ORIGINAL ARTICLE

A Comparative Appraisal of Detection of Biofilm Production Caused by Uropathogenic *Escherichia coli* in Tropical Catheterized Patients by Three Different Methods

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Abstract

Introduction: Urinary tract infections (UTIs) are very common among Indian population which can be acute or chronic. However, untreated UTIs can lead to chronic condition which ultimately causes hospitalization. **Objective:** The present study aimed to study the comparison of three different methods of detection of biofilm production by uropathogens in tropical catheterized patients. **Materials and Methods:** The study included 500 tropical catheterized patients admitted in Civil Hospital, Ambala City. All the patients were interviewed as per a pre-defined protocol, and all the required medical informations were recorded in a pre-determined format for easy extraction as well as interpretations. Samples were collected for the tests to identify the microorganisms in the isolates and also to detect the biofilm product. Identification of microorganisms was performed by routine methods. Three different methods, namely tissue culture plate method (TCPM), tube adherence method (TAM), and modified Congo red agar (MCRA) method, were used to detection the biofilm production and compared. **Results and Discussion:** The results suggested that *Escherichia coli* is the most predominant uropathogen responsible for UTIs in the catheterized patients in the present studied patient population. Of these isolates, resistant *E. coli* cases were found to be most abundant and strong biofilm producer. **Conclusion:** TCPM was found to be significant method for the detection of biofilm production in this patient population, especially when the causative organism is resistant *E. coli* followed by TAM and MCRA method.

Key words: Biofilm, catheterized patients, *Escherichia coli*, resistance, urinary tract infection, uropathogens

INTRODUCTION

Biofilms are defined as microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.[1] Within a biofilm, bacteria communicate with each other by the production of chemotactic particles or pheromones, a phenomenon called quorum sensing. Availability of key nutrients, chemotaxis toward surface, motility of bacteria, surface adhesins, and presence of surfactants are some factors which influence biofilm formation. Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1000-fold. According to a publication by the National Institutes of Health, more than 80% of all infections involve biofilms.[2] Biofilms

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are associated with many medical conditions including indwelling medical devices, dental plaque, upper respiratory tract infections, peritonitis, and urogenital infections. Both Gram-positive and Gram-negative bacteria have the capability to form biofilms. Bacteria commonly involved include *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis,* and *Pseudomonas aeruginosa*.[3] There are various methods to detect biofilm production. These include the tissue culture plate (TCP), tube method (TM), Congo red agar (CRA) method, bioluminescent assay, piezoelectric sensors, and fluorescent microscopic examination.[4] We screened 500 samples by three different methods, which could be used in a routine clinical laboratory, for determining their ability to form biofilm. Urinary tract infection (UTI) is an extremely common clinical problem. According to the 1997 National Ambulatory Medical Care Survey and National Hospital Ambulatory Medical Care Survey, UTI accounted for nearly 7 million office visits and 1 million emergency department visits, resulting in 100,000 hospitalizations. Nevertheless, it is difficult to accurately assess the incidence of UTIs because they are not reportable diseases.[5] UTIs typically occur when bacteria enter the urinary tract through the urethra and begin to multiply in the bladder.[6] The armament of therapeutic agents available to treat bacterial infections today has become restricted owing to increasing antibiotic resistance and biofilm production by the bacteria.[7] According to a recent public announcement from the National Institute of Health, "more than 60% of all microbial infections are caused by biofilm."[8,9] A biofilm is a complex aggregation of microorganisms in which cells are adhere to each other and to abiotic or biotic surface. Most urinary tract pathogens are fecal in origin, but only aerobic and facultative aerobic species such as *E. coli* or *K. pneumoniae* possess the necessary attributes to colonize the urethra. Therefore, major biofilm-producing bacteria in UTIs are *E. coli* (52.18%), followed by *K. pneumoniae* (23.91%), Proteus species (13.04%), and *Enterococcus species* (10.87%).[10,11] Biofilms are an assembly of microbial cells formed by bacterial species that are irreversibly associated with a surface and enclosed in a matrix of polysaccharide and protein material.[12,13] This confers a number of advantages such as protection from antimicrobial agents, exchange of nutrients, and exchange of genetic material.[14] Biofilms are responsible for nosocomial infections and chronic infections.[15] Biofilms may form on anatomical structures of the genitourinary tract and cause chronic UTI. This study was undertaken to study the prevalence of biofilm in specimens collected from tropical catheterized patients using tube adherence method (TAM), which is sensitive, easy to perform, and can be routinely employed. The present study aimed to study and compare the detection methods of biofilm formation by multidrug-resistant uropathogenic *E. coli* strains in tropical catheterized patients admitted in Civil Hospital, Ambala City.

