biofilm producers this probably is due to high rate of conjugation in biofilm, which facilitates transfer of drug resistant genes. Co-resistance to other non beta lactam antibiotics like, Gentamicin, Norfloxacin, Co-trimoxazole was observed among ESBL producers. According to the present study no resistance has developed against Imipenem.(100% sensitivity) Nitrofurantoin and Amikacin are the antibiotics which can be used to treat urinary tract infection with beta lactamase producing and biofilm producing *E.coli* infection with low cost.

CONCLUSION

The study result showed that ability to produce biofilm varies among strains of uropathogenic *E.coli* .Strong biofilm producers exhibit multiple drug resistance, this is probably due to non permeability of biofilm to antibiotics and increased rate of conjugation in biofilm which facilitate spread of drug resistant genes. The fimbriae which cause mannose resistant haemagglutination are one of the factor which contribute to biofilm. Haemolysin production is another virulence factor which is common among uropathogenic *E.coli*, but proteinase production is not so important in pathogenesis of UTI. There was an increased prevalence of ESBL production among *E.coli*,which reduces therapeutic options in treating UTI. According to the present study Nitrofurantoin, Amikacin and Amoxiclav are the antibiotics which can be used treat infection with uropathogenic *E.coli* with low cost.

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