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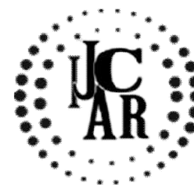

Research Article

PHENOTYPIC METHODS TO DETECT VIRULENCE FACTORS OF UROPATHOGENIC E.coli ISOLATES AND THEIR ANTIMICROBIAL SUSCEPTIBILITY PATTERN

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A B S T R A C T

Introduction: Urinary tract infection caused by uropathogenic Escherichia coli (UPEC) strains is one of the most important infections in the world. The UPEC strains harbor many genes which encode for various virulence factors, like production of biofilms, deploying toxins- hemolysin and production of various antimicrobial resistance genes.
Aim of study: To determine the prevalence and correlation of phenotypic virulence traits (biofilm and hemolysin production) and antibiotic resistance profile among the UPEC isolated from UTIs.
Material and method: A prospective study was done among patients presenting with symptoms of UTI. The uropathogenic E. coli (UPEC) isolated from samples were processed to check for biofilm production using Congo Red agar and Christensen Tube Adherence methods. Hemolysin production was checked using 5% sheep blood agar and antimicrobial susceptibility testing was done as per CLSI guidelines.
Results: out of the 100 UPEC isolates- 65% were *in vitro* positive for the biofilm production. Biofilm production by both the Congo Red agar method and the tube adherence method was seen in 52% of the total isolates, whereas 35% of the strains were found to be negative by both the methods. Hemolysin production was seen in 22 isolates and of these, 17 produced biofilms by either Congo Red Agar or tube adherence method. Antimicrobial susceptibility testing showed that overall among the 100 UPEC isolates, multidrug resistance was seen more among biofilm producers.
Conclusion: Better understanding of virulence factors using phenotypic methods in laboratories can be useful in understanding pathogenesis and thereby modify the antibiotic therapy.

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INTRODUCTION

Urinary tract infection caused by uropathogenic Escherichia coli (UPEC) strains is one of the most important infections in the world. The UPEC strains harbor many genes that encode various virulence factors, which contribute to enhanced pathogenicity. [1]

Once inside the urinary tract, UPEC preferentially colonizes the bladder and causes cystitis, but can also ascend through the ureters into the kidneys, causing pyelonephritis. [2]

The UPEC strains have virulence factors like production of biofilms, deploying toxins- hemolysin and production of various antimicrobial resistance genes. This investigation is aimed to determine the prevalence and correlation of phenotypic virulence traits and antibiotic resistance profile among the UPEC isolated from UTIs.

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As one of the virulence factors of UPEC is production of the biofilms, it remains was protected within the bacterial extracellular matrix, thus making the antimicrobial agents ineffective in eradicating the infection. [3] Bio films are the microbial communities of the surface-attached cells which are embedded in a self-produced extracellular polymeric matrix and have been associated with device associated infections. [4] Extended-spectrum beta-lactamases-producing UPEC strains, which are increasing in prevalence world- wide, have an appreciable deleterious impact on the clinical management of UTI. The purpose of this study was to evaluate the presence of different phenotypic virulence markers in UPEC isolates and determine their correlation with antibiotic resistance pattern.[5]

MATERIALS AND METHODS

Selection of the Isolates: A prospective analytical study was done in a Tertiary Care Hospital in North India over a period of 5 months (March to July 2018). Clean catch midstream urine sample was collected in a sterile container from patients

aged between 15 to 65 years, presenting with symptoms of UTI. A modified semi-quantitative technique was employed (standard wire loop method) and a standard bacteriological loopful of urine (0.01ml) was inoculated over the surface of Cystine Lactose Electrolyte Deficient (CLED) agar plate. The plates were then incubated at 37 °C for 18-24 hours. Single species count of more than 10^5 organisms per ml of urine was considered as significant. The identification of the isolates was done on the basis of the colony morphology, gram staining and the standard biochemical tests as per standard techniques.[6]

A total of 100 consecutive non-repetitive E. coli strains were isolated from specimens that showed significant bacteuria and these strains were subjected to biofilm production test and hemolysis.