MATERIALS AND METHODS

Sample Collection

A total of 500 urine samples from catheterized patients admitted in Civil Hospital, Ambala City, presenting with complaints of UTI were subjected to analysis.

Microbiological Analysis

A single bacterium in uncentrifuged urine per oil immersion field in Gram smears and more than five white blood cells per high-power field in centrifuged urine was considered as an hallmark of UTI. A set of symptoms including dysuria, frequency, incontinence, abdominal pain, and suprapubic tenderness was also evaluated. The colony count in the urine sample was done qualitatively to get the results of confirmation of significant infection.[16] The pathogens were identified using routine biochemical tests.

Biofilm Production: Detection

TAM, TCPM, and modified CRA (MCRA) method were used for the detection the biofilm production. A total of 500 clinical isolates were subjected to biofilm detection methods. All of the specimens were received from patients admitted to the hospital. Isolates were identified by standard microbiological procedures (Gram staining, colonial morphology, catalase test, cytochrome oxidase reaction, motility, and biochemical tests). Reference strain of positive biofilm producers such as *S. aureus* ATCC 35556, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 35218 was used as control. Biofilm detection was done by the following methods [Figures 2 and 3]:

TM

A loopful of test organisms from overnight culture plates was inoculated in borosilicate glass tubes containing 10 ml of Trypticase soy broth with 1% glucose. The tubes were then incubated at 37°C for 24 h aerobically. After incubation, the tubes were decanted and washed with phosphate buffer saline at pH 7.3 and dried. Tubes were then stained with crystal violet (0.1%) for 15 min. The stain was decanted, and the tubes were washed with deionized water and dried in inverted position.

Biofilm formation was considered positive when a visible film lined the walls and the bottom of the tube [Figure 1]. Formation of a stained layer at the air-liquid interface was considered negative for biofilm formation. The amount of biofilm formed was scored as shown in Table 1.

TCPM

Organisms isolated from fresh agar plates were inoculated in 10 mL of Trypticase soy broth with 1% glucose. Broths

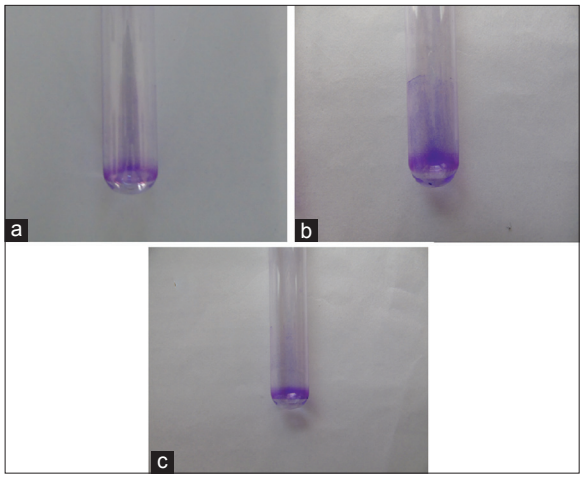

Figure. 1: Detection of biofilm production by tube adherence method. (a) Control, (b) high positive, and (c) low positive

