

Original Research Article

<https://doi.org/10.20546/ijcmas.2020.907.128>

Comparative Evaluation of Different Phenotypic Methods for Biofilm Formation among Uropathogenic *E. coli* (UPEC)

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ABSTRACT

Keywords

Biofilm, Infections, Decreased susceptibility, Phenotypic methods

Article Info

Accepted:

11 June 2020

Available Online:

10 July 2020

Biofilms have the ability to attach to both biotic and abiotic surfaces. With the advent in medical industry, use of indwelling prosthetic devices have increased abiotic surfaces providing opportunities for attachment by the biofilm forming bacteria and thus leading to infections. Treatment of infection by biofilm forming bacteria is difficult due to their decreased susceptibility to antibiotics. Hence, this study was aimed to compare the sensitivity and specificity of the three phenotypic methods for the detection of biofilms.

Introduction

Bacteria exist in two growth modes in nature, one as the planktonic form and the other as sessile aggregates called biofilm. Existence in aggregates as Biofilm confers the bacterial community certain survival advantage like evading the host immune responses, resistance to antibiotics, biofilm also entraps the nutrients for the utilizations by the microbial community^{1,2}. Biofilm is defined as a structured community of bacterial cells enclosed in a self-produced polymeric matrix which is made up of extracellular polymeric

substances (EPS).³ According to National Institute of health (NIH) 65% of microbial infections and 80% of the chronic infections are due to biofilm forming microorganisms. Biofilms have the ability to attach to both biotic and abiotic surfaces. With the advent in medical industry, use of indwelling prosthetic devices have increased abiotic surfaces providing opportunities for attachment by the biofilm forming bacteria and thus leading to infections associated with them like catheter associated blood stream infections and catheter associated urinary tract infections, ventilator associated pneumonia etc.

Treatment of infection by biofilm forming bacteria is difficult due to their decreased susceptibility to antibiotics and unresponsiveness to the standard antimicrobial susceptibility test results which are directed against the planktonic forms, eradication of biofilm associated organisms requires the determination of biofilm eradicating concentrations which are routinely not done. Thus, it is important to screen the clinical isolates for biofilm forming capacity especially in chronic infections. There are various methods for the detection of biofilm forming capacity of microorganisms which includes various phenotypic methods like Congo-red agar method (CRA), tube method (TM), tissue culture plate method (TCP), electron microscopy, confocal scanning microscopy and bioluminescent assay and molecular methods like detection of biofilm associated genes. This study was under taken to compare the sensitivity and specificity of the three phenotypic methods for the detection of biofilms.

Materials and Methods

180 *E. coli* isolated from clinically suspected cases of Urinary tract infections were evaluated for biofilm forming capacity by three different phenotypic methods namely tissue culture plate method, tube adherence test and Congo red agar method. Biofilm production by each of the methods was graded as high, moderate, and weak, only high and moderate graded isolates were considered as biofilm producers, weak and no biofilm production by each method was considered non biofilm producers. Tissue culture method was considered as the gold standard for biofilm formation.

Tissue culture plate method⁴

Fresh isolates from agar plates were inoculated into brain heart infusion (BHI)

broth with 2% sucrose and incubated for 24hrs at 37⁰ c. Broth was diluted 1:100 with fresh medium, 0.2ml of this broth was inoculated into sterile flat-bottomed microtiter plates or tissue culture plates (TCP) only medium was inoculated in a well which served as control to check sterility of the medium and nonspecific binding of media. The TCPs were incubated for 24hrs at 37⁰c. After incubation contents of each well was gently removed by taping the plates and then were washed 4 times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating “planktonic” bacteria. Biofilms formed in wells were fixed with sodium acetate (2%) for half an hour and stained with crystal violet (0.1%w/v) for half an hour. Excess stain was removed by thorough washing with deionized water and plates were kept for drying. Adherent *E. coli* formed biofilm on all side of the wells and were uniformly stained with crystal violet. Optical Density (OD) of stained wells were determined with a micro Enzyme-Linked Immunosorbent Assay auto reader at wavelength of 570 nm and were graded as per Christensen *et al.* The OD values were considered as an index of bacteria adhering to surface and forming biofilms.

Experiment was performed in triplicate; the data was then averaged and standard deviation was calculated. To compensate for background absorbance, the mean OD value obtained from media control well was deducted from all test values.

Tube adherence method⁵

Fresh isolates from agar plates were inoculated into 10 ml brain heart infusion (BHI) broth with 2% sucrose and incubated for 24hrs at 37⁰C, tubes were decanted and washed with PBS (pH 7.2) and dried. Tubes were then stained with crystal violet 0.1% for half an hour. Excess stain was removed and

tubes were washed with deionized water and dried in an inverted position and observed for Biofilm formation. Biofilm formation was considered positive when visible film lined the wall and bottom of the tube. Tubes were examined and biofilm formation was graded as absent, moderate or strong. Experiments were performed in triplicate.