Phenotypic Detection of Virulence Factors

Detection of the Biofilm Formation

The detection of the biofilms was done by two methods - tube adherence method and the Congo Red agar method.

Tube adherence method: The test strains were inoculated into glass tubes which contained Brain Heart Infusion Broth (broth) and incubated this suspension aerobically at a temperature of 35°C for 48 hours. The supernatants were discarded and the glass tubes were stained with a 4% crystal violet solution, washed with distilled water 3 times and dried. A positive result was defined as the presence of a layer of the stained material which adhered to the inner wall and bottom of the tubes. The exclusive observation of a stained ring at the liquid-air interface was considered as negative. The investigation of the biofilm production was done on the basis of the adherence of the biofilms to borosilicate test tubes, as was done by Christensen *et al*. (1982) [7]

Congo Red agar method: The test strains were inoculated into plate which contained a specially prepared solid medium- Brain Heart Infusion broth (BHI) which was supplemented with 5% sucrose and Congo Red. The medium was composed of BHI (37gms/l), sucrose (50 gms/l), agar No.1 (10 gms/l) and the Congo Red stain (0.8 gms/l). Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium constituents and was then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C. Positive result was indicated by black colonies with a dry crystalline consistency, non biofilm producing strains developed red colonies. The investigation of the biofilm production by the Congo Red agar method was proposed by Freeman *et al*. (1989). [8]

Hemolysin production: The ability of the UPEC isolates to induce hemolysis on blood agar was evaluated to detect the isolates that were hemolysin producer. The bacteria were inoculated into 5% sheep blood agar and incubated overnight at 37 C. Hemolysin production was detected by the presence of a complete clear zone of haemolysis around the colonies.

Antibiotic susceptibility testing: Antibiotic susceptibility testing was done using Kirby Bauer disc diffusion method as per CLSI guidelines.[9] The bacterial suspension was made by inoculating 4-5 isolated identical colonies in peptone water. The peptone water was then incubated at 37 ℃ for 2 hours. After 2 hours of incubation, the turbidity was standardized by using 0.5 Mc Farland standards. and inoculated plates were incubated at 37 ℃. Test organisms were streaked on Muller Hinton agar plates using sterile swab. The appropriate antibiotic disc was then placed firmly onto the surface of the dried plates using sterile forceps, depending on whether the test organism plated was a gram negative or gram-positive organism. The 6 antibiotic discs per plate were placed and plates were left at room temperature for one hour in order to allow diffusion of the antibiotics from the disc into the medium. The plates were then incubated at 37 ℃ for 18-24 hours. The plates were read the next day, zone diameters were noted and interpreted as per CLSI guidelines.[9]

The following antibiotics were used: Nitrofurantoin (300µg), Amikacin (30µg), Cotrimoxazole (25µg), Gentamicin (10µg), Norfloxacin (10µg), Ertapenem (10µg), Pipercillin/Tazobactam (100/10µg), Aztreonam (30 µg), Ceftazidime (30 µg), cefazolin (30µg), penicillin(10U), Vancomycin (30 µg), Tetracycline (30 µg), Erythromycin (15µg), Chloramphenicol (µg), high level gentamicin (120µg), linezolid (30 µg).

ESBL production: Phenotypic Confirmatory Test with Combination Disc. For this test a disc of ceftazidime 30 µg alone and a disc of ceftazidime plus clavulanic acid (30/10 µg) were used as per CLSI guidelines. The discs were placed at least 25 mm apart center to centre on a lawn culture of test isolate on MHA plate and incubated overnight at 37°C . Difference in zone diameters with and without clavulanic acid was measured. Interpretation: An increase of greater than 5 mm in inhibition zone around combination disc of ceftazidime plus clavulanic acid disc versus the inhibition zone diameter around ceftazidime disc alone- confirms ESBL production.