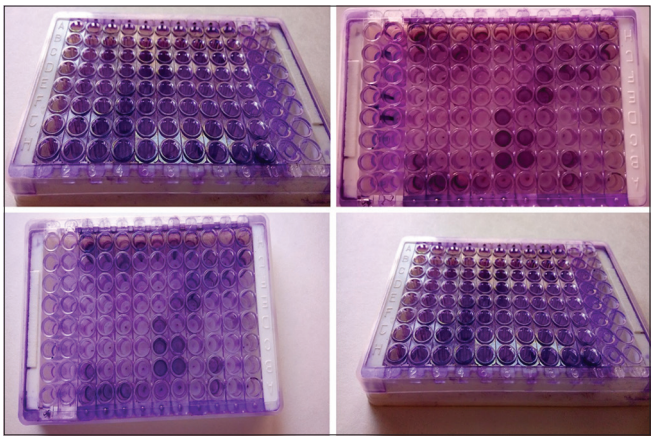

Figure 2: Detection of biofilm production by tissue culture plate method

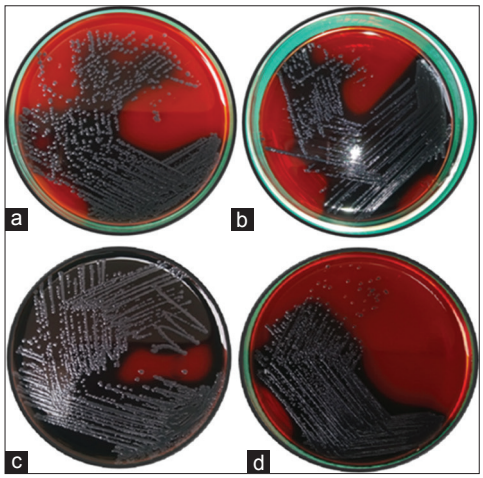

Figure 3: Detection of biofilm production by Congo red agar method (a) *Escherichia coli,* (b) *Klebsiella pneumoniae,* (c) *Enterobacter* spp., (d) *Enterococcus faecalis*

were incubated at 37°C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96-well flat bottom polystyrene tissue culture-treated plates

Table 1: Scoring of biofilm production by TAM

Score	Interpretation of results
1	Weak or none biofilm production
2	Moderate biofilm production
3	Strong or high biofilm production

TAM: Tube adherence method

Table 2: Symptom wise distribution of biofilm-producing uropathogenic *E. coli*

Symptoms of UTIs	Number of patients	Number of biofilm producer
Burning micturition	234	44
Abdominal pain	120	29
Fever	126	56
Anuria/k stone or tumor	66	21
Difficulty	83	9
Frequency	152	79
Dysuria	75	44
Hematuria	44	12
Urgency	92	60
Renal stone	69	11

χ-test=16.50 df 1, P<0.0001, Significant. UTIs: Urinary tract infections, *E. coli: Escherichia coli*

Table 3: Microorganism identified in the isolates and distribution

Organism	Isolates (%)
Resistant *E. coli*	171 (34.20)
E. coli	220 (44.00)
K. pneumoniae	112 (22.40)
P. aeruginosa	103 (20.60)
Enterobacter spp.	177 (35.40)
S. aureus	87 (17.40)

E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, K. pneumoniae: Klebsiella pneumoniae, S. aureus: Staphylococcus aureus

Table 4: Organism wise distribution of biofilm production

Organism	Total isolates	Biofilm producers (%)
Resistant *E. coli*	171	32 (18.71)
E. coli	220	-
K. pneumoniae	112	-
P. aeruginosa	103	-
Enterobacter spp.	177	-
S. aureus	87	-

E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, K. pneumoniae: Klebsiella pneumoniae, S. aureus: Staphylococcus aureus

(Sigma-Aldrich, Costar, USA) were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted, and added to TCP [Table 2]. Negative control wells contained inoculated sterile broth. The plates were incubated at 37°C for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) 4 times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells was fixed by 2% sodium acetate and stained by crystal violet (0.1%) [Table 3]. Excess stain was removed using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained using micro ELISA Autoreader (model 680, Bio-Rad, UK) at wavelength 570 nm. The experiment was performed in triplicate and repeated 3 times.

CRA Method

Bazargani and Rohloff, 2016.[9] described a simple qualitative method to detect biofilm production using CRA medium. CRA medium was prepared with brain heart infusion broth (Oxoid, UK) 37 g/L, sucrose 50 g/L, agar No. 1 (Oxoid, UK) 10 g/L, and Congo red indicator (Oxoid, UK) 8 g/L. First, Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 min) separately from the other medium constituents. Then, it was added to the autoclaved brain heart infusion agar with sucrose at 55°C. 5 CRA plates were inoculated with test organisms and incubated at 37°C for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production. The experiment was performed in triplicate and repeated 3 times.

RESULTS AND DISCUSSION

A total of 500 urine specimens from patients admitted with UTI were analyzed. Gram- negative organisms were isolated from 64 (64%) specimens and Gram-positive growth was seen in 36 (36%) specimens. *E. coli* was the most common organism isolated followed by *K. pneumoniae*. Among Gram-positive organisms, *E. faecalis* was the predominant isolate. Of 500 cases, 76 (15.2%) isolates showed biofilm formation by the TCPM, 71 (14.2%) isolates showed biofilm formation by the TAM, and 69 (13.8%) isolates showed biofilm formation by the MCRA method. Maximum biofilm production was shown by *E. coli* in isolates followed by *K. pneumoniae* and *Enterobacter* spp [Table 4].

Biofilms pose a serious problem for public health due to increased resistance of biofilm-associated organisms to antimicrobial agents and the potential of these organisms to cause infections in patients with in-dwelling medical devices. Bacteria in a biofilm survive antimicrobial agents at concentrations 1000–1500 times higher than those needed to eradicate their planktonic counterparts. Many bloodstream infections and UTIs are associated with biofilm formation. Despite good aseptic precautions, around 50% of catheterized patients develop bacteriuria in the 1st 10–14 days of catheterization.