Congo red agar method⁶

Congo red agar (CRA) was prepared by supplementing brain heart infusion broth (BHI) with 5% sucrose and Congo red. The medium was composed of BHI (37 gms/L), sucrose (50 gms/L), agar no.1 (10 gms/L) and congo red stain (0.8 gms/L). Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes and then added when the agar had cooled to 55°C. The CRA plate was inoculated with the microorganism from an overnight broth culture and incubated aerobically at 37°C for 24 to 48 hours. Black colored colonies with dry crystalline consistency was interpreted as biofilm producing strains. Red colored colonies were interpreted as non-biofilm producing isolate. Experiments were performed in triplicate.

Interpretation of biofilm formation

Mean OD	Adherence	Biofilm formation
<0.120	Non / weak	Non / weak
0.120-0.0240	Moderate	Moderate
>0.240	Strong	Strong

Results and Discussion

In the present study a total of 180 clinical isolates of *E. coli* were included for the detection of biofilm formation.

Out of the 180 UPEC *E. coli*, 76 isolates were found to strongly positive for biofilm production, 54 were found to be moderately positive and 50 were weakly positive or negative for biofilm production by TCP method, 62 isolates were strongly positive, 45 moderately positive and 73 were weakly positive or non-biofilm producers by tube method similarly 68 isolates were strongly positive, 49 moderately positive and for biofilm and 63 were weakly positive or non-biofilm producers by Congo red agar method (Table.1-3).

Only strongly positive and moderately positive and moderately positive isolates were considered as biofilm producers while weakly positive isolates were considered and non-biofilm producers.

Tissue culture plate method was considered as the gold standard method for the detection of biofilm production.

Microbial biofilm is an assemblage of microorganisms which are irreversibly associated with a surface and enclosed in a polysaccharide matrix. Biofilm producing capacity acts as an important virulence factor in microorganisms as it provides a survival advantage to the organism producing it, thus it is difficult to treat biofilm associated infections than infections caused by planktonic bacteria.

Biofilm producing microorganisms are associated with nosocomial, implant associated infections, recurrent and persistent infections, biofilm forming isolates a thus it becomes necessary to evaluate the pathogens isolated for biofilm production to prevent treatment failure.

Table.1 Biofilm production by Congo red agar method, tube method and tissue culture plate method

Method	Strongly positive	Moderately positive	Weakly positive/negative	Total
CRE	68	49	63	180
TM	62	45	73	180
TCP	76(42.22)	54(30)	50(27.77)	180

Table.2 Biofilm producers by various phenotypic methods

Method	Biofilm producers, n (%)	Biofilm non producers, n (%)	Total
Congo red agar method	117 (65%)	63 (35%)	180
Tube adherence method	107 (59.44%)	73 (23.88%)	180
Tissue culture plate method	130 (72.22%)	50 (27.77%)	180

Table.3 Sensitivity, specificity, PPV and NPV of two phenotypic method of biofilm detection

Method	Sensitivity	Specificity	PPV	NPV
CRE	81.52	78	90.59	61.90
TAM	67.69	62	82.24	42.46

In the present study, out of the 180 clinical isolates of *E. coli*, 130 isolates were found to be biofilm producers accounting to 72.22% of the isolates being biofilm producers by the TCP method which is considered as the gold standard in phenotypic method of biofilm detection^{7,8,9}, this is in correlation with a study conducted by Rashmi *et al.*,¹⁰ and Sharma *et al.*,¹¹ who reports that 69% and 67.5 % of the *E. coli* isolates from urine to be biofilm producers by TCP method.

Many surface determinants like type I fimbriae, autotransporter protein like Ag43, curlie fimbriae, F conjugative pilus and exopolysaccharide are associated with biofilm production. In the study 72.22% of the isolates were found to be biofilm producers by TCP method, 65% by Congo red agar method and 59.44% by tube adherence method. The ability of Congo red agar (65%) for biofilm detection was similar to that of

TCP (72.22%) method whereas tube adherence method detected only 59.44% of the isolates to be biofilm producers, these findings are in contrast to the findings by Taj Y *et al.*,¹² who reported only 3.47%, Bos *et al.*,¹³ who reported 6.15% and Deka *et al.*, who reported 20% ability of CRE method to detect biofilm production by microorganisms. Saroj *et al.*,¹⁴ and Munesh *et al.*,¹⁵ reported high utility of CRE with 72% and 72.9% detection rate respectively. In the present study, tube adherence method showed only 59.44% of the isolates to be producing biofilm, this could be due to subjective difference in the interpretation, where as there was better correlation between TCP method and CRA method The basis of biofilm detection by Congo red agar method is that Congo red stain binds with the extracellular adhesive amyloid fibers termed curli that mediate adhesion and promote biofilm formation in *E. coli*¹⁶, so the biofilm detection

rate is comparable with that of TCP method.

In conclusion, this study was aimed to include a simple and convenient method to screen microbial isolates for biofilm production. Considering the ease of doing the test, we conclude that both TCP method and CRA method of biofilm detection can be incorporated as a routine screening method for bio film detection.

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How to cite this article:

Rashmi P Mahale, M. N. Sumana, K. Anuradha and Adline Princy. 2020. Comparative Evaluation of Different Phenotypic Methods for Biofilm Formation among Uropathogenic *E. coli* (UPEC). *Int.J.Curr.Microbiol.App.Sci.* 9(07): 1094-1099.
doi: <https://doi.org/10.20546/ijcmas.2020.907.128>