RESULTS

Biofilm formation

In the current study, 65% strains were *in vitro* positive for the biofilm production and 35% were negative for the biofilm production. Biofilm production by both the Congo Red agar method and the tube adherence method was seen in 52% of the total isolates, whereas 35% of the strains were found to be negative by both the methods. (Table 1)

Hemolysin production: According to our results, hemolysin production was observed in 22% of the UPEC isolates. Of these 22 isolates, 17 produced biofilms by either CRA or tube adherence method. The remaining 78% isolates showed no hemolysis.

Antimicrobial resistance profile of UPEC

Antimicrobial susceptibility testing showed that overall among the 100 UPEC isolates, majority of the isolates were resistant to cotrimoxazole and norfloxacin. The susceptibility pattern has been depicted in Figure 1.

Table 1 Comparison of Congo red agar and Christensen tube adherence method

No. of isolates	Congo red agar method	Tube adherence method
52	+	+
7	+	-
6	-	+
35	-	-

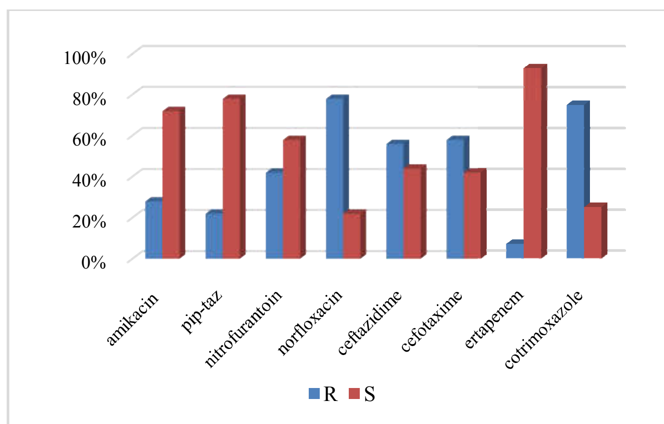

Figure 1 Antimicrobial susceptibility pattern of the 100 UPEC isolates in the study

The comparison of antimicrobial resistance pattern between the biofilm and non biofilm producing E. coli is shown in Table 2 and Figure 2. Both biofilm producer and non- biofilm producer were highly resistant to norfloxacin and trimethoprim-sulphamethoxazole. All the biofilm forming strains showed maximum resistance to norfloxacin (88%) co-trimoxazole (85%), and amikacin (70%) followed by cephotaxime (68%), ceftazidime (62%), However, resistance was comparatively higher among biofilm producer than non-biofilm producers in case of the following antibiotics- such as co-trimoxazole (85% vs. 59%), norfloxacin (88% vs. 60%) and nitrofurantoin (52% vs. 22%) and cephotaxime (68% vs 41%).

Table 2 Comparison of Antimicrobial susceptibility among Biofilm producers and non-biofilm producers

No. of isolates	Congo red agar method	Tube adherence method
52	+	+
7	+	-
6	-	+
35	-	-

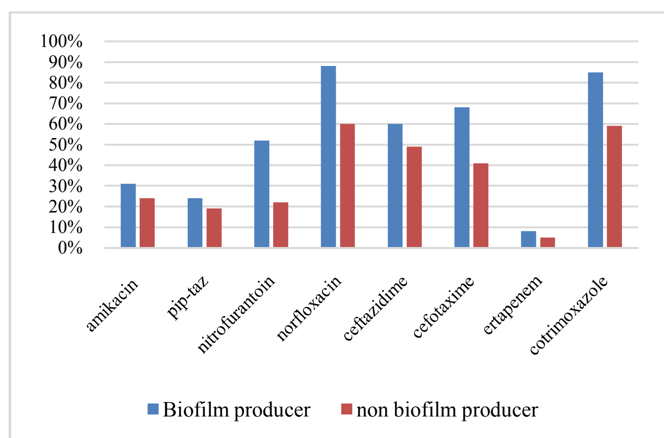

Figure 2 Comparison of antimicrobial susceptibility among Biofilm producers vs Non-biofilm producers

Double disc synergy test (DDST) showed that 28 UPEC isolates were positive for ESBL production. Out of these 28, twenty two UPEC were biofilm producers.