Of the 500 specimens analyzed, Gram-negative organisms were the predominant isolates of the total growth. *E. coli* was isolated from more than half of the urine specimens followed by *K. pneumoniae* and *P. aeruginosa*. Of the total *E. coli* isolates, 34.20% accounted for resistant *E. coli.* Previous studies in the same line of research are also in accordance with the present. Indian studies also showed *E. coli* and *K. pneumoniae* as the predominant uropathogens. The same results were found in this present study also.

The maximum biofilm production was seen in resistant *E. coli* isolates. Some previous studies showed *Enterococcus* spp. as the principal biofilm producer. The study by Praharaj *et al.* 2013 found 53% of *Enterococcus* spp. isolates to be biofilm producers. In the present study, 44.00% isolates of *E. coli* demonstrated biofilm production and 34.20% isolates of resistant *E. coli* demonstrated biofilm production. This is probably because, in the present study, the samples analyzed were from tropical patient where *E. coli* has been considered as the main causative organism associated with UTIs [Table 6].

Among 500 isolates, TCPM, the standard method, detected 76 biofilm producers. The majority of the organisms associated with biofilm production were *E. coli*, followed by *K. pneumoniae*, *S. aureus*, and *P. aeruginosa*. Strong biofilm production was reported to be caused by *E. coli* and *S. epidermidis* on Foley's urinary catheter, mainly in immunocompromised patients, sensitive predominantly to meropenem, aztreonam, vancomycin, and linezolid. *S. epidermidis* was reported to be responsible for strong biofilm production in patients with intravenous catheters, sensitive mostly to linezolid and vancomycin. By TAM, the number of strong biofilm producers was 71. Slightly different results were observed by the MCRA method, with which 69 isolates showed black colonies with crystalline appearance [Table 5].

Statistical Analysis

All the data were analyzed statistically and data were recorded in triplicate where necessary. One-way analysis of variance, Chi-square test, and Fisher's extract test were utilized where necessary to derive significance [Table 7].

Table 5: Detection of biofilm in multidrug-resistant uropathogenic *E. coli* isolates by three phenotypic methods

Methods	Number of biofilm producers (%)
TCPM	76 (15.2)
TAM	71 (14.2)
CRA method	69 (13.8)

MCRA: Modified Congo red agar, TCPM: Tissue culture plate method, TAM: Tube adherence method

Table 6: Association of risk factor with biofilm producing uropathogenic *E. coli*

Risk factor	Number of patients with UTIs	Number of biofilm producer
Immunosuppression	59	22
Prolonged cathertization	384	19
Hypertension	60	15
LSCS	110	23
Recurrent UTI	56	9
Diabeties	87	27

χ-test=11.74 df 1, *P*=0.0006, As the P value is less than the level of significance, i.e., 0.05 so the result is "Significant," UTIs: Urinary tract infections, *E. coli* : *Escherichia coli*, LSCS: Lower segment Cesarian section

Table 7: Rate of biofilm production in TAM, modified method, and TCPM

Total number of positive strains (*n*=171)	Total number of isolates (%)
MCRA+TAM+TCP	34 (19.88)
MCRA+TAM	0 (0)
MCRA+TCP	9 (5.26)
TAM+TCP	12 (7.02)
TAM	30 (17.54)

MCRA: Modified Congo red agar, TCPM: Tissue culture plate method, TAM: Tube adherence method

Table 8: Comparison of the diagnostic efficacy of the methods of the detection of biofilm formation

Methods	Number of biofilm producers	Negative	Percentage of biofilm producers
TCPM	76	96	15.2
TAM	71	103	14.2
MCRA method	69	107	13.8

MCRA: Modified Congo red agar, TCPM: Tissue culture plate method, TAM: Tube adherence method