DISCUSSION

In the current study, 65% strains were *in vitro* positive for the biofilm production and 35% were negative for the biofilm production. Similar similar rate of the biofilm production were reported by a study done in 2009 Rohtak (67.5%), 2012 in Pondicherry (60%) and in 2017 Chattisgarh (58.3%). [10,4,11] Slightly higher rates of biofilm production 70.3% and 72% were reported by authors from Tamil Nadu (2012) and Canadian authors (1992).[12,13] Higher rates of biofilm producers 80% and 92% have been reported by few authors from South India (2013 and 2007)[14,15]

Resistance to cotrimoxazole was higher in biofilm producers (85%) as compared to non biofilm producers (59%). Study done in Thailand[3] in 2015 was in agreement- biofilm formation in the cotrimoxazole resistant group was significantly higher than the cotrimoxazole sensitive group.

Our study showed that, resistance to all drugs were found more in biofilm producers than in biofilm non producers which was in agreement with study done by authors in Bangladesh (2014), Pondicherry (2012) and Chattisgarh (2017) [16,17,11]. Overall resistance to norfloxacin and cotrimoxazole was highest in both groups, which could be because these were the most commonly prescribed antibiotics for UTI in the hospital, whereas the uropathogens showed the highest sensitivity to carbapenems. Similar finding was reported by other authors.[17]

According to the results in the present study beta hemolysin production was observed in 22% of the UPEC isolates, which was in agreement with study done in Iraq in 2016.[18] The present study showed that hemolysin production by isolates was associated with a higher ability to form biofilm. This was in agreement with the study done in Iran (2015) [19]and Spain (2007) [20]whereas contrary to observations of study done by Marhova *et al* in Bulgaria (2009) [21] in the present study, 78.5 % of ESBL producing isolates demonstrated biofilm production. This finding was similar to study done in Uttarakhand[22] in 2012 that reported 81.5% of biofilm producers expressed ESBL among all of the gram negative bacteria whereas the rate was 100% in case of E.coli in the present study.

In the present study, two different methods were carried out for the detection of the biofilm production - Congo red agar method and Tube adherence method. Detection of the biofilm production by the Congo Red agar method and the tube adherence method were (59%) and (58%) respectively, in agreement with study done in 2012 in Pondicherry by Niveditha *et al* [4]. Both the methods which were based on the principle of enhancement of the exopolysaccharide production by using some enriched medium, like TSB, in case of the Christensen method [7], while the Congo Red agar method requires additional use of a highly nutritious medium- for example, the brain heart infusion broth with a 5% sucrose. Authors from Iowa, US[23] (1988) showed that the Congo Red method was rapid, more sensitive, and reproducible and that it had the advantage of the colonies remaining viable on the medium. On the contrary studies done in Pakistan[24] in 2011 and an Indian study done in Chattisgarh[11] in 2017 found that tube method is more reliable than congo red agar method. In the present study, however, both congo red agar method and Tube adherence method were were found to be equally reliable.

There is an association between biofilm production with persistent CAUTI and antibiotic therapy failure. Hence identification of infection caused by biofilm producing organisms might help to modify the antibiotic therapy and prevent infection. [11]

CONCLUSION

The incidence of device-associated infections caused by multidrug resistant isolates, predominantly forming biofilm, has increased in the past decade. The biofilm helps the UPEC isolates in their pathogenicity and virulence. The tube method is an effective screening method to test biofilm production, where sophisticated electron microscopy facilities are not available. The varying resistance pattern of organisms isolated in our setup, emphasizes the importance of studying the pattern of infection in every setting and providing antibiotic guidelines in the management of such infections.

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