DISCUSSION

Biofilm-producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. They exhibit resistance to antibiotics by various methods such as restricted penetration of antibiotic into biofilms, decreased growth rate, and expression of resistance genes [Table 8]. There are various methods for biofilm detection. In this present study, 500 isolates were evaluated by three screening methods for their ability to form biofilms. In the TCPM, the number of isolates showing biofilm formation was 76 (15.2%). We have performed the TCPM by addition of 1% glucose in Trypticase soy broth. Addition of sugar helps in biofilm formation. TAM detected 71 (14.2%) isolates as biofilm producers. This method correlated well with TCP for identifying strong biofilm producers, but it was hard to differentiate between moderate, weak, and non-biofilm producers due to the changeability in the results detected by different observers. In accordance with the preceding studies, TAM cannot be suggested as general screening test to identify biofilm-producing isolates. In another previous study noted that of 147 isolates of *S. epidermidis*, TAM detected biofilm formation in 79 (53.7%) and MCRA detected in 64 (43.5%) isolates. They showed that TAM is better for biofilm detection than MCRA. The MCRA method showed less correlation efficacy with the other methods and parameters. The present study demonstrated superior detection sensitivity for biofilm production by *E. Coli* by TCPM as compared to TAM and MCRA method.

CONCLUSION

It can be concluded from our study that TCPM is a quantitative and reliable method to detect biofilm-forming microorganisms. When compared to TAM and MCRA methods and TCPM can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories. In conclusion, biofilms are a major cause of recurrent UTIs, which lead to increased morbidity in the patient, increased duration of hospital stay, and increased economic burden on the patients. Uncomplicated UTI has rarely been studied for biofilm formation over the entire spectrum of uropathogens. The role of biofilms in the conversion of uncomplicated UTI to chronic UTI due to partial clearance of the infection needs to be studied in greater detail. TCPM is a method with good reproducibility and good specificity. This method can be used routinely in the microbiology laboratory to detect biofilm formation, especially, when the causative organism is resistant *E. coli.*

REFERENCES

1. Koopman JA, Marshall JM, Bhatiya A, Eguale T, Kwiek JJ, Gunn JS, *et al.* Inhibition of *Salmonella enterica* biofilm formation using small-molecule adenosine mimetics. Antimicrob Agents Chemother 2015;59:76-84.
2. Tapiainen T, Hanni AM, Salo J, Ikäheimo I, Uhari M. *Escherichia coli* biofilm formation and recurrences of urinary tract infections in children. Eur J Clin Microbiol Infect Dis 2014;33:111-5.
3. Teh AH, Wang Y, Dykes GA. The influence of antibiotic resistance gene carriage on biofilm formation by two *Escherichia coli* strains associated with urinary tract infections. Can J Microbiol 2014;60:105-11.
4. Xu Z, Islam S, Wood TK, Huang Z. An integrated modeling and experimental approach to study the influence of environmental nutrients on biofilm formation of *Pseudomonas aeruginosa.* BioMed Res Int 2015;2015:12.
5. Akhtar N. Urinary tract bacterial pathogens their antimicrobial susceptibility patterns at Bahawalpur. Professional 2000;7:131-7.
6. Winters JC, Dmochowski RR, Goldman HB, Herndon CD, Kobashi KC, Kraus SR, *et al.* Urodynamic studies in adults: AUA/SUFU guideline. J Urol 2012;188:2464-72.
7. Donlan RM. Biofilms and device-associated infections. Emerg Infect Dis 2001;7:277-81.
8. Soto SM, Smithson A, Horcajada JP, Martinez JA, Mensa JP, Vila J, *et al.* Implication of biofilm formation in the persistence of urinary tract infection caused by uropathogenic *Escherichia coli*. Clin Microbiol Infect 2006;12:1034-6.
9. Bazargani MM, Rohloff J. Antibiofilm activity of essential oils and plant extracts against *Staphylococcus aureus* and *Escherichia coli* biofilms. Food Control 2016;61:156-64.
10. Agarwal J, Srivastava S, Singh M. Pathogenomics of uropathogenic *Escherichia coli*. Indian J Med Microbiol 2012;30:141-9.
11. Tayal RA, Baveja SM, De Anuradha S. Analysis of biofilm formation and antibiotic susceptibility pattern of uropathogens in patients admitted in a tertiary care hospital in India. Int J Health Allied Sci 2015;4:247-52.
12. Prakash B, Veeregowda B, Krishnappa G. Biofilms: A survival strategy of bacteria. Curr Sci 2003; 85:1299-307.
13. Donlan RM, Costerton JW. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 2002;15:167-93.
14. Kokare C, Chakraborty S, Khopade A, Mahadik KR. Biofilm: Importance and applications. Indian J Biotechnol 2009;8:159-68.
15. Shirtliff ME, Peters BM, Jabra-Rizk MA. Cross-kingdom interactions: *Candida albicans* and bacteria. FEMS Microbiol Lett 2009;299:1-8.
16. Garcia L, Isenberg H. Clinical Microbiology Procedures Handbook. Washington, DC; ASM Press: 2010.

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